

# World Journal of *Stem Cells*

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2010-2015

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### EDITORIAL

- 235 SIRT1 and stem cells: In the forefront with cardiovascular disease, neurodegeneration and cancer  
*Maiese K*

### REVIEW

- 243 Substrates for clinical applicability of stem cells  
*Enam S, Jin S*
- 253 Hematopoietic stem cell-derived adipocytes and fibroblasts in the tumor microenvironment  
*Xiong Y, McDonald LT, Russell DL, Kelly RR, Wilson KR, Mehrotra M, Soloff AC, LaRue AC*
- 266 Role of nanotopography in the development of tissue engineered 3D organs and tissues using mesenchymal stem cells  
*Salmasi S, Kalaskar DM, Yoon WW, Blunn GW, Seifalian AM*
- 281 Evaluating alternative stem cell hypotheses for adult corneal epithelial maintenance  
*West JD, Dorà NJ, Collinson JM*
- 300 Imprinted *Zac1* in neural stem cells  
*Daniel G, Schmidt-Edelkraut U, Spengler D, Hoffmann A*
- 315 Induced pluripotent stem cells: Mechanisms, achievements and perspectives in farm animals  
*Kumar D, Talluri TR, Anand T, Kues WA*
- 329 Cardiac disease modeling using induced pluripotent stem cell-derived human cardiomyocytes  
*Dell'Era P, Benzoni P, Crescini E, Valle M, Xia E, Consiglio A, Memo M*
- 343 Stem cell therapy in inflammatory bowel disease: A promising therapeutic strategy?  
*Flores AI, Gómez-Gómez GJ, Masedo-González A, Martínez-Montiel MP*
- 352 "Second-generation" stem cells for cardiac repair  
*Núñez García A, Sanz-Ruiz R, Fernández Santos ME, Fernández-Avilés F*
- 368 Mesenchymal stem cells as a therapeutic tool to treat sepsis  
*Lombardo E, van der Poll T, DelaRosa O, Dalemans W*



- 380 Transplantation of stem cell-derived astrocytes for the treatment of amyotrophic lateral sclerosis and spinal cord injury  
*Nicaise C, Mitrecic D, Fahnkar A, Lepore AC*
- 399 Tooth-derived stem cells: Update and perspectives  
*Saito MT, Silvério KG, Casati MZ, Sallum EA, Nociti Jr FH*
- 408 Could cancer and infection be adverse effects of mesenchymal stromal cell therapy?  
*Arango-Rodriguez ML, Ezquer F, Ezquer M, Conget P*
- 418 Mitochondria as therapeutic targets for cancer stem cells  
*Song IS, Jeong JY, Jeong SH, Kim HK, Ko KS, Rhee BD, Kim N, Han J*
- 428 Vital roles of stem cells and biomaterials in skin tissue engineering  
*Mohd Hilmi AB, Halim AS*
- 437 Neural differentiation from embryonic stem cells *in vitro*: An overview of the signaling pathways  
*Chuang JH, Tung LC, Lin Y*
- 448 Importance of the stem cell microenvironment for ophthalmological cell-based therapy  
*Wan PX, Wang BW, Wang ZC*

# MINIREVIEWS

- 461 Using induced pluripotent stem cells as a tool for modelling carcinogenesis  
*Curry EL, Moad M, Robson CN, Heer R*
- 470 CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture  
*Álvarez-Viejo M, Menéndez-Menéndez Y, Otero-Hernández J*
- 477 Adult stem cells in neural repair: Current options, limitations and perspectives  
*Mariano ED, Teixeira MJ, Marie SKN, Lepski G*
- 483 Development of cancer-initiating cells and immortalized cells with genomic instability  
*Yoshioka K, Atsumi Y, Nakagama H, Teraoka H*
- 490 Adult stem-like cells in kidney  
*Hishikawa K, Takase O, Yoshikawa M, Tsujimura T, Nangaku M, Takato T*
- 495 Niche interactions in epidermal stem cells  
*Choi HR, Byun SY, Kwon SH, Park KC*



- 502** Neural stem cells could serve as a therapeutic material for age-related neurodegenerative diseases

*Suksuphew S, Noisa P*

- 512** Molecular mechanism of extrinsic factors affecting anti-aging of stem cells

*Wong TY, Solis MA, Chen YH, Huang LLH*

### **ORIGINAL ARTICLE**

#### **Basic Study**

- 521** Mesenchymal stem cells and collagen patches for anterior cruciate ligament repair

*Gantenbein B, Gadhari N, Chan SCW, Kohl S, Ahmad SS*



## Contents

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## SIRT1 and stem cells: In the forefront with cardiovascular disease, neurodegeneration and cancer

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proliferation for cardiac disease, vascular disorders, cancer, and neurodegenerative disorders is receiving great enthusiasm, especially those that focus upon SIRT1, a mammalian homologue of the yeast silent information regulator-2. Modulation of the cellular activity of SIRT1 can involve oversight by nicotinamide/nicotinic acid mononucleotide adenylyltransferase, mammalian forkhead transcription factors, mechanistic of rapamycin pathways, and cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma over-expressed gene family members that can impact cytoprotective outcomes. Ultimately, the ability of SIRT1 to control the programmed cell death pathways of apoptosis and autophagy can determine not only cardiac, vascular, and neuronal stem cell development and longevity, but also the onset of tumorigenesis and the resistance against chemotherapy. SIRT1 therefore has a critical role and holds exciting prospects for new therapeutic strategies that can offer reparative processes for cardiac, vascular, and nervous system degenerative disorders as well as targeted control of aberrant cell growth during cancer.

**Key words:** FoxO; Mechanistic of rapamycin; Apoptosis; Autophagy; Cardiovascular; Cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma over-expressed gene; Neurodegeneration; Progenitor stem cells; SIRT1; Cancer

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**Core tip:** SIRT1, a mammalian homologue of the yeast silent information regulator-2, holds exciting prospects for new therapeutic strategies that can offer reparative processes for cardiac, vascular, and nervous system degenerative disorders as well as targeted control of unchecked cell growth during cancer.

Maiese K. SIRT1 and stem cells: In the forefront with cardiovascular disease, neurodegeneration and cancer. *World J*

### Abstract

Cardiovascular disease, nervous system disorders, and cancer in association with other diseases such as diabetes mellitus result in greater than sixty percent of the global annual deaths. These noncommunicable diseases also affect at least one-third of the population in low and middle-income countries and lead to hypertension, elevated cholesterol, malignancy, and neurodegenerative disorders such as Alzheimer's disease and stroke. With the climbing lifespan of the world's population, increased prevalence of these disorders is expected requiring the development of new therapeutic strategies against these disabling disease entities. Targeting stem cell



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## THE IMPACT OF CARDIOVASCULAR DISEASE, CANCER, AND NEURODEGENERATIVE DISORDERS

Life expectancy is increasing in developed countries such as the United States and has been accompanied by a one percent decrease in the age-adjusted death rate from the years 2000 through 2011<sup>[1]</sup>. Yet, a number of disorders on a global scale continue to plague the population with increased morbidity and mortality from cardiovascular disease, disorders of the nervous system, and cancer. The World Health Organization reports that in 2008, greater than 60% of 57 million global deaths were primarily due to cardiovascular diseases, diabetes, cancer, and respiratory disorders<sup>[2]</sup>. Almost 80% of these noncommunicable diseases (NCDs) occur in low and middle-income countries. These NCDs affect approximately 30% of the population under 60 in low and middle-income countries. In contrast, in high-income countries, 13% of the population under 60 is affected. Hypertension and elevated cholesterol are significant risk factors for cardiovascular disease with hypertension alone contributing to approximately 13% of all deaths<sup>[3]</sup>. Disorders such as hypertension and elevated cholesterol also contribute to acute neurodegenerative disease such as stroke, the fourth leading cause of death<sup>[4,5]</sup>. With the increasing lifespan of the world's population and advancing age, it is expected that the incidence of neurodegenerative disorders also will grow. As an example, ten percent of the global population over the age of 65 is now affected with the sporadic form of Alzheimer's disease, but this is expected to increase significantly<sup>[6-8]</sup>. Continued development of new therapeutic strategies directed against the NCDs of cardiovascular disease, neurodegeneration, and cancer are necessary to increase our armamentarium against the complexity of these disease entities.

## THE SIRT1 PATHWAY

In this arsenal directed against cardiovascular disease, neurodegenerative disorders, and cancer, multiple therapeutic strategies are being advanced that involve novel stem cell applications. Targeting stem cell proliferation is being considered for cardiac disease<sup>[9]</sup>, vascular disorders<sup>[10,11]</sup>, cancer<sup>[12,13]</sup>, and neurodegenerative disease<sup>[14-16]</sup>. However, it is the investigation of stem cells that focus upon sirtuins, mammalian homologues of the yeast silent information regulator-2 (Sir2), that are proving to be extremely

exciting.

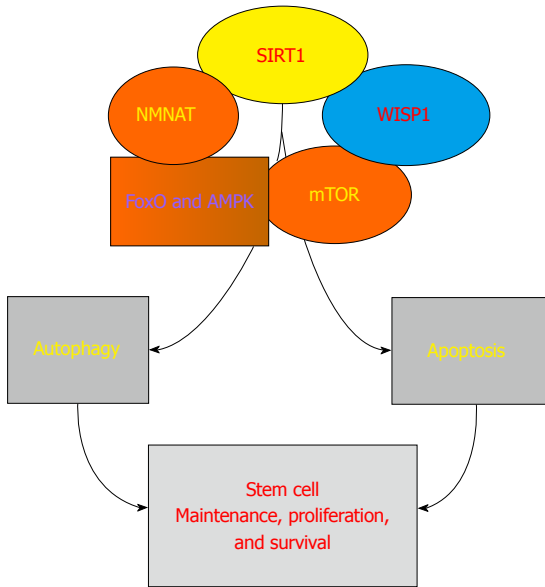
Sirtuins are histone deacetylases that transfer acetyl groups from  $\epsilon$ -N-acetyl lysine amino acids on the histones of DNA to regulate transcription<sup>[17-19]</sup>. This family of histone deacetylases also mediates post-translational changes of proteins involved with cellular proliferation, survival, and senescence<sup>[20-23]</sup>. There are seven identified mammalian homologues of Sir2 that include SIRT1 through SIRT7. Of these, SIRT1 has been tied to the modulation of multiple cellular functions that include protection against oxidative stress<sup>[24-28]</sup>, development of atherosclerosis<sup>[29,30]</sup>, modulation of vascular survival and senescence<sup>[17,20,21,31,32]</sup>, proliferation of cancer cells<sup>[33-36]</sup>, changes in diabetic cellular metabolism<sup>[33-36]</sup>, control of vascular tone through the transient receptor potential cation channel A1<sup>[37]</sup>, promotion of neuronal survival and cognitive function<sup>[21,38-41]</sup>, and the extension of lifespan<sup>[25,42,43]</sup>. Furthermore, SIRT1 appears to be necessary for efficient post-reprogramming of telomere elongation, the maintenance of pluripotency, and the modulation of differentiation in induced pluripotent stem cells<sup>[44]</sup>. In differentiated cells, SIRT1 also controls telomere length and maintenance<sup>[45]</sup>.

SIRT1 is dependent upon NAD<sup>+</sup> as substrate<sup>[17,38,46,47]</sup>. Through the salvage pathway of NAD<sup>+</sup> synthesis, nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the conversion of nicotinamide to nicotinamide mononucleotide<sup>[48]</sup>. Nicotinamide mononucleotide is subsequently converted to NAD<sup>+</sup> by enzymes in the nicotinamide/nicotinic acid mononucleotide adenyltransferase (NMNAT) family. NAMPT is a rate-limiting enzyme in mammalian NAD<sup>+</sup> biosynthesis pathway. Elevated levels of NAMPT activity increase cellular NAD levels as well as the activity of SIRT1 transcription.

The level of SIRT1 activity and its modulation in these cellular processes is considered to be an important factor in determining cell survival and protection against toxic insults. Insufficient SIRT1 activity can have a detrimental affect upon vascular cell survival<sup>[22,23,49]</sup>, protection against cardiovascular disease<sup>[31]</sup>, and prevention of neuronal injury<sup>[28,50,51]</sup>. Yet, a reduction in SIRT1 activity also may be required to promote cellular survival in systems involving trophic factors such as insulin growth factor-1<sup>[52]</sup>.

Several biological systems can control the activity of SIRT1 (Figure 1). For example, NMNAT can modulate the deacetylating activity of SIRT1. In addition, mammalian forkhead transcription factors<sup>[53]</sup> can bind to the SIRT1 promoter region that contains a cluster of five putative core binding repeat motifs (IRS-1) and a forkhead-like consensus-binding site (FKHD-L). As a result, forkhead transcription factors such as FoxO1 can govern SIRT1 transcription and increase SIRT1 expression<sup>[54]</sup>. AMP activated protein kinase (AMPK) represents another pathway for the control of SIRT1 activity. AMPK is a member of the mechanistic





**Figure 1 Schematic of SIRT1 pathways that can influence stem cell maintenance, proliferation, and survival.** Several pathways can control SIRT1. For example, NMNAT can modulate the deacetylating activity of SIRT1, FoxO1 can govern SIRT1 transcription and increase SIRT1 expression, and AMPK can increase the cellular NAD<sup>+</sup>/NADH ratio leading to the deacetylation of downstream SIRT1 targets. SIRT1 subsequently can depress mTOR pathways and promote autophagy to preserve stem cell integrity during oxidant stress as well as promote neuronal growth. In addition, SIRT1 is necessary to initiate autophagy and transition cells from a quiescence state to an active state. WISP1 increases SIRT1 activity to protect cells from oxidative stress and apoptotic injury and blocks SIRT1 caspase degradation. NMNAT: Nicotinamide/nicotinic acid mononucleotide adenylyltransferase; mTOR: Mechanistic of rapamycin; AMPK: AMP activated protein kinase.

of rapamycin (mTOR) pathway that phosphorylates tuberous sclerosis protein 2 and inhibits the activity of mTORC1<sup>[55,56]</sup>. AMPK can increase the cellular NAD<sup>+</sup>/NADH ratio leading to the deacetylation of downstream SIRT1 targets that include the peroxisome proliferator-activated receptor- $\gamma$  coactivator 1  $\alpha$ , FoxO1, and FoxO3a<sup>[57]</sup>. AMPK also can increase NAMPT during glucose restriction that results in increased NAD<sup>+</sup> and decreased levels of nicotinamide<sup>[58]</sup>, an inhibitor of SIRT1<sup>[59]</sup>. Resveratrol, a SIRT1 activator, also has been shown to activate AMPK through SIRT1 dependent or independent mechanisms<sup>[57,60]</sup>.

## STEM CELLS, SIRT1, APOPTOSIS, AND AUTOPHAGY

The impact of SIRT1 on cellular function is intimately associated with programmed cell death pathways that involve apoptosis and autophagy<sup>[61-63]</sup>. Apoptosis leads to DNA degradation and caspase activation through an early phase that involves the loss of plasma membrane lipid phosphatidylserine (PS) asymmetry and a later phase that results in genomic DNA degradation<sup>[64]</sup>. During the early phase of apoptosis, prevention of membrane PS externalization in injured cells is necessary to block the loss of functional cells that may

be removed by activated inflammatory cells<sup>[56]</sup>. SIRT1 activation limits external membrane PS exposure during the early phases of apoptosis in mature cells<sup>[22,23,65,66]</sup>. In endothelial progenitor cells, SIRT1 activity can counteract the “pro-apoptotic” effects of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>[67]</sup>. During exposure to TNF- $\alpha$ , SIRT1 also has been shown to protect skeletal myoblast survival<sup>[68]</sup>. Loss of SIRT1 activity in human mesenchymal stem cells yields a reduction of proliferation rate with increased apoptosis<sup>[69]</sup>. During aging in the mouse auditory system, loss of SIRT1 in cochlear neurons and in the auditory cortex is associated with hearing loss<sup>[70]</sup>. In addition, endothelial progenitor cell dysfunction with apoptotic cell death that can occur in smokers and chronic obstructive disease patients has been associated with the loss of SIRT1 expression<sup>[71]</sup>.

Stem cell survival with SIRT1 can be reliant upon forkhead transcription factors and mTOR (Figure 1). Although several studies involving differentiated cells support the premise that down-regulation of forkhead transcription factors by SIRT1 activation can protect against apoptotic cell death especially during oxidant stress<sup>[22,23,65,72,73]</sup>, other studies in embryonic stem cells suggest that SIRT1 down-regulation can lead to the acetylation/phosphorylation of forkhead transcription factor pathways such as FoxO1, and in association with phosphatase and tensin homolog (PTEN) and c-Jun N-terminal kinase (JNK), block oxidant stress induced apoptosis<sup>[74]</sup>. However, in embryonic stem cells, the presence of SIRT1 also can be protective and appears to have an inverse relationship with mTOR<sup>[35]</sup>. SIRT1 can depress mTOR mediated pathways as well as promote autophagy to preserve the integrity of embryonic stem cells during oxidant stress<sup>[75]</sup>. SIRT1 can foster inhibition of mTOR signaling to promote neuronal growth<sup>[76]</sup>. In addition, during high glucose exposure to mesangial cells, the loss of SIRT1 activity is necessary for mTOR to arrest mesangial cell senescence<sup>[77]</sup>.

It is important to note that during apoptotic cell injury with the induction of caspase activity, SIRT1 is susceptible to degradation by caspases. Although SIRT1 degradation also may be mediated by apoptotic pathways associated with p38<sup>[78]</sup> and JNK1<sup>[79]</sup>, loss of SIRT1 activity can be the result of caspase degradation of the SIRT1 protein<sup>[80]</sup> that can then accelerate further activation of caspases<sup>[80,81]</sup>. In some systems that involve the cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma over-expressed gene (CCN) family (defined by the first three members of the family that include cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma over-expressed gene)<sup>[12]</sup>, the CCN member WISP1 increases SIRT1 activity to protect cells from oxidative stress and apoptotic injury<sup>[28]</sup> (Figure 1). WISP1 also prevents SIRT1 degradation and oversees forkhead transcription activity with SIRT1 similar to other



cytoprotective pathways<sup>[20,73,82]</sup> to block FoxO3a activity and prevent caspase activation that would otherwise lead to the degradation of SIRT1<sup>[28,83-85]</sup>.

In contrast to apoptosis, autophagy promotes tissue remodeling by recycling cytoplasmic components and eliminating no longer useful organelles<sup>[62]</sup>. Macroautophagy is the classification of autophagy most commonly described<sup>[86]</sup>. It involves the sequestration of cytoplasmic proteins into autophagosomes that fuse with lysosomes for degradation and are eventually recycled. In most cases, SIRT1 activation with the induction of autophagy appears to be vital to promote cell survival in mature cells. In differentiated chondrocytes during oxidant stress, knockdown of the forkhead transcription factors FoxO1 and FoxO3 result in cell death with decreased SIRT1 activity and reduced autophagic related proteins, suggesting that SIRT1 with the activation of autophagy is necessary for cellular protection<sup>[24]</sup>. SIRT1 also plays a role in autophagic flux and promoting autophagy in mitochondria<sup>[87]</sup> that may be required to maintain a healthy pool of mitochondria<sup>[88]</sup>. In endothelial cells exposed to oxidized low density lipoproteins that can lead to atherosclerosis, SIRT1 up-regulation in conjunction with AMPK results in autophagy that is necessary for cellular protection<sup>[89]</sup>. In models of cognitive loss with chronic intermittent hypoxia hypercapnia exposure, SIRT1 activation is able to block apoptotic cell injury, up-regulate autophagy, and improve cognitive performance<sup>[90]</sup>. However, in pulmonary models of oxidant stress such as the exposure to cigarette smoke in bronchial epithelial cells, SIRT1 has been shown to prevent cell injury through the inhibition of autophagy<sup>[91,92]</sup>. In regards to stem cells and the autophagic pathway, stem cells rely upon SIRT1 to modulate autophagic flux<sup>[93]</sup>. In muscle stem cells, SIRT1 is necessary to initiate autophagy and transition muscle stem cells from a quiescence state to an active state<sup>[94]</sup>. In endothelial progenitor cells, SIRT1 blocks apoptotic cell injury during oxidative stress through the induction of autophagy<sup>[95]</sup>.

## STEM CELLS, SIRT1, AND THE CARDIOVASCULAR SYSTEM

In the cardiovascular system, SIRT1 expression can affect not only the survival of stem cells, but also the ability of stem cells to differentiate and the efficacy of these cells for therapeutic applications. Increased SIRT1 expression can improve the survival of cardiomyoblasts<sup>[96]</sup> and prevent senescence and impaired differentiation in endothelial progenitor cells<sup>[97]</sup>. In regards to treatment efficacy, mesenchymal stem cells that are subjected to SIRT1 over-expression exhibit increased blood vessel density in the area of cardiac infarcts, reduced cardiac remodeling, and improved cardiac performance in rodent models<sup>[98]</sup>, factors that may be associated with cardiac stem migration that is vital to tissue repair<sup>[99]</sup>. SIRT1 also

can limit expression of aged mesenchymal stem cell phenotypes<sup>[98]</sup>. Loss of SIRT1 in circulating endothelial progenitor cells that can occur during tobacco exposure or chronic obstructive pulmonary disease may lead to increased senescence and apoptotic cell death that presents increased risk for vascular disease or cardiac disease<sup>[71]</sup>. SIRT1 also may improve the function of aged stem cells that are senescent. Aged mesenchymal stem cells that were exposed to pre-conditioning with glucose depletion exhibited increased expression of SIRT1 in addition to other proliferative entities such as growth factors and resulted in increased cardiac performance<sup>[100]</sup>.

## STEM CELLS, SIRT1, AND NEURODEGENERATION

In the nervous system, SIRT1 has been tied to the differentiation, maturation, and maintenance of neurons. Loss of SIRT1 expression with the concurrent induction of heat shock protein-70 promotes neural differentiation, maturation of embryonic cortical neurons<sup>[101]</sup>, and the differentiation of human embryonic stem cells into motor neurons<sup>[102]</sup>. SIRT1 also is considered a negative regulator of subventricular zone and hippocampal neural precursors in murine animal models. Knockdown of SIRT1 does not eliminate neural precursor numbers or proliferation but increases the production of neurons in the subventricular zone and the hippocampus<sup>[103]</sup>. In the mouse cerebral cortex, repression of SIRT1 by the oncogene BCL6 leads to the conversion of neural stem cell/progenitor cells to become neurons<sup>[104]</sup>. Neural stem cell differentiation also can be controlled through alternate pathways that involve SIRT1. In mouse neural stem cells, neuronal differentiation can be driven through the microRNA miR-34a that leads to decreased SIRT1 expression and DNA-binding of p53<sup>[105]</sup>. Interestingly, in these studies, increased expression of SIRT1 enhanced the astrocytic subpopulation of cells<sup>[105]</sup>.

## STEM CELLS, SIRT1, AND CANCER

The cellular proliferative effects of SIRT1 also play a critical role in tumorigenesis. For example, SIRT1 activity can maintain acute myeloid leukemia stem cells and confer resistance against chemotherapy<sup>[106]</sup>, stimulate endometrial cell tumor growth through lipogenesis<sup>[107]</sup>, maintain neural stem cells and promote oncogenic transformation<sup>[108]</sup>, and foster hepatocellular carcinoma<sup>[109]</sup>. As a result, SIRT1 and agents that modulate SIRT1 activity may represent new therapeutic strategies against tumorigenesis. For example, down-regulation of endoglin, a protein over-expressed in tumor associated endothelial cells, leads to apoptotic cell death, DNA damage, inhibition of several DNA repair genes including SIRT1, and enhanced chemotherapy sensitivity<sup>[110]</sup>. In addition, pathways linked to SIRT1



also may provide new strategies against cancer. Activation of p53 through SIRT1 inhibition can result in apoptotic cell death for quiescent leukemia stem cells in chronic myelogenous leukemia<sup>[111]</sup>. In breast cancer, estrogen receptor- $\alpha$  can lead to SIRT1 expression that activates pro-survival genes in breast cancer cells, such as catalase and glutathione peroxidase, and inhibits tumor suppressor genes, such as cyclin G2 (CCNG2) and p53. In these breast cancer cells, if SIRT1 is inhibited, estrogen receptor-induced breast cell growth is blocked through apoptotic cell death<sup>[112]</sup>.

## FUTURE CONSIDERATIONS

Cardiac disease, vascular disorders, neurodegenerative disease, and cancer lead to significant disability and death in the global population. Development of stem cell strategies for these disorders and the targeting of SIRT1 to drive stem cell viability and function holds great promise for the future. In the cardiovascular system, SIRT1 through stem cell proliferation can drive angiogenesis, improve cardiac performance following ischemic injury, limit cell senescence, and enhance the function of aged stem cells. In the nervous system, SIRT1 can be a negative modulator of neural precursors with the loss of SIRT1 leading to differentiation and maturation of embryonic stem cells in the nervous system. During tumorigenesis, SIRT1 foster the development of acute myeloid leukemia stem cells, promote oncogenic transformation of neural stem cells, and lead to hepatocellular cancer. Vital to the clinical outcomes controlled by SIRT1 is its level of activity overseen by pathways that include NMNAT, mammalian forkhead transcription factors, mTOR, and CCN family members such as WISP1 that determine cell survival through apoptosis and autophagy. Future work that can target SIRT1 and navigate stem cell proliferation under required conditions to either cellular proliferation or cellular death can open new avenues for the treatment of cardiovascular disorders, neurodegenerative disease, and cancer.

## REFERENCES

- 1 **Miniño AM.** Death in the United States, 2011. *NCHS Data Brief* 2013; **(115)**: 1-8 [PMID: 23742756]
- 2 **World Health Organization.** Description of the global burden of ncds, their risk factors and determinants. USA: Global status report on noncommunicable diseases, 2010, 2011: 1-176
- 3 **Sivaraman V, Yellon DM.** Pharmacologic therapy that simulates conditioning for cardiac ischemic/reperfusion injury. *J Cardiovasc Pharmacol Ther* 2014; **19**: 83-96 [PMID: 24038018 DOI: 10.1177/1074248413499973]
- 4 **Maiese K.** Cutting through the complexities of mTOR for the treatment of stroke. *Curr Neurovasc Res* 2014; **11**: 177-186 [PMID: 24712647 DOI: 10.2174/1567202611666140408104831]
- 5 **Pergola PE, White CL, Szychowski JM, Talbert R, Brutto OD, Castellanos M, Graves JW, Matamala G, Pretell EJ, Yee J, Rebello R, Zhang Y, Benavente OR.** Achieved blood pressures in the secondary prevention of small subcortical strokes (SPS3) study: challenges and lessons learned. *Am J Hypertens* 2014; **27**: 1052-1060 [PMID: 24610884 DOI: 10.1093/ajh/hpu027]
- 6 **Maiese K.** Taking aim at Alzheimer's disease through the mammalian target of rapamycin. *Ann Med* 2014; **46**: 587-596 [PMID: 25105207 DOI: 10.3109/07853890.2014.941921]
- 7 **Maiese K.** Driving neural regeneration through the mammalian target of rapamycin. *Neural Regen Res* 2014; **9**: 1413-1417 [PMID: 25317149 DOI: 10.4103/1673-5374.139453]
- 8 **Schluesener JK, Zhu X, Schluesener HJ, Wang GW, Ao P.** Key network approach reveals new insight into Alzheimer's disease. *IET Syst Biol* 2014; **8**: 169-175 [PMID: 25075530 DOI: 10.1049/iet-syb.2013.0047]
- 9 **Jung DW, Kim WH, Williams DR.** Reprogram or reboot: small molecule approaches for the production of induced pluripotent stem cells and direct cell reprogramming. *ACS Chem Biol* 2014; **9**: 80-95 [PMID: 24245936 DOI: 10.1021/cb400754f]
- 10 **Fraineau S, Pal CG 2nd, Allan DS, Brand M.** Epigenetic Regulation of Endothelial Cell-mediated Vascular Repair. *FEBS J* 2014 Dec 24; Epub ahead of print [PMID: 25546332 DOI: 10.1111/febs.13183]
- 11 **Puthanveetil P, Wan A, Rodrigues B.** FoxO1 is crucial for sustaining cardiomyocyte metabolism and cell survival. *Cardiovasc Res* 2013; **97**: 393-403 [PMID: 23263330 DOI: 10.1093/cvr/cvs426]
- 12 **Maiese K.** WISP1: Clinical insights for a proliferative and restorative member of the CCN family. *Curr Neurovasc Res* 2014; **11**: 378-389 [PMID: 25219658]
- 13 **Teschendorff AE, West J, Beck S.** Age-associated epigenetic drift: implications, and a case of epigenetic thrift? *Hum Mol Genet* 2013; **22**: R7-R15 [PMID: 23918660 DOI: 10.1093/hmg/ddt375]
- 14 **Maiese K.** The challenges for drug development: cytokines, genes, and stem cells. *Curr Neurovasc Res* 2012; **9**: 231-232 [PMID: 23030554]
- 15 **Mittal D, Ali A, Md S, Baboota S, Sahni JK, Ali J.** Insights into direct nose to brain delivery: current status and future perspective. *Drug Deliv* 2014; **21**: 75-86 [PMID: 24102636 DOI: 10.3109/10717544.2013.838713]
- 16 **Yi BR, Kim SU, Choi KC.** Development and application of neural stem cells for treating various human neurological diseases in animal models. *Lab Anim Res* 2013; **29**: 131-137 [PMID: 24106507 DOI: 10.5625/lar.2013.29.3.131]
- 17 **Chong ZZ, Shang YC, Wang S, Maiese K.** SIRT1: new avenues of discovery for disorders of oxidative stress. *Expert Opin Ther Targets* 2012; **16**: 167-178 [PMID: 22233091 DOI: 10.1517/14728222.2012.648926]
- 18 **Oblong JE.** The evolving role of the NAD<sup>+</sup>/nicotinamide metabolome in skin homeostasis, cellular bioenergetics, and aging. *DNA Repair (Amst)* 2014; **23**: 59-63 [PMID: 24794404 DOI: 10.1016/j.dnarep.2014.04.005]
- 19 **Patel SA, Velingkaar NS, Kondratov RV.** Transcriptional control of antioxidant defense by the circadian clock. *Antioxid Redox Signal* 2014; **20**: 2997-3006 [PMID: 24111970 DOI: 10.1089/ars.2013.5671]
- 20 **Arunachalam G, Samuel SM, Marei I, Ding H, Trigg CR.** Metformin modulates hyperglycaemia-induced endothelial senescence and apoptosis through SIRT1. *Br J Pharmacol* 2014; **171**: 523-535 [PMID: 24372553 DOI: 10.1111/bph.12496]
- 21 **Gong H, Pang J, Han Y, Dai Y, Dai D, Cai J, Zhang TM.** Age-dependent tissue expression patterns of Sirt1 in senescence-accelerated mice. *Mol Med Rep* 2014; **10**: 3296-3302 [PMID: 25323555 DOI: 10.3892/mmr.2014.2648]
- 22 **Hou J, Chong ZZ, Shang YC, Maiese K.** Early apoptotic vascular signaling is determined by Sirt1 through nuclear shuttling, forkhead trafficking, bad, and mitochondrial caspase activation. *Curr Neurovasc Res* 2010; **7**: 95-112 [PMID: 20370652]
- 23 **Hou J, Wang S, Shang YC, Chong ZZ, Maiese K.** Erythropoietin employs cell longevity pathways of SIRT1 to foster endothelial vascular integrity during oxidant stress. *Curr Neurovasc Res* 2011; **8**: 220-235 [PMID: 21722091]
- 24 **Akasaki Y, Alvarez-Garcia O, Saito M, Caramés B, Iwamoto Y, Lotz MK.** FoxO Transcription Factors Support Oxidative Stress



- Resistance in Human Chondrocytes. *Arthritis Rheumatol* 2014; **66**: 3349-3358 [PMID: 25186470 DOI: 10.1002/art.38868]
- 25 **Balan V**, Miller GS, Kaplun L, Balan K, Chong ZZ, Li F, Kaplun A, VanBerkum MF, Arking R, Freeman DC, Maiese K, Tzivion G. Life span extension and neuronal cell protection by Drosophila nicotinamidase. *J Biol Chem* 2008; **283**: 27810-27819 [PMID: 18678867 DOI: 10.1074/jbc.M804681200]
- 26 **Chong ZZ**, Maiese K. Enhanced tolerance against early and late apoptotic oxidative stress in mammalian neurons through nicotinamidase and sirtuin mediated pathways. *Curr Neurovasc Res* 2008; **5**: 159-170 [PMID: 18691073 DOI: 10.2174/156720208785425666]
- 27 **Li J**, Feng L, Xing Y, Wang Y, Du L, Xu C, Cao J, Wang Q, Fan S, Liu Q, Fan F. Radioprotective and antioxidant effect of resveratrol in hippocampus by activating Sirt1. *Int J Mol Sci* 2014; **15**: 5928-5939 [PMID: 24722566 DOI: 10.3390/ijms15045928]
- 28 **Wang S**, Chong ZZ, Shang YC, Maiese K. WISP1 neuroprotection requires FoxO3a post-translational modulation with autoregulatory control of SIRT1. *Curr Neurovasc Res* 2013; **10**: 54-69 [PMID: 23151077]
- 29 **Kedenko L**, Lamina C, Kedenko I, Kollerits B, Kiesslich T, Iglseider B, Kronenberg F, Paulweber B. Genetic polymorphisms at SIRT1 and FOXO1 are associated with carotid atherosclerosis in the SAPHIR cohort. *BMC Med Genet* 2014; **15**: 112 [PMID: 25273948 DOI: 10.1186/s12881-014-0112-7]
- 30 **Stein S**, Matter CM. Protective roles of SIRT1 in atherosclerosis. *Cell Cycle* 2011; **10**: 640-647 [PMID: 21293192]
- 31 **Kilic U**, Gok O, Bacaksiz A, Izmirli M, Elibol-Can B, Uysal O. SIRT1 gene polymorphisms affect the protein expression in cardiovascular diseases. *PLoS One* 2014; **9**: e90428 [PMID: 24587358 DOI: 10.1371/journal.pone.0090428]
- 32 **Liu J**, Wu X, Wang X, Zhang Y, Bu P, Zhang Q, Jiang F. Global Gene Expression Profiling Reveals Functional Importance of Sirt2 in Endothelial Cells under Oxidative Stress. *Int J Mol Sci* 2013; **14**: 5633-5649 [PMID: 23478437 DOI: 10.3390/ijms14035633]
- 33 **Audrito V**, Vaisitti T, Rossi D, Gottardi D, D'Arena G, Laurenti L, Gaidano G, Malavasi F, Deaglio S. Nicotinamide blocks proliferation and induces apoptosis of chronic lymphocytic leukemia cells through activation of the p53/miR-34a/SIRT1 tumor suppressor network. *Cancer Res* 2011; **71**: 4473-4483 [PMID: 21565980 DOI: 10.1158/0008-5472.can-10-4452]
- 34 **Knight JR**, Allison SJ, Milner J. Active regulator of SIRT1 is required for cancer cell survival but not for SIRT1 activity. *Open Biol* 2013; **3**: 130130 [PMID: 24258275 DOI: 10.1098/rsob.130130]
- 35 **Maiese K**, Chong ZZ, Shang YC, Wang S. Novel directions for diabetes mellitus drug discovery. *Expert Opin Drug Discov* 2013; **8**: 35-48 [PMID: 23092114 DOI: 10.1517/17460441.2013.736485]
- 36 **Zhang JG**, Zhao G, Qin Q, Wang B, Liu L, Liu Y, Deng SC, Tian K, Wang CY. Nicotinamide prohibits proliferation and enhances chemosensitivity of pancreatic cancer cells through deregulating SIRT1 and Ras/Akt pathways. *Pancreatol* 2013; **13**: 140-146 [PMID: 23561972 DOI: 10.1016/j.pan.2013.01.001]
- 37 **Pazienza V**, Pomara C, Cappello F, Calogero R, Carrara M, Mazzoccoli G, Vinciguerra M. The TRPA1 channel is a cardiac target of mIGF-1/SIRT1 signaling. *Am J Physiol Heart Circ Physiol* 2014; **307**: H939-H944 [PMID: 25108014 DOI: 10.1152/ajpheart.00150.2014]
- 38 **Maiese K**, Chong ZZ, Shang YC, Wang S. Translating cell survival and cell longevity into treatment strategies with SIRT1. *Rom J Morphol Embryol* 2011; **52**: 1173-1185 [PMID: 22203920]
- 39 **Martin A**, Tegla CA, Cudrici CD, Kruszewski AM, Azimzadeh P, Boodhoo D, Mekala AP, Rus V, Rus H. Role of SIRT1 in autoimmune demyelination and neurodegeneration. *Immunol Res* 2015; **61**: 187-197 [PMID: 25281273 DOI: 10.1007/s12026-014-8557-5]
- 40 **Paraíso AF**, Mendes KL, Santos SH. Brain activation of SIRT1: role in neuropathology. *Mol Neurobiol* 2013; **48**: 681-689 [PMID: 23615921 DOI: 10.1007/s12035-013-8459-x]
- 41 **Zhao Y**, Luo P, Guo Q, Li S, Zhang L, Zhao M, Xu H, Yang Y, Poon W, Fei Z. Interactions between SIRT1 and MAPK/ERK regulate neuronal apoptosis induced by traumatic brain injury in vitro and in vivo. *Exp Neurol* 2012; **237**: 489-498 [PMID: 22828134 DOI: 10.1016/j.expneurol.2012.07.004]
- 42 **Moroz N**, Carmona JJ, Anderson E, Hart AC, Sinclair DA, Blackwell TK. Dietary restriction involves NAD(+) -dependent mechanisms and a shift toward oxidative metabolism. *Aging Cell* 2014; **13**: 1075-1085 [PMID: 25257342 DOI: 10.1111/accel.12273]
- 43 **Wang Y**, Liang Y, Vanhoutte PM. SIRT1 and AMPK in regulating mammalian senescence: a critical review and a working model. *FEBS Lett* 2011; **585**: 986-994 [PMID: 21130086 DOI: 10.1016/j.febslet.2010.11.047]
- 44 **De Bonis ML**, Ortega S, Blasco MA. SIRT1 is necessary for proficient telomere elongation and genomic stability of induced pluripotent stem cells. *Stem Cell Reports* 2014; **2**: 690-706 [PMID: 24936455 DOI: 10.1016/j.stemcr.2014.03.002]
- 45 **Palacios JA**, Herranz D, De Bonis ML, Velasco S, Serrano M, Blasco MA. SIRT1 contributes to telomere maintenance and augments global homologous recombination. *J Cell Biol* 2010; **191**: 1299-1313 [PMID: 21187328 DOI: 10.1083/jcb.201005160]
- 46 **Duan W**. Sirtuins: from metabolic regulation to brain aging. *Front Aging Neurosci* 2013; **5**: 36 [PMID: 23888142 DOI: 10.3389/fnagi.2013.00036]
- 47 **Srivastava S**, Haigis MC. Role of sirtuins and calorie restriction in neuroprotection: implications in Alzheimer's and Parkinson's diseases. *Curr Pharm Des* 2011; **17**: 3418-3433 [PMID: 21902666 DOI: 10.2174/138161211798072526]
- 48 **Maiese K**, Chong ZZ, Hou J, Shang YC. The vitamin nicotinamide: translating nutrition into clinical care. *Molecules* 2009; **14**: 3446-3485 [PMID: 19783937 DOI: 10.3390/molecules14093446]
- 49 **Marampon F**, Gravina GL, Scarsella L, Festuccia C, Lovat F, Ciccarelli C, Zani BM, Polidoro L, Grassi D, Desideri G, Evangelista S, Ferri C. Angiotensin-converting-enzyme inhibition counteracts angiotensin II-mediated endothelial cell dysfunction by modulating the p38/Sirt1 axis. *J Hypertens* 2013; **31**: 1972-1983 [PMID: 23868084 DOI: 10.1097/HJH.0b013e3283638b32]
- 50 **Kim DW**, Kim YM, Kang SD, Han YM, Pae HO. Effects of Resveratrol and trans-3,5,4'-Trimethoxystilbene on Glutamate-Induced Cytotoxicity, Heme Oxygenase-1, and Sirtuin 1 in HT22 Neuronal Cells. *Biomol Ther (Seoul)* 2012; **20**: 306-312 [PMID: 24130928 DOI: 10.4062/biomolther.2012.20.3.306]
- 51 **Zhang J**, Feng X, Wu J, Xu H, Li G, Zhu D, Yue Q, Liu H, Zhang Y, Sun D, Wang H, Sun J. Neuroprotective effects of resveratrol on damages of mouse cortical neurons induced by  $\beta$ -amyloid through activation of SIRT1/Akt1 pathway. *Biofactors* 2013; **40**: 258-267 [PMID: 24132831 DOI: 10.1002/biof.1149]
- 52 **Sansone L**, Reali V, Pellegrini L, Villanova L, Aventaggiato M, Marfe G, Rosa R, Nebbioso M, Tafani M, Fini M, Russo MA, Pucci B. SIRT1 silencing confers neuroprotection through IGF-1 pathway activation. *J Cell Physiol* 2013; **228**: 1754-1761 [PMID: 23359486 DOI: 10.1002/jcp.24334]
- 53 **Maiese K**, Chong ZZ, Shang YC. OutFOXing disease and disability: the therapeutic potential of targeting FoxO proteins. *Trends Mol Med* 2008; **14**: 219-227 [PMID: 18403263 DOI: 10.1016/j.molmed.2008.03.002]
- 54 **Xiong C**, Salazar G, Patrushev N, Alexander RW. FoxO1 mediates an autocrine feedback loop regulating SIRT1 expression. *J Biol Chem* 2011; **286**: 5289-5299 [PMID: 21149440 DOI: 10.1074/jbc.M110.163667]
- 55 **Maiese K**, Chong ZZ, Shang YC, Wang S. mTOR: on target for novel therapeutic strategies in the nervous system. *Trends Mol Med* 2013; **19**: 51-60 [PMID: 23265840 DOI: 10.1016/j.molmed.2012.11.001]
- 56 **Maiese K**, Chong ZZ, Wang S, Shang YC. Oxidant stress and signal transduction in the nervous system with the PI 3-K, Akt, and mTOR cascade. *Int J Mol Sci* 2012; **13**: 13830-13866 [PMID: 23203037 DOI: 10.3390/ijms131113830]
- 57 **Cantó C**, Auwerx J. Caloric restriction, SIRT1 and longevity. *Trends Endocrinol Metab* 2009; **20**: 325-331 [PMID: 19713122 DOI: 10.1016/j.tem.2009.03.008]
- 58 **Fulco M**, Cen Y, Zhao P, Hoffman EP, McBurney MW, Sauve



- AA, Sartorelli V. Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Namp1. *Dev Cell* 2008; **14**: 661-673 [PMID: 18477450 DOI: 10.1016/j.devcel.2008.02.004]
- 59 **Maiese K**, Chong ZZ, Shang YC, Hou J. Novel avenues of drug discovery and biomarkers for diabetes mellitus. *J Clin Pharmacol* 2011; **51**: 128-152 [PMID: 20220043 DOI: 10.1177/0091270010362904]
- 60 **Herranz D**, Serrano M. SIRT1: recent lessons from mouse models. *Nat Rev Cancer* 2010; **10**: 819-823 [PMID: 21102633 DOI: 10.1038/nrc2962]
- 61 **Chong ZZ**, Shang YC, Wang S, Maiese K. Shedding new light on neurodegenerative diseases through the mammalian target of rapamycin. *Prog Neurobiol* 2012; **99**: 128-148 [PMID: 22980037 DOI: 10.1016/j.pneurobio.2012.08.001]
- 62 **Maiese K**, Chong ZZ, Shang YC, Wang S. Targeting disease through novel pathways of apoptosis and autophagy. *Expert Opin Ther Targets* 2012; **16**: 1203-1214 [PMID: 22924465 DOI: 10.1517/14728222.2012.719499]
- 63 **Shah N**, Morsi Y, Manasseh R. From mechanical stimulation to biological pathways in the regulation of stem cell fate. *Cell Biochem Funct* 2014; **32**: 309-325 [PMID: 24574137 DOI: 10.1002/cbf.3027]
- 64 **Chong ZZ**, Li F, Maiese K. Oxidative stress in the brain: novel cellular targets that govern survival during neurodegenerative disease. *Prog Neurobiol* 2005; **75**: 207-246 [PMID: 15882775]
- 65 **Fong Y**, Lin YC, Wu CY, Wang HM, Lin LL, Chou HL, Teng YN, Yuan SS, Chiu CC. The antiproliferative and apoptotic effects of sirtinol, a sirtuin inhibitor on human lung cancer cells by modulating Akt/ $\beta$ -catenin-Foxo3a axis. *ScientificWorldJournal* 2014; **2014**: 937051 [PMID: 25184156 DOI: 10.1155/2014/937051]
- 66 **Wang T**, Cui H, Ma N, Jiang Y. Nicotinamide-mediated inhibition of SIRT1 deacetylase is associated with the viability of cancer cells exposed to antitumor agents and apoptosis. *Oncol Lett* 2013; **6**: 600-604 [PMID: 24137378 DOI: 10.3892/ol.2013.1400]
- 67 **Du G**, Song Y, Zhang T, Ma L, Bian N, Chen X, Feng J, Chang Q, Li Z. Simvastatin attenuates TNF- $\alpha$ -induced apoptosis in endothelial progenitor cells via the upregulation of SIRT1. *Int J Mol Med* 2014; **34**: 177-182 [PMID: 24718722 DOI: 10.3892/ijmm.2014.1740]
- 68 **Saini A**, Al-Shanti N, Sharples AP, Stewart CE. Sirtuin 1 regulates skeletal myoblast survival and enhances differentiation in the presence of resveratrol. *Exp Physiol* 2012; **97**: 400-418 [PMID: 22125309 DOI: 10.1113/expphysiol.2011.061028]
- 69 **Chiara B**, Ilaria C, Antonietta C, Francesca C, Marco M, Lucia A, Gilda C. SIRT1 inhibition affects angiogenic properties of human MSCs. *Biomed Res Int* 2014; **2014**: 783459 [PMID: 25243179 DOI: 10.1155/2014/783459]
- 70 **Xiong H**, Dai M, Ou Y, Pang J, Yang H, Huang Q, Chen S, Zhang Z, Xu Y, Cai Y, Liang M, Zhang X, Lai L, Zheng Y. SIRT1 expression in the cochlea and auditory cortex of a mouse model of age-related hearing loss. *Exp Gerontol* 2014; **51**: 8-14 [PMID: 24365660 DOI: 10.1016/j.exger.2013.12.006]
- 71 **Paschalaki KE**, Starke RD, Hu Y, Mercado N, Margariti A, Gorgoulis VG, Randi AM, Barnes PJ. Dysfunction of endothelial progenitor cells from smokers and chronic obstructive pulmonary disease patients due to increased DNA damage and senescence. *Stem Cells* 2013; **31**: 2813-2826 [PMID: 23897750 DOI: 10.1002/stem.1488]
- 72 **Chen CJ**, Yu W, Fu YC, Wang X, Li JL, Wang W. Resveratrol protects cardiomyocytes from hypoxia-induced apoptosis through the SIRT1-FoxO1 pathway. *Biochem Biophys Res Commun* 2009; **378**: 389-393 [PMID: 19059213]
- 73 **Wang W**, Yan C, Zhang J, Lin R, Lin Q, Yang L, Ren F, Zhang J, Ji M, Li Y. SIRT1 inhibits TNF- $\alpha$ -induced apoptosis of vascular adventitial fibroblasts partly through the deacetylation of FoxO1. *Apoptosis* 2013; **18**: 689-701 [PMID: 23479127 DOI: 10.1007/s10495-013-0833-7]
- 74 **Chae HD**, Broxmeyer HE. SIRT1 deficiency downregulates PTEN/JNK/FOXO1 pathway to block reactive oxygen species-induced apoptosis in mouse embryonic stem cells. *Stem Cells Dev* 2011; **20**: 1277-1285 [PMID: 21083429 DOI: 10.1089/scd.2010.0465]
- 75 **Ou X**, Lee MR, Huang X, Messina-Graham S, Broxmeyer HE. SIRT1 positively regulates autophagy and mitochondria function in embryonic stem cells under oxidative stress. *Stem Cells* 2014; **32**: 1183-1194 [PMID: 24449278 DOI: 10.1002/stem.1641]
- 76 **Guo W**, Qian L, Zhang J, Zhang W, Morrison A, Hayes P, Wilson S, Chen T, Zhao J. Sirt1 overexpression in neurons promotes neurite outgrowth and cell survival through inhibition of the mTOR signaling. *J Neurosci Res* 2011; **89**: 1723-1736 [PMID: 21826702 DOI: 10.1002/jnr.22725]
- 77 **Zhang S**, Cai G, Fu B, Feng Z, Ding R, Bai X, Liu W, Zhuo L, Sun L, Liu F, Chen X. SIRT1 is required for the effects of rapamycin on high glucose-inducing mesangial cells senescence. *Mech Ageing Dev* 2012; **133**: 387-400 [PMID: 22561310 DOI: 10.1016/j.mad.2012.04.005]
- 78 **Hong EH**, Lee SJ, Kim JS, Lee KH, Um HD, Kim JH, Kim SJ, Kim JJ, Hwang SG. Ionizing radiation induces cellular senescence of articular chondrocytes via negative regulation of SIRT1 by p38 kinase. *J Biol Chem* 2010; **285**: 1283-1295 [PMID: 19887452 DOI: 10.1074/jbc.M109.058628]
- 79 **Gao Z**, Zhang J, Kheterpal I, Kennedy N, Davis RJ, Ye J. Sirtuin 1 (SIRT1) protein degradation in response to persistent c-Jun N-terminal kinase 1 (JNK1) activation contributes to hepatic steatosis in obesity. *J Biol Chem* 2011; **286**: 22227-22234 [PMID: 21540183 DOI: 10.1074/jbc.M111.228874]
- 80 **Kozako T**, Aikawa A, Shoji T, Fujimoto T, Yoshimitsu M, Shirasawa S, Tanaka H, Honda S, Shimeno H, Arima N, Soeda S. High expression of the longevity gene product SIRT1 and apoptosis induction by sirtinol in adult T-cell leukemia cells. *Int J Cancer* 2012; **131**: 2044-2055 [PMID: 22322739 DOI: 10.1002/ijc.27481]
- 81 **Balaiya S**, Ferguson LR, Chalam KV. Evaluation of sirtuin role in neuroprotection of retinal ganglion cells in hypoxia. *Invest Ophthalmol Vis Sci* 2012; **53**: 4315-4322 [PMID: 22669716 DOI: 10.1167/iovs.11-9259]
- 82 **Lai CS**, Tsai ML, Badmaev V, Jimenez M, Ho CT, Pan MH. Xanthigen suppresses preadipocyte differentiation and adipogenesis through down-regulation of PPAR $\gamma$  and C/EBPs and modulation of SIRT-1, AMPK, and FoxO pathways. *J Agric Food Chem* 2012; **60**: 1094-1101 [PMID: 22224971 DOI: 10.1021/jf204862d]
- 83 **Dong L**, Zhou S, Yang X, Chen Q, He Y, Huang W. Magnolol protects against oxidative stress-mediated neural cell damage by modulating mitochondrial dysfunction and PI3K/Akt signaling. *J Mol Neurosci* 2013; **50**: 469-481 [PMID: 23404573 DOI: 10.1007/s12031-013-9964-0]
- 84 **Qi XF**, Li YJ, Chen ZY, Kim SK, Lee KJ, Cai DQ. Involvement of the FoxO3a pathway in the ischemia/reperfusion injury of cardiac microvascular endothelial cells. *Exp Mol Pathol* 2013; **95**: 242-247 [PMID: 23948278 DOI: 10.1016/j.yexmp.2013.08.003]
- 85 **Yang Y**, Su Y, Wang D, Chen Y, Wu T, Li G, Sun X, Cui L. Tanshinol attenuates the deleterious effects of oxidative stress on osteoblastic differentiation via Wnt/FoxO3a signaling. *Oxid Med Cell Longev* 2013; **2013**: 351895 [PMID: 24489983 DOI: 10.1155/2013/351895]
- 86 **Maiese K**. Therapeutic targets for cancer: current concepts with PI 3-K, Akt, & mTOR. *Indian J Med Res* 2013; **137**: 243-246 [PMID: 23563366]
- 87 **Jang SY**, Kang HT, Hwang ES. Nicotinamide-induced mitophagy: event mediated by high NAD<sup>+</sup>/NADH ratio and SIRT1 protein activation. *J Biol Chem* 2012; **287**: 19304-19314 [PMID: 22493485 DOI: 10.1074/jbc.M112.363747]
- 88 **Fang EF**, Scheibye-Knudsen M, Brace LE, Kassahun H, SenGupta T, Nilsen H, Mitchell JR, Croteau DL, Bohr VA. Defective mitophagy in XPA via PARP-1 hyperactivation and NAD(+)/SIRT1 reduction. *Cell* 2014; **157**: 882-896 [PMID: 24813611 DOI: 10.1016/j.cell.2014.03.026]
- 89 **Jin X**, Chen M, Yi L, Chang H, Zhang T, Wang L, Ma W, Peng X, Zhou Y, Mi M. Delphinidin-3-glucoside protects human umbilical vein endothelial cells against oxidized low-density lipoprotein-induced injury by autophagy upregulation via the AMPK/SIRT1 signaling pathway. *Mol Nutr Food Res* 2014; **58**: 1941-1951 [PMID: 24489983 DOI: 10.1155/2013/351895]



- 25047736 DOI: 10.1002/mnfr.201400161]
- 90 **Min JJ**, Huo XL, Xiang LY, Qin YQ, Chai KQ, Wu B, Jin L, Wang XT. Protective effect of DI-3n-butylphthalide on learning and memory impairment induced by chronic intermittent hypoxia-hypercapnia exposure. *Sci Rep* 2014; **4**: 5555 [PMID: 24990154 DOI: 10.1038/srep05555]
- 91 **Chun P**. Role of sirtuins in chronic obstructive pulmonary disease. *Arch Pharm Res* 2015; **38**: 1-10 [PMID: 25304127 DOI: 10.1007/s12272-014-0494-2]
- 92 **Shi J**, Yin N, Xuan LL, Yao CS, Meng AM, Hou Q. Vam3, a derivative of resveratrol, attenuates cigarette smoke-induced autophagy. *Acta Pharmacol Sin* 2012; **33**: 888-896 [PMID: 22705731 DOI: 10.1038/aps.2012.73]
- 93 **Mazzocchi G**, Tevy MF, Borghesan M, Delle Vergini MR, Vinciguerra M. Caloric restriction and aging stem cells: the stick and the carrot? *Exp Gerontol* 2014; **50**: 137-148 [PMID: 24211426 DOI: 10.1016/j.exger.2013.10.014]
- 94 **Tang AH**, Rando TA. Induction of autophagy supports the bioenergetic demands of quiescent muscle stem cell activation. *EMBO J* 2014; **33**: 2782-2797 [PMID: 25316028 DOI: 10.15252/embj.201488278]
- 95 **Chen J**, Xavier S, Moskowitz-Kassai E, Chen R, Lu CY, Sanduski K, Špes A, Turk B, Goligorsky MS. Cathepsin cleavage of sirtuin 1 in endothelial progenitor cells mediates stress-induced premature senescence. *Am J Pathol* 2012; **180**: 973-983 [PMID: 22234173 DOI: 10.1016/j.ajpath.2011.11.033]
- 96 **Passariello CL**, Zini M, Nassi PA, Pignatti C, Stefanelli C. Upregulation of SIRT1 deacetylase in phenylephrine-treated cardiomyoblasts. *Biochem Biophys Res Commun* 2011; **407**: 512-516 [PMID: 21414296 DOI: 10.1016/j.bbrc.2011.03.049]
- 97 **Lemarié CA**, Shbat L, Marchesi C, Angulo OJ, Deschênes ME, Blostein MD, Paradis P, Schiffrin EL. Mthfr deficiency induces endothelial progenitor cell senescence via uncoupling of eNOS and downregulation of SIRT1. *Am J Physiol Heart Circ Physiol* 2011; **300**: H745-H753 [PMID: 21169404 DOI: 10.1152/ajpheart.00321.2010]
- 98 **Liu X**, Chen H, Zhu W, Chen H, Hu X, Jiang Z, Xu Y, Zhou Y, Wang K, Wang L, Chen P, Hu H, Wang C, Zhang N, Ma Q, Huang M, Hu D, Zhang L, Wu R, Wang Y, Xu Q, Yu H, Wang J. Transplantation of SIRT1-engineered aged mesenchymal stem cells improves cardiac function in a rat myocardial infarction model. *J Heart Lung Transplant* 2014; **33**: 1083-1092 [PMID: 25034794 DOI: 10.1016/j.healun.2014.05.008]
- 99 **Liang SX**, Phillips WD. Migration of resident cardiac stem cells in myocardial infarction. *Anat Rec (Hoboken)* 2013; **296**: 184-191 [PMID: 23225361 DOI: 10.1002/ar.22633]
- 100 **Choudhery MS**, Khan M, Mahmood R, Mohsin S, Akhtar S, Ali F, Khan SN, Riazuddin S. Mesenchymal stem cells conditioned with glucose depletion augments their ability to repair-infarcted myocardium. *J Cell Mol Med* 2012; **16**: 2518-2529 [PMID: 22435530 DOI: 10.1111/j.1582-4934.2012.01568.x]
- 101 **Liu DJ**, Hammer D, Komlos D, Chen KY, Firestein BL, Liu AY. SIRT1 knockdown promotes neural differentiation and attenuates the heat shock response. *J Cell Physiol* 2014; **229**: 1224-1235 [PMID: 24435709 DOI: 10.1002/jcp.24556]
- 102 **Zhang Y**, Wang J, Chen G, Fan D, Deng M. Inhibition of Sirt1 promotes neural progenitors toward motoneuron differentiation from human embryonic stem cells. *Biochem Biophys Res Commun* 2011; **404**: 610-614 [PMID: 21144831 DOI: 10.1016/j.bbrc.2010.12.014]
- 103 **Saharan S**, Jhaveri DJ, Bartlett PF. SIRT1 regulates the neurogenic potential of neural precursors in the adult subventricular zone and hippocampus. *J Neurosci Res* 2013; **91**: 642-659 [PMID: 23404532 DOI: 10.1002/jnr.23199]
- 104 **Tiberi L**, van den Ameel J, Dimidschstein J, Piccirilli J, Gall D, Herpoel A, Bilheu A, Bonnefont J, Iacovino M, Kyba M, Bouschet T, Vanderhaeghen P. BCL6 controls neurogenesis through Sirt1-dependent epigenetic repression of selective Notch targets. *Nat Neurosci* 2012; **15**: 1627-1635 [PMID: 23160044 DOI: 10.1038/nn.3264]
- 105 **Aranha MM**, Santos DM, Solá S, Steer CJ, Rodrigues CM. miR-34a regulates mouse neural stem cell differentiation. *PLoS One* 2011; **6**: e21396 [PMID: 21857907 DOI: 10.1371/journal.pone.0021396]
- 106 **Li L**, Osdal T, Ho Y, Chun S, McDonald T, Agarwal P, Lin A, Chu S, Qi J, Li L, Hsieh YT, Dos Santos C, Yuan H, Ha TQ, Popa M, Hovland R, Bruserud O, Gjertsen BT, Kuo YH, Chen W, Lain S, McCormack E, Bhatia R. SIRT1 activation by a c-MYC oncogenic network promotes the maintenance and drug resistance of human FLT3-ITD acute Myeloid Leukemia stem cells. *Cell Stem Cell* 2014; **15**: 431-446 [PMID: 25280219 DOI: 10.1016/j.stem.2014.08.001]
- 107 **Lin L**, Zheng X, Qiu C, Dongol S, Lv Q, Jiang J, Kong B, Wang C. SIRT1 promotes endometrial tumor growth by targeting SREBP1 and lipogenesis. *Oncol Rep* 2014; **32**: 2831-2835 [PMID: 25270091 DOI: 10.3892/or.2014.3521]
- 108 **Lee JS**, Park JR, Kwon OS, Lee TH, Nakano I, Miyoshi H, Chun KH, Park MJ, Lee HJ, Kim SU, Cha HJ. SIRT1 is required for oncogenic transformation of neural stem cells and for the survival of "cancer cells with neural stemness" in a p53-dependent manner. *Neuro Oncol* 2015; **17**: 95-106 [PMID: 25096191 DOI: 10.1093/neuonc/nou145]
- 109 **Mao B**, Hu F, Cheng J, Wang P, Xu M, Yuan F, Meng S, Wang Y, Yuan Z, Bi W. SIRT1 regulates YAP2-mediated cell proliferation and chemoresistance in hepatocellular carcinoma. *Oncogene* 2014; **33**: 1468-1474 [PMID: 23542177 DOI: 10.1038/onc.2013.88]
- 110 **Ziebarth AJ**, Newshean S, Steg AD, Shah MM, Katre AA, Dobbin ZC, Han HD, Lopez-Berestein G, Sood AK, Conner M, Yang ES, Landen CN. Endoglin (CD105) contributes to platinum resistance and is a target for tumor-specific therapy in epithelial ovarian cancer. *Clin Cancer Res* 2013; **19**: 170-182 [PMID: 23147994 DOI: 10.1158/1078-0432.ccr-12-1045]
- 111 **Li L**, Wang L, Li L, Wang Z, Ho Y, McDonald T, Holyoake TL, Chen W, Bhatia R. Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. *Cancer Cell* 2012; **21**: 266-281 [PMID: 22340598 DOI: 10.1016/j.ccr.2011.12.020]
- 112 **Elangovan S**, Ramachandran S, Venkatesan N, Ananth S, Gnana-Prakasam JP, Martin PM, Browning DD, Schoenlein PV, Prasad PD, Ganapathy V, Thangaraju M. SIRT1 is essential for oncogenic signaling by estrogen/estrogen receptor  $\alpha$  in breast cancer. *Cancer Res* 2011; **71**: 6654-6664 [PMID: 21920899 DOI: 10.1158/0008-5472.can-11-1446]

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## Substrates for clinical applicability of stem cells

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as a type of substrate that can bring the benefits of regenerative medicine to clinical settings.

**Key words:** Human pluripotent stem cells; Extracellular matrix protein; Synthetic substrate; Peptide; Polymer; Scaffold; Hydrogel

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**Core tip:** This review article highlights numerous extracellular matrix proteins, peptide and polymer based substrates, scaffolds and hydrogels that have been pioneered for human pluripotent stem cell self-renewal for stem cell-based therapy. The benefits and shortcomings of these substrates as well as future direction that can bring the benefits of regenerative medicine to clinical settings are discussed.

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### Abstract

The capability of human pluripotent stem cells (hPSCs) to differentiate into a variety of cells in the human body holds great promise for regenerative medicine. Many substrates exist on which hPSCs can be self-renewed, maintained and expanded to further the goal of clinical application of stem cells. In this review, we highlight numerous extracellular matrix proteins, peptide and polymer based substrates, scaffolds and hydrogels that have been pioneered. We discuss their benefits and shortcomings and offer future directions as well as emphasize commercially available synthetic peptides

### INTRODUCTION

Ever since the derivation of human embryonic stem cells (hESCs) from the inner mass of the blastocyst<sup>[1]</sup>, great effort has been placed on extending the benefits of these pluripotent cells to regenerative medicine<sup>[2,3]</sup>. However, human pluripotent stem cells (hPSCs) which include human induced pluripotent stem cells (hiPSCs) and hESCs, have traditionally been cultured on mouse fibroblast feeder layers in the presence of animal cell-conditioned media or on Matrigel that pose inherent risk of pathogenic contamination<sup>[4]</sup> as well as the presence of non-human immunogenic epitopes such as N-glycolylneuraminic acid (Neu5Gc)<sup>[5]</sup>. Consequently, research has focused on developing xeno-free, chemically defined media and substrates that are



compliant with current good manufacturing practice (cGMP), scalable, maintain high degree of hPSC purity, colony homogeneity and pluripotency. Here, we review substrates that have been developed for maintenance of hPSCs for clinical applications.

## EXTRACELLULAR MATRIX PROTEINS

Feeder free cultures for hPSC expansion rely on Matrigel which constitutes the basement membrane components derived from mouse Engelbreth-Holm Swarm (EHS) tumor. The EHS tumor can easily yield a hundred grams of basement membrane components that are abundantly found in Matrigel such as collagen IV, laminin, heparin sulfate proteoglycans and nidogen/entactin<sup>[6]</sup>. In addition, minor components can also be found in Matrigel which include proteases such as 72 kDa matrix metalloproteinase-2, 92 kDa matrix metalloproteinase-9, urokinase, tissue-type plasminogen activator as well as growth factors, like transforming growth factor beta, fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, insulin-like growth factors, and proteins (Amylase, Transferrin, Clusterin) in an undefined composition. Matrigel has been used as a substrate to study the long-term stability (40 passages) of hESCs<sup>[7]</sup> as well as to derive and maintain new hESC lines for over 70 passages<sup>[8]</sup>. Other studies have used Matrigel as an extracellular matrix (ECM) to induce hepatic differentiation of hESCs using hepatic growth hormone and activin A<sup>[9]</sup>, to expand hESCs on Matrigel-coated cellulose microcarriers in 3D suspension cultures<sup>[10]</sup> and to study the effects of bone morphogenetic protein 4 on hESCs differentiation<sup>[11]</sup>. As Table 1 highlights, the batch-to-batch variability and xenogeneic-origin makes Matrigel unsuitable for quality control or cGMP production of hPSCs. Consequently, various ECM protein components have been sought as alternatives to Matrigel.

### Vitronectin

The N-terminal of the 75 kDa vitronectin protein consists of the binding sites for integrins *via* the Arg-Gly-Asp RGD sequence, which allows cell-vitronectin interaction<sup>[12]</sup>. Braam *et al*<sup>[13]</sup> have shown that recombinant vitronectin is as effective as plasma purified vitronectin for hESC expansion and mediates cell attachment *via* the  $\alpha$ V $\beta$ 5 integrins. In mTeSR1 medium, recombinant vitronectin supported several hESC lines for five passages while maintaining normal morphology, karyotype, differentiation potential and cell surface markers for stemness. However, other ECM proteins such as laminin, collagen I, collagen IV and fibronectin were only able to support cell growth in mouse embryonic fibroblast-conditioned media (MEF-CM)<sup>[13]</sup>. The use of mouse myeloma cell line NS0 to obtain the recombinant vitronectin holds potential risk of sialic acid contamination but others have been

able to produce recombinant vitronectin in *Escherichia colivia* plasmid vectors<sup>[14]</sup>. Prowse *et al*<sup>[14]</sup> obtained recombinant somatomedin B (SMB) domain along with the RGD sequence of vitronectin (rVN SMB) and anchored the substrate to tissue culture plate through vitronectin's polyhistidine linker sequence.

Recently, a fully-defined, serum- and feeder-free medium called StemPro was developed for hPSC expansion and differentiation. The hESCs grown in an ascorbate (vitamin C) free StemPro media for over 10 passages showed similar binding affinity to another ECM protein purified from EHS tumor (called Geltrex) and pluripotency markers comparable to cultures grown on whole purified vitronectin and Geltrex<sup>[14]</sup>. The surface density of human purified plasma vitronectin necessary for long term (> 30 passages) hESCs attachment, proliferation and differentiation was determined by Yap *et al*<sup>[15]</sup> to be 250 ng/cm<sup>2</sup>. However, the hESCs cultured on purified plasma vitronectin in mTeSR media showed slower (35 h) doubling time than Matrigel (25 h) but expressed normal pluripotency markers and karyotype<sup>[15]</sup>. Another study examined the scale up potential of vitronectin and laminin-coated three dimensional (3D) substrates from traditional two dimensional (2D) tissue culture polystyrenes (TCPs) to 3D polystyrene microcarriers (MCs)<sup>[16]</sup>. In this study, vitronectin was adsorbed onto TCPs and reached a surface density saturation of 510 ng/cm<sup>2</sup> compared with laminin at 850 ng/cm<sup>2</sup>. Coating vitronectin and laminin to MCs allowed for a surface density saturation of 450 and 650 ng/cm<sup>2</sup>, respectively. Even though, the two extracellular matrices showed similar adhesion and growth kinetics, the scale up to 3D culture resulted in a slower growth kinetics on the polystyrene MCs. This was explained by the hydrophobicity of MCs as well as the formation of large compact cell aggregates which deprive essential nourishments to the cells that are located in the center.

### Laminins

Laminin provides adequate growth for hESCs *via* the integrin  $\alpha$ 6 $\beta$ 1 laminin specific receptor since it is the major type of integrin present on hESCs<sup>[17,18]</sup>. The effect of various recombinant human laminin (rhLM) isoforms on hESC attachment and proliferation has been investigated. Miyazaki *et al*<sup>[18]</sup> showed that hESC line KhES-1 cells adhere markedly to rhLM-332 as well as to rhLM-511 and rhLM-111 (least adherence) when compared to non-adherence on rhLM-211 and rhLM-411. In the study, KhES-3 cells were able to adhere to rhLM-211 and rhLM-411 but their growth rate was markedly lower on rhLM-332, rhLM-511 and rhLM-111<sup>[18]</sup>. The primary reason for such a marked difference in adherence and growth is because rhLM-211 and rhLM-411 possess affinity for  $\alpha$ 3 $\beta$ 1 and  $\alpha$ 7 $\beta$ 1 which are laminin-binding integrin isoforms that are expressed on hESCs in a relatively low amount compared to  $\alpha$ 6 $\beta$ 1 integrins<sup>[18]</sup>. This receptor specificity



**Table 1** Benefits and shortcomings of Matrigel, extracellular matrix proteins, synthetic peptides, synthetic polymers and hydrogels

Substrate	Advantages	Disadvantages
Matrigel	Allows feeder-free cell culture Inexpensive Long-term hESCs culture <sup>[7,8]</sup>	Xenogeneic origin <sup>[6]</sup> Undefined components <sup>[6]</sup> Pathogenic contamination risk <sup>[4]</sup> Neu5Gc immunogenic epitope <sup>[5]</sup> Batch-to-batch variability <sup>[6]</sup> Batch-to-batch variability
ECM proteins	See subsections below	Degradation upon sterilization
Vitronectin		Pathogenic contamination risk
rhLM-332		Not-Scalable <sup>[30]</sup>
LM-E8		High production cost <sup>[30]</sup>
rh E-cadherins-Fc protein		Immunogenicity risk <sup>[17]</sup>
Fibronectin		Degradation upon sterilization
Vitronectin	Long-term hESC culture (> 30 passages) <sup>[15]</sup> $\alpha$ V $\beta$ 5 integrin receptor mediated cell attachment <sup>[13]</sup>	Not-Scalable <sup>[16,30]</sup> High production cost <sup>[30]</sup>
rhLM-332	High $\alpha$ 6 $\beta$ 1 integrin affinity <sup>[18]</sup>	
LM-E8	Smaller, easily purified, higher purity <i>vs</i> 780 ku laminins <sup>[20]</sup> Better stem cell adhesion than Matrigel and intact laminins <sup>[20]</sup> ROCK inhibitor Y-27632 not needed <sup>[20]</sup>	Not-Scalable <sup>[30]</sup> High production cost <sup>[30]</sup>
rh E-cadherins-Fc protein	hESC self-renewal, maintenance and pluripotency comparable to Matrigel <sup>TM</sup> <sup>[26]</sup>	Low cell adherence <i>vs</i> Matrigel <sup>[26]</sup>
Synthetic peptides	No batch-to-batch variation <sup>[36]</sup> Immunogenicity risk avoided <sup>[37]</sup> Since chemically synthesized Long-term hESCs culture <sup>[32,37,44]</sup>	High production costs <sup>[47,48]</sup> Sterilization difficulties <sup>[47]</sup> Easily degradable <sup>[47]</sup> Labor intensive cell passaging Limited scale-up potential of 2D platform <sup>[51]</sup>
Synthemax surface	Gamma irradiation sterilization <sup>[39]</sup> 2 yr shelf-life <sup>[39]</sup> hESCs cryopreserved and thawed on substrate <sup>[38]</sup> Scalable <sup>[38]</sup> Long-term hESCs culture <sup>[38-40]</sup>	
Synthetic polymers	Inexpensive <sup>[45,47]</sup>	Limited scale-up potential of 2D platform
PMVE-alt-MA	Easy and rapid fabrication <sup>[45,49]</sup>	
PMEDSAH	Highly manipulable <sup>[47]</sup>	
APMAAm	Long-term substrate stability <sup>[46]</sup>	
Polyacrylates		
Chitosan-alginate polymers		
(pDTEc) polymer scaffolds		
Hydrogels	<i>In-vivo</i> 3D type environment <sup>[58]</sup> Thermoresponsive and pH sensitive properties <sup>[54,55,58]</sup>	Difficult to analyze cells embedded in hydrogels
(AETMA-Cl)-DEAEA based		Enzymatic release of cells from hydrogel <sup>[57]</sup>
PDEAAm-based		
HA-based		
Alginate-collagen based		
PEG-based		
PPP-based		

hESC: Human embryonic stem cell; ECM: Extracellular matrix; LM: laminins; PMVE-alt-MA : Poly(methyl vinyl ether-alt-maleic anhydride); PMEDSAH: poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]; APMAAm: Aminopropyl methacrylamide; pDTEc: Poly(desaminotyrosyl tyrosine ethyl ester carbonate); AETMA-Cl: 2-(acryloyloxyethyl) trimethylammonium chloride; HA: Hyaluronic acid; PDEAAm: Poly(N,N-diethylacrylamide); PEG: Polyethylene glycol; PPP: Platelet poor plasma; 2D: Two dimensional.

is further established by the fact that although laminin-332 is not expressed on hESCs, rhLM-332 was an adequate substrate for hESCs adherence and proliferation due to its affinity for  $\alpha$ 6 $\beta$ 1 integrins<sup>[18]</sup>.

Since laminin isoform-511/-521 are the major laminins expressed on hESCs, another study investigated hESC self-renewal of cell lines HS420, HS207 and HS401 on a rhLM-511 substrate in the mTeSR1 variant (O3) and xeno-free TeSR1 variant (H3) media<sup>[19]</sup>. The cells proliferated for 28 passages and normal karyotype was observed after 20 passages while exhibiting OCT4, SOX2 and Nanog pluripotency markers at a higher level than Matrigel and comparable

to those grown on feeder cells. Importantly, rhLM-511 affinity of the cells was higher than cell-cell adhesion as demonstrated by cell spreading when the cells were passaged as individual clumps and this highlights the role of rhLM-511 in cell motility.

Further refinement to laminin-coated substrates has been achieved by using laminin E8 fragments (LM-E8) that are composed of the  $\alpha$ ,  $\beta$  and  $\gamma$  chains' C-terminal regions<sup>[20]</sup>. These truncated proteins are smaller, easily purified in a short time period and yield a higher purity compared with the 780 kDa LMs. Miyazaki *et al.*<sup>[20]</sup> used LM-511-E8 and LM 332-E8 to achieve greater hESC and hiPSC adhesion than Matrigel<sup>TM</sup> and intact LM-511



and 332 in a TeSR2 medium. The three hESC lines and two hiPSC lines cultured on LM-E8-coated substrates underwent proliferation for 30 and 10 passages, respectively, where disassociated cells attached strongly to LM-E8 substrates as compared to weak adhesion on Matrigel, fibronectin and vitronectin substrates<sup>[20]</sup>. Importantly, LM-E8 substrates did not require the use of ROCK inhibitor  $\gamma$ -27632 which is needed for some recombinant protein-based substrates.

### **E-cadherin**

E-cadherin, which is a transmembrane glycoprotein is involved in calcium-dependent cell-cell adhesion in epithelial as well as embryonic stem cells<sup>[21]</sup>. It is essential for the maintenance of pluripotent state of stem cells due to their critical role in cell-cell adhesions<sup>[22]</sup> as well as cell survival and renewal<sup>[23]</sup>. This E-cadherin mediated pluripotency is quickly lost, as measured by the levels of Oct4, Nanog and Sox2, when siRNA is used to suppress E-cadherin expression on cells<sup>[24]</sup>. This in turn causes a decrease in hESC proliferation<sup>[25]</sup> and emphasizes the critical role that E-cadherins play in cell-cell adhesion, survival, renewal, proliferation and pluripotency. Nagaoka *et al*<sup>[26]</sup> used recombinant E-cadherin-Fc-coated culture dishes (rhE-cad-Fc) where E-cadherins are fused with an IgG-Fc domain to coat tissue culture plates on which hESCs were examined for their pluripotency and propagation characteristics. The study demonstrated that both hESCs and hiPSCs that were cultured on rhE-cad-Fc protein-coated surface for > 13 passages in mTeSR1 medium were indistinguishable from those that were cultured on Matrigel as far as the rate of proliferation, cell morphology, maintenance of pluripotency and the ability to differentiate into multiple cell types were concerned. However, the ability of cells to adhere was decreased as a result of protease digestion cocktails using Accutase. This problem can be circumvented by using enzyme-free Cell Dissociation Buffer for passaging the cells. Therefore, rhE-cad-Fc can be considered superior to Matrigel as a substrate because of the use of more defined chemical conditions that yield similar results for hESC self-renewal, maintenance and pluripotency. E-cadherins have also been linked with matrix rigidity where hESCs on rigid substrates were shown to co-express E-cadherins and were OCT4<sup>+</sup>. In contrast, the softer substrates had large hESC aggregates that had an increased tendency to differentiate. Furthermore, hESCs on rigid substrates exhibit increased cytoskeleton contractility related to E-cadherin expression among cells<sup>[27]</sup>.

### **Fibronectin**

The 220 kDa fibronectin dimer interacts with hESCs *via* the  $\alpha$ 5 $\beta$ 1 integrin receptor such that only 25 percent of adsorbed fibronectin is needed for hESC renewal<sup>[28]</sup>. However, the 120 kDa central-binding domain of fibronectin is necessary for attachment

and maintenance of hESCs pluripotency. Using a high throughput screening of various ECM proteins, Brafman *et al*<sup>[29]</sup> discovered that high concentrations of fibronectin and laminin (500  $\mu$ g/mL) supported hESC proliferation commensurate with Matrigel in a concentration dependent manner and fibronectin, collagen I and laminin were each able to support a high degree of pluripotency.

## **SYNTHETIC PEPTIDES**

Even though, the protein based substrates such as recombinant vitronectin, laminin and rhE-cad-Fc coated tissue plates may be feeder free, they are not xeno-free and as a result pose significant immunogenic and pathogenic hazards<sup>[4]</sup>. Additionally, such substrates are not scalable and have significant manufacturing costs associated with them<sup>[30]</sup>. Being protein-based, they can also undergo degradation following common sterilization techniques. Consequently, peptide-based substrates offer better alternatives for maintaining hPSCs pluripotency, self-renewal, growth, scalability and clinical applicability.

Heparin binding peptides promote cell adhesion and spreading through their interaction with cell-surface glycosaminoglycans (GAGs) which are involved in cell-cell recognition and adhesion, cell-matrix interactions and receptor-signal complexes<sup>[31]</sup>. In a study conducted to determine the optimal peptides for hESC self-renewal, Klim *et al*<sup>[32]</sup> discovered that surfaces which display heparin binding peptide GKKQRFHRNRKG, derived from vitronectin, supported adhesion and self-renewal of hESCs at the lowest peptide surface density (0.5%) while other surface presenting various heparin binding peptides did not allow for cell attachment. These heparin binding peptides exhibit increased levels of Oct-4 and SSEA-4 expression, exhibit similar rates of growth, long term stem cell propagation (17 passages) and differentiation capabilities that are equal to Matrigel. The heparin binding peptide GKKQRFHRNRKG has been used in polyacrylamide hydrogels of varying stiffness where H9 hESCs were cultured for 12 passages in mTeSR1 medium supplemented with 10  $\mu$ mol/L ROCK inhibitor Y-27632<sup>[33]</sup>. Not only was the hESC self-renewal confirmed but it was found that GAG binding on stiffer materials conveyed mechanosensing information that allowed for F-actin polymerization and enabled cell adhesion. Additionally, the nuclear localization of transcriptional co-activators Yes-associated protein and transcriptional coactivator with PDZ-binding motif (TAZ) on stiffer matrices allowed for hESCs pluripotency<sup>[33]</sup>.

Self-assembled monolayers (SAMs) with peptide sequence TVKHPDALHPQ and LTTAPKLPKVTR have also been shown to promote hESC adhesion and proliferation for 3 passages<sup>[34]</sup>. However, non-clonal abnormalities were detected in cells cultured on TVKHPDALHPQ SAMs while LTTAPKLPKVTR SAMs



did not exhibit karyotypic abnormalities. Similarly, 18 different laminin-derived peptides were also screened and five (including RNIAEIIKDI) were capable of supporting hESCs proliferation and pluripotency comparable to Matrigel<sup>TM</sup>[35]. The presence of relatively low receptor concentration on cells requires the presentation of laminin-derived peptides at a higher concentration despite being presented in a uniform density and specific orientation.

More recently, Au-coated glasses have been modified with poly[oligo(ethylene glycol) methacrylate] polymer brushes through surface initiated polymerization and peptides that are derived from vitronectin<sup>[36]</sup>. A 0.75 mmol/L vitronectin peptide concentration is deemed necessary for hiPSC expansion for up to 10 passages in mTeSR1 medium. Using micro-contact printed polydimethylsiloxane stamp, cell adhesion was easily regulated only on the vitronectin peptide and pluripotency markers and self-renewal capabilities were observed to be similar to Matrigel. A more potent version of the RGD sequence in the form of cyclic RGD peptide has been used to modify surfaces on which hESCs can adhere more prominently than on linear RGD peptides at a 4 fold higher rate<sup>[37]</sup>. Normal pluripotency markers, karyotype and differentiation potential similar to Matrigel have been observed for cells cultured for 10 passages as well as in mTeSR1 medium.

### Peptide acrylate surfaces

Peptide acrylate surfaces (PAS) are synthetic surfaces that are composed of acrylates which are conjugated to biologically active peptides<sup>[38]</sup>. One such platform is the so called Synthemax Surface where the synthetic surface is manufactured by covalently linking the vitronectin-derived RGD sequence to the acrylate surface<sup>[39]</sup>. Melkounian *et al*<sup>[38]</sup> have used this technique to deposit various peptide sequences, as well as the vitronectin sequence, onto acrylate surfaces in a process that deposits carboxylic acid containing acrylate onto the surfaces of cultures and then conjugates amine-containing peptides to these surfaces using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide/*N*-hydroxysuccinimide. Here, bone sialoprotein-PAS and vitronectin-PAS were used to culture hESC line H7 for more than 10 passages in a defined medium. The authors concluded that the hESCs grown on PAS system were not different to the cells that were grown on Matrigel when comparing cell viability, colony morphology, doubling time and hESC marker expression of OCT4, TRA-1-60 and SSEA-4. Furthermore, hESCs can be cryopreserved and thawed onto PAS in addition to their ability to undergo directed differentiation into functional cardiomyocytes. This research demonstrated the scalability of feeder free, xeno-free and chemically defined PAS substrate that are suitable for clinical applications. Jin *et al*<sup>[40]</sup> further examined the expansion and differentiation capabilities of hiPSCs on synthemax surface. The authors concluded

that there is no significant difference between the doubling time and pluripotency marker expressions for hiPSCs cultured on Matrigel and Synthemax substrate. However, the cells cultured on the Synthemax substrate showed more compact morphology than Matrigel. The researchers were able to demonstrate definitive endoderm differentiation of hiPSCs and were able to decipher the specific binding of  $\alpha V\beta 5$  receptors for Synthemax Surface while the Matrigel substrate bound to various integrin receptors. Lastly, cell-cell interfaces on Synthemax Surface showed broad and dense actin filaments as well as the up-regulation of zyxin which demonstrates the involvement of various cytoskeletal proteins in hiPSCs attachment and proliferation.

Synthemax Surface has also been used for retinal differentiation of hiPSCs<sup>[41]</sup> and to show efficient single cell passaging of hPSCs, using ROCK inhibitor Y-27632, similar to the results obtained on LM-E8 substrates<sup>[42]</sup>. It has been used to obtain oligodendrocyte progenitor cells (OPCs) from hESCs that express higher levels of OPC specific proteoglycan, NG2, as compared to OPCs grown on xenogeneic-derived ECM<sup>[43]</sup>. The researchers concluded that Synthemax Surface can replace the xenogeneic-derived ECM for hESCs differentiation into OPCs. Recently, Synthemax Surface was used to expand hESCs for 10 passages and to obtain insulin-producing  $\beta$  cells within 21 d in a defined serum-free media<sup>[44]</sup>.

Nevertheless, the high production costs, degradation upon sterilization, labor intensive cell passaging and limited scale-up potential of 2D peptide surfaces have caused researchers to seek alternative substrates in the synthetic polymer arena.

## SYNTHETIC POLYMERS

### Poly(methyl vinyl ether-alt-maleic anhydride)

Brafman *et al*<sup>[45]</sup> used a high throughput screening approach to identify supports for hPSCs self-renewal as well as optimal conditions for pluripotency and proliferation for hPSCs. The authors examined 90 polymers with different functional groups, chemical compositions and molecular weights. They discovered that poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA) is most capable of supporting both short and long term maintenance of hESCs for HUES1 and HUES9, as well as one hiPSC line for over five passages. PMVE-alt-MA and poly(acrylic acid) cause greatest hPSC proliferation rates at discrete molecular weights of  $1.25 \times 10^6$  Da and  $4.5 \times 10^5$  Da, respectively. Of the 16 polymers, cells on PMVE-alt-MA did not exhibit detachment or differentiation. Furthermore, one hiPSC line and two HUES 1/9 lines were successfully cultured for over five passages with expression of normal pluripotency markers and differentiation capabilities and showed an increased expression of  $\alpha 5$  and  $\alpha v$  integrin receptors. Importantly, the authors showed that hPSCs can



further secrete endogenous ECM proteins in the absence of exogenous ECM proteins in defined medium thereby highlighting the role of ECM proteins in hPSC self-renewal.

#### ***Poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]***

Long term hESC growth has been demonstrated on poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] (PMEDSAH)-coated TCPs *via* surface initiated graft polymerization technique that grafts PMEDSAH onto ozone-activated surfaces of TCPs<sup>[46]</sup>. For 25 passages, H9 and BG01 cells that were grown on PMEDSAH attached, proliferated, exhibited long term growth along with normal genetic and proteomic makeup as exhibited through Nanog, OCT3/4 and SOX2 expression and possessed differentiation potential. H9 cells exhibited higher cell-aggregate adhesions when grown in human cell conditioned medium as compared to BG01 cells. However, both these cell lines showed comparable cell doubling times, pluripotency and karyotype when compared with cells grown on Matrigel<sup>[46]</sup>. Consequently, variability exists in the response of these cell lines to various media conditions. H9 hESCs grown on PMEDSAH were able to be cultured on chemically defined StemPro media for 10 passages. Additionally, hiPSCs grown on PMEDSAH have also been shown to proliferate in defined xeno-free conditions for 15 passages while retaining pluripotency markers, normal morphology and karyotype<sup>[47]</sup>. However, the hiPSC-derived mesenchymal stem cell unequally preferred osteogenic and chondrogenic differentiation rather than adipogenic lineage.

#### ***Aminopropyl methacrylamide***

Other synthetic polymer substrates such as the methacrylamide-based aminopropyl methacrylamide have been used to culture hESCs for over 20 passages in mTeSR1 media<sup>[48]</sup>. The H9-hOct4-pGZs cells showed higher expression of pluripotency markers than Matrigel as well as higher proliferation rate at passage 1 and 22 but the proliferation rate slowed down at latter passages. Importantly, bovine serum albumin (BSA) remains crucial for hESC attachment, growth and proliferation since it was shown to be adsorbed onto the APMAA surfaces in an unfolded state<sup>[48]</sup>.

#### ***Polyacrylates***

One study applied a high throughput screening approach to high-density polymer microarrays to obtain polymers with either 2-carboxyethyl acrylate or 2-(methylthio) ethyl methacrylate containing monomers that allowed cell adhesion at specific ratios<sup>[49]</sup>. Furthermore, an equal ratio of 4-tert-butylcyclohexyl acrylate and n-butyl methacrylate was found in polymers supporting greatest adhesion. The study demonstrated short term (5 passages) pluripotency potential of hESCs cultured on these surfaces.

#### ***Chitosan-alginate polymers***

As natural polymers that mimic GAG structure, chitosan-alginate (CA) 3D scaffolds have been used for hESC self-renewal<sup>[50]</sup>. The cells grown on these scaffolds exhibit three times higher alkaline phosphatase activity and the expression of pluripotency markers were similar to hESCs grown on human fibroblast feeder layers. Additionally, hESCs which are recovered *via* EDTA and K<sub>2</sub>HPO<sub>4</sub> solution express over 95% cell viability and the CA scaffolds allow for easy passaging of cells. Another study used alginate-chitin polymer based scaffold, which mimic the GAGs units of the ECM<sup>[51]</sup>. Combined conditions of high cell seeding density, avoidance of fluid shear stresses *via* encapsulation as well as increased surface area to volume ratio of encapsulated cells, and cryopreservation with high cell viability (> 75%) allows for long term culture (10 passages) and quick non-cytotoxic harvesting of cells through enzymatic disassociation of microfibers with further scalability.

#### ***Poly(desaminotyrosyl tyrosine ethyl ester carbonate) polymer scaffolds***

Synthetic scaffolds composed of poly(desaminotyrosyl tyrosine ethyl ester carbonate)<sup>[52]</sup>, a tyrosine derived polycarbonate polymer<sup>[53]</sup>, have been used to study the effects of geometries on hESCs self-renewal and proliferation. Carlson *et al.*<sup>[52]</sup> discovered that microfibrillar architecture of poly-D-lysine pretreated scaffolds supports hESC survival and colony formation as this geometry allows cell-cell and cell-matrix contact and extensive ECM deposition of laminin and collagen IV with some collagen I deposition. The endogenous production of laminin was an essential factor for hESC adhesion and survival.

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## **HYDROGELS**

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Although synthetic polymers represent a more economical approach with more quality control over the manufacturing process, the 2D topography does not mimic the *in vivo* cell-cell and cell-ECM interactions. Hydrogels, serve as viable alternatives to 2D cultures that hold the potential for clinical scale hESC production. Using acrylate and acrylamide monomers, one study used 2-(acryloyloxyethyl) trimethylammonium chloride (AETMA-Cl) and 2-(diethylamino) ethyl acrylate (DEAEA) in a 3:1 ratio to construct thermoresponsive hydrogels<sup>[54]</sup>. The undifferentiated colonies can be passaged without enzymatic disassociation by reducing the temperature from 37 °C to 15 °C for 30 min, followed by gentle pipetting since hydrogel swelling alone was not sufficient for hESC removal. The hydrogel adsorbed BSA and cells that were grown on the hydrogel demonstrated slower growth and lower total expansion rate over 5 d compared to Matrigel. Additionally, microdeletions and duplications on some chromosomes were present in both Matrigel and hydrogel culture conditions. Nonetheless, hESCs were able to be cultured



for over 20 passages in mTeSR1 medium on glass coverslips coated with the hydrogel and expressed pluripotency markers.

Another acrylamide-based Poly(N,N-diethylacrylamide) (PDEAAm) thermoresponsive hydrogel uses pentapeptide YIGSR-NH<sub>2</sub> to mimic B1 chain amino acid sequence of laminin<sup>[55]</sup>. Here, the hydrogel was further modified with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> salt to yield highly porous interconnected (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-PDEAAm hydrogels that were able to support adhesion and growth of hESCs better than large NaCl generated random pores. The polyacrylamide hydrogels can also be used to direct the differentiation to specific lineages in microwells since microwells accumulate molecules above the hydrogel cut off range of 40 to 70 kDa<sup>[56]</sup>. As a result, microwells direct stem cells to be differentiated into neural, ectodermal and endodermal lineages while relatively small mesodermal inducing factors diffuse away and large mesodermal inducing factors accumulate in the microwells.

Hyaluronic acid (HA) based hydrogels were used by Gerecht *et al.*<sup>[57]</sup> to culture hESCs in MEF-CM. The encapsulated hESCs formed colonies of varying sizes and maintained doubling time similar to 2D cultures. After 20 d, undifferentiated colony morphology was observed but a high cell seeding density between 5-10 × 10<sup>6</sup> is necessary to prevent apoptosis. The hydrogel allowed for enzymatic release (hyaluronidase) of cells that achieved cell viability of 76.5% ± 8%<sup>[57]</sup>. Additionally, Ikonen *et al.*<sup>[58]</sup> used HA with hydrophilic pH-sensitive hydrogels to demonstrate adhesion and expansion of hESC cardiomyocytes. Here, the collagen-mimicking hydrogel nanofibers allowed for cell adhesion and growth due to their hydrophilicity but HA further augmented the cell survival and provided a more structurally sound hydrogel. Furthermore, the thinnest nanofibers (4.2 nm) were supportive of hESC cardiomyocytes culture.

Clinical scale applications require microenvironments that not only self-renew hESCs but are also able to direct their differentiation. To this end, Dixon *et al.*<sup>[59]</sup> have used alginate-collagen hydrogels such that self-renewal of hESCs is sustained on alginate dominated state but differentiation can be induced by "switching" to a collagen predominant microenvironment via EDTA/sodium citrate based treatment. This process further changes the elasticity of the matrix from approximately 21.37 ± 5.37 kPa to ~4.87 ± 1.64 kPa. Moreover, the early switching (day 3) correlates with ectodermal differentiation while day 5 switching results in mesodermal and endodermal commitment. This hydrogel configuration offers advantages, namely the preservation of hydrogel structure and the relatively high hESC cell density (approximately 2 × 10<sup>7</sup>) obtained before differentiation<sup>[59]</sup>.

Polyethylene glycol (PEG)-based hydrogel functionalized with vinyl sulfone (VS) macromers with multiarms have been shown to maintain hESC self-renewal where the 8 multiarm PEG-VS hydrogel (10% PEG) proved to be ideal for hESC self-renewal and stemness expression<sup>[60]</sup>. However, the hydrogel needs to be optimized for specific

cell lines since some of the cells lines demonstrated weak stemness markers. Another PEG-based thermoresponsive substrate utilized recombinant protein factors as a poly(N-isopropylacrylamide)-co-poly(ethylene glycol) (PNIPAAm-PEG) hydrogel<sup>[61]</sup>. Here, 10<sup>72</sup> fold expansion over 60 passages was achieved in hPSCs using single cell passaging and stem cells were able to be further differentiated into dopaminergic neurons at even higher numbers.

Another novel hydrogel is derived from human platelet poor plasma (PPP) gelled in the presence of DMEM media, which contains calcium ions<sup>[62]</sup>. The resulting coagulation cascade forms a stable hydrogel. hESCs were able to be cultured for 25 passages and fibronectin was speculated to play a role in hESC adhesion via α5β1 integrins<sup>[63]</sup>. Despite being relatively inexpensive, the PPP-based hydrogel was not xeno-free due to the N2 and B27 supplements in the media. Furthermore, scalability may be an issue being that the system is donor dependent.

## CONCLUSION

Although many substrates exist for the self-renewal and expansion of hPSCs, most of the substrates possess limitations hinder their clinical applicability. As summarized in Table 1, Matrigel is xenogeneic in origin, contains undefined components and can be immunogenic. The relatively high production costs, immunogenicity risks and difficulties with sterilization<sup>[16,17,30]</sup> of ECM proteins limit their scalability potential while synthetic polymers, although being inexpensive and easily fabricated, have shown limited 3D scale-up capabilities. Hydrogels address the need for 3D *in vivo*-like environment but are not easily scalable.

Lastly, synthetic peptides are easily degradable<sup>[47]</sup> and difficulties with sterilization and high production costs exist<sup>[47,48]</sup>. Nevertheless, Synthemax Surface has shown to be capable for clinical applications since it can be sterilized via gamma irradiation, has a long shelf life of two years and can be stored at room temperature<sup>[39]</sup>. Furthermore, hESCs can be cryopreserved and thawed on substrate and studies have demonstrated its capability for long term hESCs self-renewal and maintenance<sup>[38-40]</sup>. Its scalability has also been demonstrated in T75 flasks<sup>[38]</sup>. Therefore, we conclude that Synthemax Surface is an ideal substrate for clinical applicability.

## FUTURE DIRECTION

Although feeder free, xeno-free and chemically defined media has been developed, the clinical applicability of hESCs depends on synthetic substrates that can easily and economically be manufactured, can undergo common sterilization methods without degradation (*i.e.*, reusability) and yield large number of cells as required for transplantation dosage (2 × 10<sup>8</sup> cells/kg per dose)<sup>[64]</sup>. Microcarriers and suspension cultures have



been used to meet these demands but their limitations in controlling aggregate size, passaging challenges, shear forces in stirred cultures and difficult cell extraction from microcarrier have caused researchers to seek alternatives. As a result, significant need exists for developing scalable synthetic substrates like Synthemax that can work with multiple cell lines (without conferring epigenetic modifications to cells), easily cryopreserved, are non-labor intensive (*i.e.*, automation), adaptable to induce differentiation conditions and require fewer exogenous factors to maintain hPSCs self-renewal and expansion.

## REFERENCES

- 1 **Thomson JA**, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: 9804556 DOI: 10.1126/science.282.5391.1145]
- 2 **Serra M**, Brito C, Correia C, Alves PM. Process engineering of human pluripotent stem cells for clinical application. *Trends Biotechnol* 2012; **30**: 350-359 [PMID: 22541338 DOI: 10.1016/j.tibtech.2012.03.003]
- 3 **Segers VF**, Lee RT. Stem-cell therapy for cardiac disease. *Nature* 2008; **451**: 937-942 [PMID: 18288183 DOI: 10.1038/nature06800]
- 4 **Carlson Scholz JA**, Garg R, Compton SR, Allore HG, Zeiss CJ, Uchio EM. Poliomyelitis in MuLV-infected ICR-SCID mice after injection of basement membrane matrix contaminated with lactate dehydrogenase-elevating virus. *Comp Med* 2011; **61**: 404-411 [PMID: 22330347]
- 5 **Martin MJ**, Muotri A, Gage F, Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med* 2005; **11**: 228-232 [PMID: 15685172 DOI: 10.1038/nm1181]
- 6 **Kleinman HK**, Martin GR. Matrigel: basement membrane matrix with biological activity. *Semin Cancer Biol* 2005; **15**: 378-386 [PMID: 15975825 DOI: 10.1016/j.semcancer.2005.05.004]
- 7 **Navarro-Alvarez N**, Soto-Gutierrez A, Yuasa T, Yamatsuji T, Shirakawa Y, Nagasaka T, Sun SD, Javed MS, Tanaka N, Kobayashi N. Long-term culture of Japanese human embryonic stem cells in feeder-free conditions. *Cell Transplant* 2008; **17**: 27-33 [PMID: 18468232 DOI: 10.3727/000000008783906900]
- 8 **Lagarkova MA**, Eremeev AV, Svetlakov AV, Rubtsov NB, Kiselev SL. Human embryonic stem cell lines isolation, cultivation, and characterization. *In Vitro Cell Dev Biol Anim* 2010; **46**: 284-293 [PMID: 20178000 DOI: 10.1007/s11626-010-9282-6]
- 9 **Ishii T**, Fukumitsu K, Yasuchika K, Adachi K, Kawase E, Suemori H, Nakatsuji N, Ikai I, Uemoto S. Effects of extracellular matrixes and growth factors on the hepatic differentiation of human embryonic stem cells. *Am J Physiol Gastrointest Liver Physiol* 2008; **295**: G313-G321 [PMID: 18535293 DOI: 10.1152/ajpgi.00072.2008]
- 10 **Chen AK**, Chen X, Choo AB, Reuveny S, Oh SK. Expansion of human embryonic stem cells on cellulose microcarriers. *Curr Protoc Stem Cell Biol* 2010; **Chapter 1**: Unit 1C.11 [PMID: 20814936 DOI: 10.1002/9780470151808.sc01c11s14]
- 11 **Amita M**, Adachi K, Alexenko AP, Sinha S, Schust DJ, Schulz LC, Roberts RM, Ezashi T. Complete and unidirectional conversion of human embryonic stem cells to trophoblast by BMP4. *Proc Natl Acad Sci USA* 2013; **110**: E1212-E1221 [PMID: 23493551 DOI: 10.1073/pnas.1303094110]
- 12 **Schvartz I**, Seger D, Shaltiel S. Vitronectin. *Int J Biochem Cell Biol* 1999; **31**: 539-544 [PMID: 10399314 DOI: 10.1016/S1357-2725(99)00005-9]
- 13 **Braam SR**, Zeinstra L, Litjens S, Ward-van Oostwaard D, van den Brink S, van Laake L, Lebrin F, Kats P, Hochstenbach R, Passier R, Sonnenberg A, Mummery CL. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. *Stem Cells* 2008; **26**: 2257-2265 [PMID: 18599809 DOI: 10.1634/stemcells.2008-0291]
- 14 **Prowse AB**, Doran MR, Cooper-White JJ, Chong F, Munro TP, Fitzpatrick J, Chung TL, Haylock DN, Gray PP, Wolvetang EJ. Long term culture of human embryonic stem cells on recombinant vitronectin in ascorbate free media. *Biomaterials* 2010; **31**: 8281-8288 [PMID: 20674971 DOI: 10.1016/j.biomaterials.2010.07.037]
- 15 **Yap LY**, Li J, Phang IY, Ong LT, Ow JZ, Goh JC, Nurcombe V, Hobley J, Choo AB, Oh SK, Cool SM, Birch WR. Defining a threshold surface density of vitronectin for the stable expansion of human embryonic stem cells. *Tissue Eng Part C Methods* 2011; **17**: 193-207 [PMID: 20726687 DOI: 10.1089/ten.TEC.2010.0328]
- 16 **Heng BC**, Li J, Chen AK, Reuveny S, Cool SM, Birch WR, Oh SK. Translating human embryonic stem cells from 2-dimensional to 3-dimensional cultures in a defined medium on laminin- and vitronectin-coated surfaces. *Stem Cells Dev* 2012; **21**: 1701-1715 [PMID: 22034857 DOI: 10.1089/scd.2011.0509]
- 17 **Xu C**, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 2001; **19**: 971-974 [PMID: 11581665 DOI: 10.1038/nbt1001-971]
- 18 **Miyazaki T**, Futaki S, Hasegawa K, Kawasaki M, Sanzen N, Hayashi M, Kawase E, Sekiguchi K, Nakatsuji N, Suemori H. Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells. *Biochem Biophys Res Commun* 2008; **375**: 27-32 [PMID: 18675790 DOI: 10.1016/j.bbrc.2008.07.111]
- 19 **Rodin S**, Domogatskaya A, Ström S, Hansson EM, Chien KR, Inzunza J, Hovatta O, Tryggvason K. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. *Nat Biotechnol* 2010; **28**: 611-615 [PMID: 20512123 DOI: 10.1038/nbt.1620]
- 20 **Miyazaki T**, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M, Hayashi M, Kumagai H, Nakatsuji N, Sekiguchi K, Kawase E. Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. *Nat Commun* 2012; **3**: 1236 [PMID: 23212365 DOI: 10.1038/ncomms2231]
- 21 **Oda H**, Takeichi M. Evolution: structural and functional diversity of cadherin at the adherens junction. *J Cell Biol* 2011; **193**: 1137-1146 [PMID: 21708975 DOI: 10.1083/jcb.201008173]
- 22 **Chou YF**, Chen HH, Eijpe M, Yabuuchi A, Chenoweth JG, Tesar P, Lu J, McKay RD, Geijsen N. The growth factor environment defines distinct pluripotent ground states in novel blastocyst-derived stem cells. *Cell* 2008; **135**: 449-461 [PMID: 18984157 DOI: 10.1016/j.cell.2008.08.035]
- 23 **Ohgushi M**, Matsumura M, Eiraku M, Murakami K, Aramaki T, Nishiyama A, Muguruma K, Nakano T, Suga H, Ueno M, Ishizaki T, Suemori H, Narumiya S, Niwa H, Sasai Y. Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells. *Cell Stem Cell* 2010; **7**: 225-239 [PMID: 20682448 DOI: 10.1016/j.stem.2010.06.018]
- 24 **Li D**, Zhou J, Wang L, Shin ME, Su P, Lei X, Kuang H, Guo W, Yang H, Cheng L, Tanaka TS, Leckband DE, Reynolds AB, Duan E, Wang F. Integrated biochemical and mechanical signals regulate multifaceted human embryonic stem cell functions. *J Cell Biol* 2010; **191**: 631-644 [PMID: 20974810 DOI: 10.1083/jcb.201006094]
- 25 **Eastham AM**, Spencer H, Soncin F, Ritson S, Merry CL, Stern PL, Ward CM. Epithelial-mesenchymal transition events during human embryonic stem cell differentiation. *Cancer Res* 2007; **67**: 11254-11262 [PMID: 18056451 DOI: 10.1158/0008-5472.can-07-2253]
- 26 **Nagaoka M**, Si-Tayeb K, Akaike T, Duncan SA. Culture of human pluripotent stem cells using completely defined conditions on a recombinant E-cadherin substratum. *BMC Dev Biol* 2010; **10**: 60 [PMID: 20525219 DOI: 10.1186/1471-213x-10-60]
- 27 **Sun Y**, Villa-Diaz LG, Lam RH, Chen W, Krebsbach PH, Fu J. Mechanics regulates fate decisions of human embryonic stem cells. *PLoS One* 2012; **7**: e37178 [PMID: 22615930 DOI: 10.1371/



- journal.pone.0037178]
- 28 **Kalaskar DM**, Downes JE, Murray P, Edgar DH, Williams RL. Characterization of the interface between adsorbed fibronectin and human embryonic stem cells. *J R Soc Interface* 2013; **10**: 20130139 [PMID: 23554347 DOI: 10.1098/rsif.2013.0139]
  - 29 **Brafman DA**, Shah KD, Fellner T, Chien S, Willert K. Defining long-term maintenance conditions of human embryonic stem cells with arrayed cellular microenvironment technology. *Stem Cells Dev* 2009; **18**: 1141-1154 [PMID: 19327010 DOI: 10.1089/scd.2008.0410]
  - 30 **Mahlstedt MM**, Anderson D, Sharp JS, McGilvray R, Muñoz MD, BATTERY LD, Alexander MR, Rose FR, Denning C. Maintenance of pluripotency in human embryonic stem cells cultured on a synthetic substrate in conditioned medium. *Biotechnol Bioeng* 2010; **105**: 130-140 [PMID: 19718698 DOI: 10.1002/bit.22520]
  - 31 **Chevalier F**, Lavergne M, Negroni E, Ferratge S, Carpentier G, Gilbert-Sirieix M, Siñeriz F, Uzan G, Albanese P. Glycosaminoglycan mimetic improves enrichment and cell functions of human endothelial progenitor cell colonies. *Stem Cell Res* 2014; **12**: 703-715 [PMID: 24681520 DOI: 10.1016/j.scr.2014.03.001]
  - 32 **Klim JR**, Li L, Wrighton PJ, Piekarczyk MS, Kiessling LL. A defined glycosaminoglycan-binding substratum for human pluripotent stem cells. *Nat Methods* 2010; **7**: 989-994 [PMID: 21076418 DOI: 10.1038/nmeth.1532]
  - 33 **Musah S**, Morin SA, Wrighton PJ, Zwick DB, Jin S, Kiessling LL. Glycosaminoglycan-binding hydrogels enable mechanical control of human pluripotent stem cell self-renewal. *ACS Nano* 2012; **6**: 10168-10177 [PMID: 23005914 DOI: 10.1021/nn3039148]
  - 34 **Derda R**, Musah S, Orner BP, Klim JR, Li L, Kiessling LL. High-throughput discovery of synthetic surfaces that support proliferation of pluripotent cells. *J Am Chem Soc* 2010; **132**: 1289-1295 [PMID: 20067240 DOI: 10.1021/ja906089g]
  - 35 **Derda R**, Li L, Orner BP, Lewis RL, Thomson JA, Kiessling LL. Defined substrates for human embryonic stem cell growth identified from surface arrays. *ACS Chem Biol* 2007; **2**: 347-355 [PMID: 17480050 DOI: 10.1021/cb700032u]
  - 36 **Deng Y**, Zhang X, Zhao X, Li Q, Ye Z, Li Z, Liu Y, Zhou Y, Ma H, Pan G, Pei D, Fang J, Wei S. Long-term self-renewal of human pluripotent stem cells on peptide-decorated poly(OEGMA-co-HEMA) brushes under fully defined conditions. *Acta Biomater* 2013; **9**: 8840-8850 [PMID: 23891809 DOI: 10.1016/j.actbio.2013.07.017]
  - 37 **Kolhar P**, Kotamraju VR, Hikita ST, Clegg DO, Ruoslahti E. Synthetic surfaces for human embryonic stem cell culture. *J Biotechnol* 2010; **146**: 143-146 [PMID: 20132848 DOI: 10.1016/j.jbiotec.2010.01.016]
  - 38 **Melkounian Z**, Weber JL, Weber DM, Fadeev AG, Zhou Y, Dolley-Sonneville P, Yang J, Qiu L, Priest CA, Shogbon C, Martin AW, Nelson J, West P, Beltzer JP, Pal S, Brandenberger R. Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. *Nat Biotechnol* 2010; **28**: 606-610 [PMID: 20512120 DOI: 10.1038/nbt.1629]
  - 39 **Weber JL**, Dolley-Sonneville P, Weber DM, Fadeev AG, Zhou Y, Yang J, Priest CA, Brandenberger R, Melkounian Z. Corning[reg] synthemax[trade] surface: A tool for feeder-free, xeno-free culture of human embryonic stem cells. *Nat Meth* 2010; **7**: 12
  - 40 **Jin S**, Yao H, Weber JL, Melkounian ZK, Ye K. A synthetic, xeno-free peptide surface for expansion and directed differentiation of human induced pluripotent stem cells. *PLoS One* 2012; **7**: e50880 [PMID: 23226418 DOI: 10.1371/journal.pone.0050880]
  - 41 **Tucker BA**, Anfinson KR, Mullins RF, Stone EM, Young MJ. Use of a synthetic xeno-free culture substrate for induced pluripotent stem cell induction and retinal differentiation. *Stem Cells Transl Med* 2013; **2**: 16-24 [PMID: 23283489 DOI: 10.5966/sctm.2012-0040]
  - 42 **Kawase E**. Efficient expansion of dissociated human pluripotent stem cells using a synthetic substrate. *Methods Mol Biol* 2014 May 30; Epub ahead of print [PMID: 24875248 DOI: 10.1007/978-1-4939-821-8\_2]
  - 43 **Li Y**, Gautam A, Yang J, Qiu L, Melkounian Z, Weber J, Telukuntla L, Srivastava R, Whiteley EM, Brandenberger R. Differentiation of oligodendrocyte progenitor cells from human embryonic stem cells on vitronectin-derived synthetic peptide acrylate surface. *Stem Cells Dev* 2013; **22**: 1497-1505 [PMID: 23249362 DOI: 10.1089/scd.2012.0508]
  - 44 **Lin PY**, Hung SH, Yang YC, Liao LC, Hsieh YC, Yen HJ, Lu HE, Lee MS, Chu IM, Hwang SM. A synthetic peptide-acrylate surface for production of insulin-producing cells from human embryonic stem cells. *Stem Cells Dev* 2014; **23**: 372-379 [PMID: 24083371 DOI: 10.1089/scd.2013.0253]
  - 45 **Brafman DA**, Chang CW, Fernandez A, Willert K, Varghese S, Chien S. Long-term human pluripotent stem cell self-renewal on synthetic polymer surfaces. *Biomaterials* 2010; **31**: 9135-9144 [PMID: 20817292 DOI: 10.1016/j.biomaterials.2010.08.007]
  - 46 **Villa-Diaz LG**, Nandivada H, Ding J, Nogueira-de-Souza NC, Krebsbach PH, O'Shea KS, Lahann J, Smith GD. Synthetic polymer coatings for long-term growth of human embryonic stem cells. *Nat Biotechnol* 2010; **28**: 581-583 [PMID: 20512122 DOI: 10.1038/nbt.1631]
  - 47 **Villa-Diaz LG**, Brown SE, Liu Y, Ross AM, Lahann J, Parent JM, Krebsbach PH. Derivation of mesenchymal stem cells from human induced pluripotent stem cells cultured on synthetic substrates. *Stem Cells* 2012; **30**: 1174-1181 [PMID: 22415987 DOI: 10.1002/stem.1084]
  - 48 **Irwin EF**, Gupta R, Dashti DC, Healy KE. Engineered polymer-media interfaces for the long-term self-renewal of human embryonic stem cells. *Biomaterials* 2011; **32**: 6912-6919 [PMID: 21774983 DOI: 10.1016/j.biomaterials.2011.05.058]
  - 49 **Hansen A**, Mjoseng HK, Zhang R, Kalloudis M, Koutsos V, de Sousa PA, Bradley M. High-density polymer microarrays: identifying synthetic polymers that control human embryonic stem cell growth. *Adv Healthc Mater* 2014; **3**: 848-853 [PMID: 24353271 DOI: 10.1002/adhm.201300489]
  - 50 **Li Z**, Leung M, Hopper R, Ellenbogen R, Zhang M. Feeder-free self-renewal of human embryonic stem cells in 3D porous natural polymer scaffolds. *Biomaterials* 2010; **31**: 404-412 [PMID: 19819007 DOI: 10.1016/j.biomaterials.2009.09.070]
  - 51 **Lu HF**, Narayanan K, Lim SX, Gao S, Leong MF, Wan AC. A 3D microfibrillar scaffold for long-term human pluripotent stem cell self-renewal under chemically defined conditions. *Biomaterials* 2012; **33**: 2419-2430 [PMID: 22196900 DOI: 10.1016/j.biomaterials.2011.11.077]
  - 52 **Carlson AL**, Florek CA, Kim JJ, Neubauer T, Moore JC, Cohen RI, Kohn J, Grumet M, Moghe PV. Microfibrillar substrate geometry as a critical trigger for organization, self-renewal, and differentiation of human embryonic stem cells within synthetic 3-dimensional microenvironments. *FASEB J* 2012; **26**: 3240-3251 [PMID: 22542683 DOI: 10.1096/fj.11-192732]
  - 53 **Meechaisue C**, Dubin R, Supaphol P, Hoven VP, Kohn J. Electrospun mat of tyrosine-derived polycarbonate fibers for potential use as tissue scaffolding material. *J Biomater Sci Polym Ed* 2006; **17**: 1039-1056 [PMID: 17094641 DOI: 10.1163/156856206778365988]
  - 54 **Zhang R**, Mjoseng HK, Hoeve MA, Bauer NG, Pells S, Besseling R, Velugotla S, Tourniaire G, Kishen RE, Tsenkina Y, Armit C, Duffy CR, Helfen M, Edenhofer F, de Sousa PA, Bradley M. A thermoresponsive and chemically defined hydrogel for long-term culture of human embryonic stem cells. *Nat Commun* 2013; **4**: 1335 [PMID: 23299885 DOI: 10.1038/ncomms2341]
  - 55 **Horák D**, Matulka K, Hlídková H, Lapčíková M, Beneš MJ, Jaroš J, Hampl A, Dvořák P. Pentapeptide-modified poly(N,N-diethylacrylamide) hydrogel scaffolds for tissue engineering. *J Biomed Mater Res B Appl Biomater* 2011; **98**: 54-67 [PMID: 21563303 DOI: 10.1002/jbm.b.31832]
  - 56 **Gioffe GG**, Zagallo M, Riello M, Serena E, Masi G, Barzon L, Di Camillo B, Elvassore N. Confined 3D microenvironment regulates early differentiation in human pluripotent stem cells. *Biotechnol Bioeng* 2012; **109**: 3119-3132 [PMID: 22674472 DOI: 10.1002/bit.24571]
  - 57 **Gerecht S**, Burdick JA, Ferreira LS, Townsend SA, Langer R, Vunjak-Novakovic G. Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. *Proc*



- Natl Acad Sci USA* 2007; **104**: 11298-11303 [PMID: 17581871 DOI: 10.1073/pnas.0703723104]
- 58 **Ikonen L**, Kerkelä E, Metselaar G, Stuart MC, de Jong MR, Aalto-Setälä K. 2D and 3D self-assembling nanofiber hydrogels for cardiomyocyte culture. *Biomed Res Int* 2013; **2013**: 285678 [PMID: 23573513 DOI: 10.1155/2013/285678]
- 59 **Dixon JE**, Shah DA, Rogers C, Hall S, Weston N, Parmenter CD, McNally D, Denning C, Shakesheff KM. Combined hydrogels that switch human pluripotent stem cells from self-renewal to differentiation. *Proc Natl Acad Sci USA* 2014; **111**: 5580-5585 [PMID: 24706900 DOI: 10.1073/pnas.1319685111]
- 60 **Jang M**, Lee ST, Kim JW, Yang JH, Yoon JK, Park JC, Ryoo HM, van der Vlies AJ, Ahn JY, Hubbell JA, Song YS, Lee G, Lim JM. A feeder-free, defined three-dimensional polyethylene glycol-based extracellular matrix niche for culture of human embryonic stem cells. *Biomaterials* 2013; **34**: 3571-3580 [PMID: 23422594 DOI: 10.1016/j.biomaterials.2013.01.073]
- 61 **Lei Y**, Schaffer DV. A fully defined and scalable 3D culture system for human pluripotent stem cell expansion and differentiation. *Proc Natl Acad Sci USA* 2013; **110**: E5039-E5048 [PMID: 24248365 DOI: 10.1073/pnas.1309408110]
- 62 **Lewis FC**, Bryan N, Hunt JA. A feeder-free, human plasma-derived hydrogel for maintenance of a human embryonic stem cell phenotype in vitro. *Cell Regen (Lond)* 2012; **1**: 6 [PMID: 25408869 DOI: 10.1186/2045-9769-1-6]
- 63 **Marshall JF**, Rutherford DC, McCartney AC, Mitjans F, Goodman SL, Hart IR. Alpha v beta 1 is a receptor for vitronectin and fibrinogen, and acts with alpha 5 beta 1 to mediate spreading on fibronectin. *J Cell Sci* 1995; **108** (Pt 3): 1227-1238 [PMID: 7542669]
- 64 **Ra JC**, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, Kim YJ, Jo JY, Yoon EJ, Choi HJ, Kwon E. Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev* 2011; **20**: 1297-1308 [PMID: 21303266 DOI: 10.1089/scd.2010.0466]

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## Hematopoietic stem cell-derived adipocytes and fibroblasts in the tumor microenvironment

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### Abstract

The tumor microenvironment (TME) is complex and constantly evolving. This is due, in part, to the crosstalk between tumor cells and the multiple cell types that comprise the TME, which results in a heterogeneous population of tumor cells and TME cells. This review will focus on two stromal cell types, the cancer-associated adipocyte (CAA) and the cancer-associated fibroblast (CAF). In the clinic, the presence of CAAs and CAFs in the TME translates to poor prognosis in multiple tumor types. CAAs and CAFs have an activated phenotype and produce growth factors, inflammatory factors, cytokines, chemokines, extracellular matrix components, and proteases in an accelerated and aberrant fashion. Through this activated state, CAAs and CAFs remodel the TME, thereby driving all aspects of tumor progression, including tumor growth and survival, chemoresistance, tumor vascularization, tumor invasion, and tumor cell metastasis. Similarities in the tumor-promoting functions of CAAs and CAFs suggest that a multipronged therapeutic approach may be necessary to achieve maximal impact on disease. While CAAs and CAFs are thought to arise from tissues adjacent to the tumor, multiple alternative origins for CAAs and CAFs have recently been identified. Recent studies from our lab and others suggest that the hematopoietic stem cell, through the myeloid lineage, may serve as a progenitor for CAAs and CAFs. We hypothesize that the multiple origins of CAAs and CAFs may contribute to the heterogeneity seen in the TME. Thus, a better understanding of the origin of CAAs and CAFs, how this origin impacts their functions in the TME, and the



temporal participation of uniquely originating TME cells may lead to novel or improved anti-tumor therapeutics.

**Key words:** Hematopoietic stem cell; Cancer associated adipocyte; Mesenchymal stromal cell; Tumor progression; Cancer associated fibroblast; Plasticity; Metastasis; Fibrocyte

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**Core tip:** This review examines the roles of cancer-associated adipocytes (CAAs) and cancer-associated fibroblasts (CAFs) in remodeling of the tumor microenvironment (TME), presents evidence for a unique hematopoietic stem cell origin for both CAAs and CAFs, and discusses potential therapeutic implications of this novel origin. Studies highlighted herein emphasize the necessity of developing an understanding of the origins of cells in the TME and the importance of multipronged therapeutic targets directed at preventing both the incorporation and effects of stromal remodeling by the cells of the TME.

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## INTRODUCTION: THE TUMOR MICROENVIRONMENT

The “seed and soil” hypothesis suggests that an appropriate host microenvironment (“soil”) must be present for the optimal growth of tumor cells (“seed”)<sup>[1-3]</sup>. Although this paradigm was initially proposed by Stephen Paget in 1889, research efforts have predominantly focused on the epithelial component of solid tumors and tumor cell-intrinsic factors leading to tumorigenicity. However, in the last decade, Paget’s hypothesis has again come to focus and it has been recognized that the epithelial “seed” and stromal “soil” components co-evolve and interact during tumor progression<sup>[4]</sup>. Seminal works from Weinberg’s group have shown that this stromal compartment, often referred to as the reactive stroma or tumor microenvironment (TME), directly and indirectly supports tumor survival, growth, vascularization, escape from immune surveillance, drug resistance, and metastasis *via* extracellular matrix (ECM) remodeling and production of growth factors, cytokines, and chemokines (reviewed in<sup>[5-7]</sup>). The TME is comprised of a variety of cell types including endothelial cells, perivascular cells, immune cells, adipocytes, and fibroblasts/myofibroblasts.

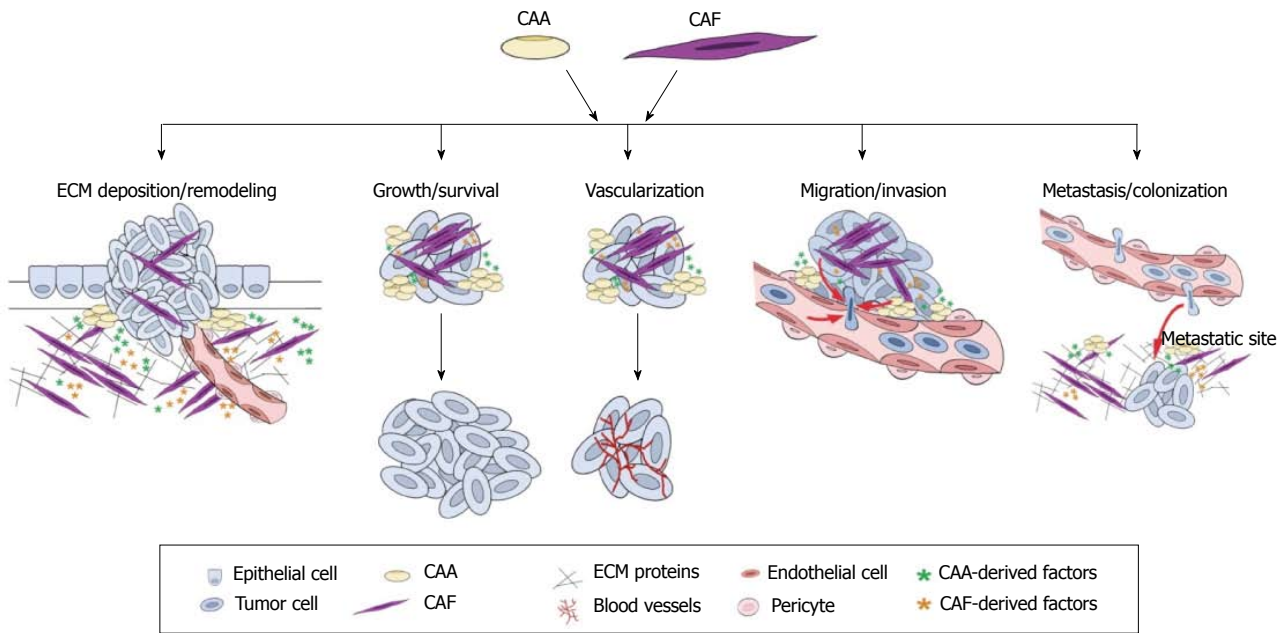
These cells interact with one another as well as with tumor cells to create an intricate network of cellular crosstalk and bidirectional regulation.

This crosstalk results in a heterogeneous population of tumor cells exhibiting varying degrees of differentiation, unregulated proliferation, the capacity to migrate and invade through surrounding tissue, and the ability to establish a dense irregular and leaky vascular network, all critical steps in metastatic tumor progression. Concomitantly, this crosstalk leads to changes in the local stromal populations, contributing to the heterogeneity of TME cells. The heterogeneity of the cells of the TME, the factors they contribute and their broad functional ability to promote all aspects of tumor progression make the “soil” a challenging and complex therapeutic target. Many factors contribute to the heterogeneity of these cell types, including exposure to the local tumor milieu, the plasticity between cells of the TME, and the multiple potential origins of each cellular population. Understanding the mechanisms behind this heterogeneity could lead to the identification of novel therapeutic targets for cancer. This review will focus on two stromal cell types, the cancer-associated adipocyte (CAA) and the cancer-associated fibroblast (CAF). The adipocyte is a stromal cell type that has recently been implicated in tumor initiation, growth, and metastasis (reviewed in<sup>[8]</sup>). Several epidemiologic studies have linked obesity with multiple types of cancer<sup>[9-11]</sup>. Recent clinical studies have reported a positive correlation between the presence of CAAs at the tumor margin and poor patient outcome, suggesting that CAAs contribute to the permissive pro-TME, particularly in adipocyte-rich tissues, such as the mammary gland<sup>[12,13]</sup> (and reviewed in<sup>[14]</sup>). CAFs, the most abundant cellular component of the TME in solid tumors, have a significant impact on tumor progression during multiple stages<sup>[5-7]</sup>. While more extensively studied than CAAs, the numerous roles of CAFs in tumor progression and metastasis are still under investigation. Like CAAs, CAFs have clinically been correlated with tumorigenesis and poor prognosis in many cancer types<sup>[15-18]</sup>. Similarities in the pro-tumorigenic functions of CAAs and CAFs suggest that these TME cell types may act in concert to promote tumor progression, indicating that therapeutic targeting of the TME may need to encompass both cell types. Herein, we will examine the phenotype and function of CAAs and CAFs in remodeling of the TME, present evidence for a unique hematopoietic stem cell origin for both CAAs and CAFs, and discuss potential therapeutic implications of this novel origin.

## CONTRIBUTIONS OF CAAS AND CAFS TO TME REMODELING

Cancer has been likened to a perpetual wound





**Figure 1 Multifactorial contributions of cancer-associated adipocytes and cancer-associated fibroblasts to tumor progression and metastasis.** Research in the last decade has highlighted the importance of the tumor microenvironment in cancer progression. While there are numerous stromal cell types that contribute to the tumor microenvironment, this illustration depicts roles for cancer-associated adipocytes (CAAs) and cancer-associated fibroblasts (CAFs) in promoting the multiple stages of tumor progression and metastasis. ECM: Extracellular matrix.

healing process<sup>[19]</sup> since both processes begin with the formation of a reactive stroma. During wound healing the reactive stroma resolves rapidly, but, during cancer progression, this actively remodeling, inflammatory state is perpetuated. CAAs and CAFs have been shown to play a role in a variety of tumor promoting processes including ECM deposition/degradation, inflammation and immune surveillance, tumor growth and survival, angiogenesis, invasion, and metastasis<sup>[5,6,20-24]</sup>, suggesting similarities in the pro-tumorigenic functions of these cells. As summarized in Figure 1, this section will discuss the CAA and CAF phenotypes and their roles in generating and maintaining the reactive stroma associated with cancer progression and metastasis.

#### **The activated phenotype of CAAs and CAFs**

Adipocytes, surrounded by fibroblasts, preadipocytes, pluripotent stem cells, endothelial cells, and immune cells, are the major components of the adipose tissue. Apart from their traditional function in energy storage, adipocytes are also considered endocrine cells, producing hormones, growth factors, cytokines and adipokines, including leptin, adiponectin, resistin, vascular endothelial growth factor (VEGF), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-6 (IL-6)<sup>[25]</sup>. During interaction with cancer cells, adipocytes acquire phenotypic changes and are reprogrammed to an activated state during which they are referred to as CAAs<sup>[26]</sup>. CAAs are generally located at the invasive front of tumors<sup>[21]</sup>. Several reports suggest that cancer cells can induce metabolic changes in adipocytes,

resulting in enhanced lipolytic activity and an inability to properly store triglyceride<sup>[27]</sup>. Moreover, transforming growth factor- $\beta$  (TGF- $\beta$ ), secreted by cancer cells or local stroma, is a potent inhibitor of adipocyte differentiation<sup>[28]</sup>. Thus, the associated morphological changes upon activation include loss of lipid content (delipidation) and acquisition of a fibroblast-like/preadipocyte phenotype (de-differentiation)<sup>[21]</sup>. Functional alterations in CAAs include loss of terminal adipocyte markers and products (adiponectin, resistin, fatty acid binding protein-4 (FABP4), hormone sensitive lipase (HSL), and CCAAT/enhancer binding protein- $\alpha$  (C/EBP $\alpha$ )) and an increased production of pro-inflammatory cytokines IL-6, IL-1 $\beta$ , plasminogen activator inhibitor-1 (PAI-1)<sup>[21]</sup>. As detailed below, in this activated state, CAAs produce adipokines and inflammatory factors that have been shown to promote tumor progression in adipocyte-rich environments.

In non-malignant tissues, fibroblasts provide structure and ECM scaffolding for tissues. In a wound environment, these fibroblasts become activated, produce increasing amounts of ECM proteins and migrate to wound interfaces to cause wound contraction and closure. In both wound healing and the TME, fibroblast activation is marked by increased  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) protein expression, along with increased expression of vimentin, desmin, fibroblast specific protein, platelet-derived growth factor receptor  $\alpha$  and  $\beta$  (PDGFR $\alpha$  and  $\beta$ ), fibroblast activation protein (FAP), or a combination of these markers. TGF- $\beta$  is present in both the wound and TMEs and has been shown to both induce and suppress differentiation and



tumorigenesis in a dose and context specific manner<sup>[29]</sup>. With respect to fibroblasts, TGF- $\beta$  has been shown to upregulate  $\alpha$ SMA expression<sup>[30,31]</sup>, induce expression of FAP<sup>[32]</sup>, and promote collagen synthesis<sup>[33]</sup>, hallmarks of activated fibroblasts. Like CAAs, activated fibroblasts in the TME are also characterized by increased and altered production of inflammatory cytokines, chemokines, ECM components, and growth factors. The clinical importance of this activated phenotype is highlighted by molecular profiling studies of CAFs and matched normal fibroblasts. Studies in non-small cell lung cancer<sup>[16]</sup> and breast cancer<sup>[34]</sup> revealed that the cancer-associated gene signatures in CAFs correlated to disease outcome.

#### **CAAs and CAFs in ECM remodeling and establishment of a reactive stroma**

CAAs have been shown to play an important role in stromal remodeling during tumorigenesis. Type VI collagen, a soluble ECM protein, was reportedly up-regulated in peritumoral adipocytes during tumorigenesis<sup>[22]</sup> and was shown to promote early mammary tumor progression *in vivo*<sup>[23]</sup>. The  $\alpha 3$  chain cleavage product of type VI collagen, endotrophin, augmented fibrosis, angiogenesis and inflammation through recruitment of macrophages and endothelial cells<sup>[23]</sup>. Rio and colleagues found that the native  $\alpha 3$  chain of type VI collagen constituted a specific substrate for matrix metalloproteinase (MMP)-11, whose collagenolytic activity was functional in fat tissue ontogenesis as well as during cancer invasive steps<sup>[24]</sup>. Interestingly, they also reported that invasive breast cancer cells induced the expression of MMP-11 in the neighboring CAAs<sup>[20]</sup>, suggesting that exposure to tumor cells promotes stromal remodeling abilities of CAAs.

CAF function to generate and remodel ECM through production of collagens, fibronectin, and laminin<sup>[30,35]</sup>, and proteases such as MMPs<sup>[36,37]</sup>. Collagens, fibronectin, and laminin contribute to the stiffness and density of the stroma, give structural support to the tumor cells, and provide important mechano-signals in the TME. Additionally, the expression of  $\alpha$ SMA by activated fibroblasts was shown to promote matrix contraction<sup>[38-40]</sup>, suggesting a direct effect on matrix stiffness. Like CAAs, CAFs also produce MMPs that degrade matrix collagens, fibronectins, and proteoglycans, profoundly contributing to structural remodeling of the TME. It has also been demonstrated *in vitro* that fibroblast overexpression of FAP, a serine protease selectively produced by CAFs, remodeled the ECM by increasing expression levels of  $\alpha$ SMA, fibronectin, and collagen I<sup>[41]</sup>. These data indicate that, orchestrated by the tumor-stroma crosstalk, CAAs and CAFs actively remodel the ECM to favor local tumor progression.

In addition to elaboration and remodeling of matrix, CAAs and CAFs may also promote changes in the local stroma by contributing to the inflammatory

state of the TME. As described above, CAAs have been shown to produce a variety of inflammatory cytokines including IL-6, IL-8, IL-1 $\beta$  and TNF- $\alpha$ <sup>[8,26]</sup>. Through production of factors such as IL-1 $\beta$ , IL-23, TGF- $\beta$ , IL-6, and IL-8<sup>[42-44]</sup>, CAFs exert considerable influence over the inflammatory state of the TME. CAF production of ECM components such as hyaluronic acid further drive the inflammatory state by recruiting tumor-associated macrophages<sup>[45]</sup> that promote tumor vascularization and proliferation. While the interactions between CAAs, CAFs and immune cells are only beginning to be explored, it is clear that cellular cross-talk modulates the inflammatory state of the TME, potentially influencing tumor-specific immunity.

#### **Tumor growth and survival**

Both CAAs and CAFs aid the tumor in meeting requirements for rapid growth by providing structural matrix as described above as well as directly promoting tumor cell proliferation and survival. Rapid, unchecked proliferation is characteristic of tumor cells, and as the cells of the TME remodel the reactive stroma, tumor cell proliferation is further accelerated. In addition to proteases and ECM constituents, CAAs provide their high energy content lipids to cancer cells resulting in accelerated tumor progression<sup>[46]</sup>. In support of this, morphologically, CAAs at the tumor invasive front are smaller than those observed at a distance, which implies lipolysis. In the case of ovarian cancer, the cancer cell-adipocyte interaction initiated HSL-mediated lipolysis in the adipocytes, releasing fatty acids, which were then taken up by the ovarian cancer cells for energy production through  $\beta$ -oxidation<sup>[46]</sup>. In a PC-3 model of prostate cancer, the translocation of lipid from adipocytes to prostate cancer cells was visualized by Fourier transform infrared spectroscopy<sup>[47]</sup>. Together these studies suggest that in multiple cancer types, CAAs supply the TME with energy rich lipids that may act to promote tumor growth by supplying tumor cells with essential metabolites.

Furthermore, adipocytes secrete adipokines into the TME, such as TNF $\alpha$ , IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1/CCL2), and leptin which have been shown to enhance tumor growth locally and systemically in a variety of cancer types (reviewed in<sup>[8]</sup>) including prostate<sup>[48,49]</sup> and breast<sup>[14,21]</sup> cancers. Another aspect of adipocyte pro-tumorigenic activity is their contribution to chemo/radio-therapy resistance. The antitumor effect of vincristine, daunorubicin and dexamethasone to acute lymphoblastic leukemia (ALL) was impaired under the influence of adipocytes, which augmented ALL cell survival due to increased expressions of Bcl-2 and Pim-2<sup>[50]</sup>. Likewise, breast tumor cells co-cultivated with adipocytes or recombinant IL-6 exhibited radioresistance, an increase in the effector



kinase Chk1, and a decrease in cell death<sup>[51]</sup>. Taking into account that CAAs secrete elevated levels of IL-6 in the TME, CAAs could promote chemoresistance *via* multiple mechanisms. These studies shed light on a new role of CAAs in fostering a chemo/radio-resistant phenotype in cancers and suggest targeting CAAs may increase effectiveness of conventional chemotherapy treatments.

CAFs also promote tumor growth through the production of factors that have been shown to be involved in proliferation of tumor cells, including TGF- $\alpha$ <sup>[52]</sup>, TGF- $\beta$ <sup>[53]</sup>, hepatocyte growth factor (HGF)<sup>[54]</sup>, and others (reviewed in<sup>[44,55]</sup>). Through production of MMPs, CAFs act to release stored growth factors from within the matrix, further contributing to an enriched host microenvironment and promoting proliferation of tumor cells (reviewed in<sup>[37]</sup>). Studies have identified a novel mechanism of cellular respiration, coined the “reverse Warburg effect” that involves an interplay and exchange between tumor cells and stromal cells whereby tumor cells take up energy-rich metabolites from CAAs<sup>[8]</sup> and CAFs<sup>[56]</sup> for use in the mitochondrial TCA cycle. This may contribute to the rapid proliferation of tumor cells by directing cellular energy towards cell division rather than cellular respiration. CAFs, like CAAs, have been implicated in promoting tumor chemoresistance. The expression and organization of collagen type I has been inversely correlated with intratumoral uptake of chemotherapeutic agents *in vivo* as it contributes to increased interstitial fluid pressure, forming a barrier to trans-capillary transport of agents<sup>[57]</sup>. DNA vaccine targeting of FAP on CAFs led to decreased deposition of a collagen I rich matrix and improved chemotherapeutic drug uptake in pre-clinical animal studies<sup>[58]</sup>. Direct targeting of CAF-derived FAP also suppressed primary tumor cell growth in a pre-clinical murine model of multi-drug resistant breast cancer<sup>[58]</sup>; however, this finding has not held in Phase II clinical trials. *In vitro* studies revealed that adherence of melanoma cells to fibroblast monolayers allowed for reduction of the cytotoxic effects of cisplatin, supporting a role for the CAF-induced ECM<sup>[59]</sup> in tumor cell survival. CAFs from melanoma and prostate cancer were found to be less sensitive to etoposide and vincristine due to expression of a non-mutated but functionally deficient form of p53<sup>[60]</sup>. Together, these findings suggest CAFs promote tumor growth and survival through multiple mechanisms and these effects may contribute to both the primary tumor and metastatic site.

### **Tumor vascularization**

Angiogenesis is a critical step in tumor progression, without which tumors cannot maintain growth beyond 1-2 mm<sup>3</sup><sup>[61]</sup>. Many of the key factors required to initiate the angiogenic switch in solid tumors are produced by CAAs and CAFs. Adipocytes are known to produce multiple angiogenic factors [VEGF, fibroblast growth

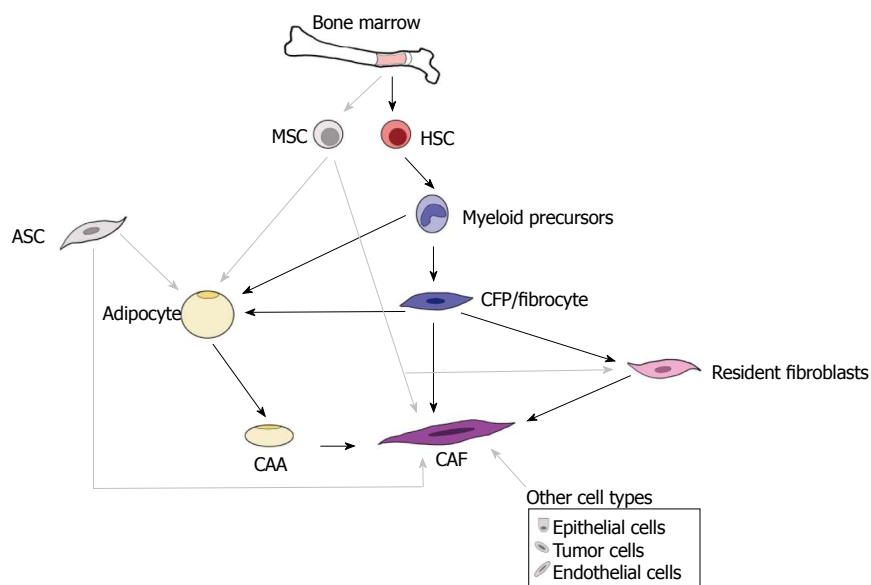
factor-2 (FGF2)], adipokines (leptin, adiponectin, resistin) and cytokines (IL-6), all of which stimulate angiogenesis and contribute to an overall pro-angiogenic microenvironment for tumor progression<sup>[62]</sup>. CAFs have been shown to produce angiogenic factors including stromal derived factor-1 (SDF-1)<sup>[63]</sup>, TGF- $\beta$ <sup>[64]</sup>, IL-6<sup>[65]</sup> and VEGF<sup>[66,67]</sup>, which support endothelial cell proliferation and tumor vascularization. In addition to their production of angiogenic cytokines, CAFs may play a more direct role in tumor vascularization through their ability to serve as vascular support cells. CAF and myofibroblast expression of  $\alpha$ SMA closely links this cell type with the pericyte, a fibroblast-like cell, which plays a supportive role for endothelial cells in both normal and tumor systems. Thus, CAAs and CAFs are a source for critical angiogenic factors and may be involved in flipping the hypothetical switch to a vascularized tumor site, thereby acting to support an essential early step in tumorigenesis.

### **Invasion and metastasis**

One of the initial steps in metastasis of solid tumor is the migration and invasion of the tumor cell through the ECM and through the basement membrane. This is followed by intravasation of tumor cells into a local blood vessel and the extravasation of the tumor cell to colonize and proliferate at a distant site. CAAs secrete similar levels of MMP-2 and MMP-9 compared to normal adipocytes<sup>[21]</sup>, whereas MMP-11 is highly expressed by CAAs in the proximity of invading cancer cells, but not in normal resting adipocytes<sup>[24]</sup>. The role of MMP-11 (stomelysin-3) in tumor biology is still unclear (reviewed in<sup>[37,68]</sup>). However, MMP-11 has been shown to promote tumorigenesis and function through its proteolytic activity<sup>[69,70]</sup>, but only weakly degrades matrix molecules. CAFs have been shown to produce a variety of matrix metalloproteinases (MMP), including MMP-1, MMP-2, MMP-3, MMP-9, MMP-11, MMP-13, and MMP-14 (reviewed in<sup>[36,37]</sup>). Degradation of ECM allows tumor cells to cross the structural barrier of the basement membrane, a key step in tumor metastasis (reviewed in<sup>[37]</sup>). In an elegant imaging study, Gaggioli<sup>[35]</sup>, demonstrated that CAFs are able to degrade matrix to form tracks through the ECM that allow invading tumor cells to efficiently follow behind.

CAAs and CAFs also directly affect the migratory and invasive abilities of tumor cells through production of adipokines, cytokines, and chemokines. Adipocyte/CAA-secreted IL-6 has been shown to play a key role in mediating adipocyte-dependent invasive activity of both breast cancer cells and melanoma cells<sup>[21,71]</sup>. FAP production by fibroblasts was linked to the increased invasion of pancreatic cancer cells in a  $\beta_1$ -integrin/FAK mediated fashion<sup>[41]</sup>. In breast cancer, CAFs were shown to increase the invasive ability of DCIS epithelial cells and this was related to their production of MMP-9 and MMP-14<sup>[72,73]</sup>. In addition, these factors secreted by CAAs and CAFs may flood





**Figure 2** Origins of cancer-associated adipocytes and cancer-associated fibroblasts. This drawing illustrates the complex and ever-growing understanding of the origins for cancer-associated adipocytes (CAAs) and cancer-associated fibroblasts (CAFs). CAAs and CAFs are generally thought to arise from resident tissue cells, the adipose stem cell (ASC) and resident fibroblast, respectively. However, alternative sources for adipocytes and CAAs have been demonstrated including cells of the bone marrow, specifically those of the myeloid lineage (e.g., macrophages, CFPs, and fibrocytes). In addition to resident fibroblasts, CAFs have been shown to be derived from myeloid progenitors (CFPs, fibrocytes), mesenchymal stromal cells (MSCs), ASCs, CAAs, epithelial cells, tumor cells, and endothelial cells. It is possible that these multiple sources are reflected in the morphological, phenotypic, and functional heterogeneity described for adipocytes and for CAFs. HSC: Hematopoietic stem cell; CFPs: Circulating fibroblast precursors.

the circulation with signals for distant metastatic sites to initiate their own expression of chemokines that will aid the tumor cell in homing to the metastatic site and preparing the site for colonization once the tumor cell arrives<sup>[74]</sup>. Research conducted on human omental adipocytes indicates they secrete IL-6 and IL-8 and that antibody-mediated inhibition of either factor resulted in reduced homing of ovarian cancer cells *in vitro* and *in vivo*, although inhibition of IL-8 was more efficient at reducing homing of cancer cells *in vivo*<sup>[46]</sup>. Once ovarian tumors are established on the omentum, they may convert adjacent adipocytes into CAAs, which results in a positive feedback loop leading to increased IL-6 production and further recruitment of cancer cells to the omentum. It has also been suggested that tumor cells do not metastasize alone, rather, they “travel” with stromal cells. Studies from Duda *et al.*<sup>[75]</sup> demonstrated by cannulating primary tumor bearing mice, that tumor cells are shed with stromal cells in heterotypic “clumps” from the primary tumor. The stromal component, which included fibroblasts, acted to support the viability of the tumor cells while traveling through the circulation to the metastatic site. Together, these studies suggest that CAAs and CAFs promote the migratory and invasive phenotype in a variety of solid tumors and highlight the importance of elucidating mechanisms to target both CAAs and CAFs.

## ORIGINS OF CAAS AND CAFs IN THE TME

Both CAAs and CAFs are generally thought to arise from tissues adjacent to the tumor; however, recent studies have begun to demonstrate alternative sources, including other resident stromal cells, epithelial cells, and bone marrow. This complex and ever-growing understanding of the origins for CAAs and CAFs can be appreciated in Figure 2. It is possible that these

multiple sources are reflected in the morphological, phenotypic, and functional heterogeneity described for adipocytes from different fat depots<sup>[76]</sup> and for CAFs<sup>[77,78]</sup>. Given that this heterogeneity is a significant hurdle in therapeutically targeting the TME populations, it will be essential to elucidate the multiple origins for these cells as well as to examine the impact these origins may have on cellular function.

### Origins of CAAs

The expansion of adipose tissue is achieved *via* increases in size (hypertrophy) and/or number (hyperplasia) of adipocytes. Mature adipocytes are postmitotic, therefore, adipocyte hyperplasia requires new adipocytes be produced from their adipogenic precursors. A long-standing paradigm of adipocyte generation is that all adipocytes are differentiated from mesenchymal progenitor cells resident in the vascular stroma, referred to as adipose stem cells (ASCs), where the regional fat depots eventually form<sup>[79,80]</sup>. However, these progenitor cells are found associated with adipose vessels<sup>[79]</sup>, bringing up the possibility that circulating progenitors, such as those provided by bone marrow, home to adipose tissue through the bloodstream followed by extravasation across the endothelium of blood vessels, subsequently undergoing adipogenic conversion. To test this hypothesis, several groups transplanted GFP-labeled bone marrow into wild-type mice<sup>[81-83]</sup>. Two of these groups detected GFP-expressing adipocytes in the major adipose depots<sup>[81,82]</sup>, while one failed to detect these cells<sup>[83]</sup>, perhaps due to low marker expression or limited engraftment. When engrafted mice were treated with rosiglitazone or a high fat diet that stimulated adipogenesis, the number of GFP-expressing adipocytes was elevated, and cells were often found in clusters, suggesting clonal growth from bone marrow-derived progenitors<sup>[81]</sup>. While these studies support a bone marrow origin for CAAs, it is unclear which bone



marrow stem cell serves as the CAA progenitor.

It is commonly held that the bone marrow contains two types of stem cells, the mesenchymal stromal cell (MSC) and the hematopoietic stem cell (HSC). MSCs are defined by their adherence to plastic and potential to differentiate into mesenchymal tissue cells such as bone, fat, muscle, cartilage, and fibroblasts<sup>[84-87]</sup>. HSCs are defined by their capability of hematopoietic reconstitution *in vivo* and have also been shown to give rise to other tissue cell types including mast cells and osteoclasts. Our laboratory has developed a method for transplantation of a clonal population from a single sorted HSC defined as an EGFP<sup>+</sup>Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>hi</sup>CD34<sup>-</sup> cell. Using this single cell transplantation model, we have demonstrated that HSCs give rise to a variety of mesenchymal cell types including adipocytes<sup>[88]</sup>, osteocytes and chondrocytes<sup>[89]</sup>, cardiac valve interstitial cells<sup>[90]</sup>, circulating fibroblast precursors<sup>[91]</sup>, and fibroblasts and myofibroblasts in multiple tissues<sup>[92-94]</sup> (reviewed in<sup>[95]</sup>). Further use of our unique clonal cell transplantation model revealed the generation of adipocytes *in vivo* from clonally derived bone marrow HSCs<sup>[88]</sup>. Similar to findings from Crossno *et al.*<sup>[81]</sup>, we found that rosiglitazone stimulated adipogenesis from the HSC<sup>[88]</sup>. *In vitro*, clones giving rise to monocytes/macrophages under hematopoietic conditions were also able to generate adipocytes under adipogenic conditions, suggesting a differentiation pathway from HSCs-myeloid precursors-adipocytes<sup>[88]</sup>. Similar results were obtained from the Klemm group who confirmed the *de novo* generation of a subset of adipocytes from bone marrow myeloid progenitor cells using a non-transplant transgenic mouse model in which LacZ expression was restricted to the myeloid lineage<sup>[96]</sup>. Moreover, Sterieter and colleagues reported that circulating fibrocytes were capable of adipogenic differentiation both *in vitro* and *in vivo*<sup>[97]</sup>. Due to the dual hematopoietic/mesenchymal nature of fibrocytes, they may be considered as an intermediate for myeloid-derived adipocyte population. These studies demonstrate the ability of the HSC to give rise to adipocytes through the myeloid lineage. While studies have not yet directly examined the role of HSC-derived CAAs in tumor progression, findings from our laboratory suggest that they can enhance tumor growth and tumor cell motility in breast cancer and melanoma models (unpublished data).

### Origins of CAFs

Traditionally, CAFs are thought to arise from resident tissue fibroblasts<sup>[98]</sup>. However, recent studies have suggested alternative sources including other resident stromal cells, epithelial cells, epithelial-mesenchymal transition or endothelial-mesenchymal transdifferentiation (EndoMT), and adipose tissue. Several studies have also suggested a bone marrow origin for myofibroblasts and CAFs<sup>[99]</sup> (and reviewed in<sup>[100-102]</sup>). As described above, the bone marrow provides a rich source for both MSCs and HSCs. Using

our model for transplantation of a clonal population from a single sorted HSC in conjunction with a variety of solid tumor models, we have demonstrated the presence of HSC-derived fibroblasts in tumor sections from mice transplanted with a clonal population of cells derived from a single, sorted HSC<sup>[91,103]</sup>. Analysis of sections from Lewis lung carcinoma (LLC) and melanoma (K1735-M2) tumors harvested from clonally engrafted animals showed the presence of HSC-derived CAFs<sup>[91,103]</sup>. These EGFP-expressing cells had a fibroblastic morphology and constituted 8%-28% of the tumor stromal cells<sup>[103]</sup>. Characterization of these HSC-derived cells indicated that they were activated fibroblasts, based on expression of  $\alpha$ SMA and mRNA expression of collagen I<sup>[103]</sup>. Also prevalent in the specimens were EGFP<sup>+</sup> pericyte-like perivascular cells, suggesting that HSCs contribute to tumor vasculature<sup>[103]</sup>.

Work from our laboratory has also identified a population of circulating fibroblast precursors (CFPs) that express markers of both hematopoietic cells (CD34, CD45) and fibroblasts [collagen I (Col I), discoidin domain receptor-2 (DDR2; a collagen 1 receptor)]<sup>[91]</sup> (and unpublished data). The CD45<sup>+</sup>DDR2<sup>+</sup> population was shown to differentiate along the monocyte/macrophage lineage, contain the CD34<sup>+</sup>Col I<sup>+</sup> fibrocyte and rapidly differentiate to collagen 1<sup>+</sup>,  $\alpha$ SMA<sup>+</sup> cells with fibroblastic morphology. Using our clonal hematopoietic stem cell transplantation model, we conducted an *in vitro* examination of CFPs/fibrocytes derived from peripheral blood cells of clonally engrafted mice<sup>[92]</sup>. In these studies, nucleated blood cells were cultured and the appearance of EGFP<sup>+</sup> (HSC-derived), spindle-shaped or polygonal cells was detected by the seventh day. Flow cytometric time course analysis of the cultured cells demonstrated decreasing CD45 expression and increasing DDR2 expression. Our studies have demonstrated that CFPs may be stimulated to express markers of activated fibroblasts including collagen, vimentin, and  $\alpha$ SMA by exposure to tumor conditioned media. This demonstrates a possible differentiation pathway from the HSCs-myeloid precursors-CFP and with exposure to tumor, HSCs-myeloid precursors-CFP-CAF<sup>[91]</sup>. These findings are supported by our *in vivo* data demonstrating the activated fibroblast phenotype of HSC-derived cells recruited from the bone marrow to the tumor stroma<sup>[91]</sup>.

### CAA and CAF common origins and plasticity

As summarized in Figure 2, multiple origins for CAAs and CAFs have been proposed. Evidence also suggests that CAAs and CAFs may share a common origin. Data from our laboratory using clonal cell lineage tracing demonstrated a monocyte lineage origin for adipocytes, specifically the Mac1<sup>lo</sup> fraction of bone marrow<sup>[88]</sup>. Similarly, lineage and gene expression analyses demonstrated that adipocytes and adipocyte progenitors arise from the hematopoietic stem cell *via* the myeloid lineage<sup>[96]</sup>. Our studies of CAFs



and their circulating precursors demonstrated that these cells originate in the Mac1<sup>hi</sup> population of peripheral blood and that their participation in tumor may be regulated by MCP-1<sup>[91]</sup>. Additional evidence suggests plasticity between preadipocytes and macrophages, with preadipocytes being a source for macrophages<sup>[104]</sup> and tissue macrophages being a source for preadipocytes<sup>[105]</sup>. Histological evidence of a high ratio of adipocytes to fibroblasts at the tumor invasive front and an extremely high fibroblast-like cell to adipocyte ratio observed at the tumor center, suggests that CAAs may transition and/or give rise to CAFs as tumor progresses<sup>[14]</sup>. Breast cancer cells were also shown to induce de-differentiation of adipocytes to a more fibroblastic phenotype<sup>[21]</sup>. Human adipose tissue derived stem cells (hASCs) were found to give rise to CAF-like cells when cultured with conditioned media from MDA-MB-231 or MCF-7 breast cancer cell lines<sup>[106]</sup>. Under tumor conditions, the hASC-derived CAF-like cells were shown to have a myofibroblastic phenotype, with increased expression of  $\alpha$ SMA and tenascin C. This change in phenotype was found to be dependent upon TGF $\beta$  signaling in the hASCs. Studies have yet to directly demonstrate a CAF to CAA conversion, however, we have observed *in vitro* that non-adherent bone marrow cells, enriched for hematopoietic progenitors, cultured in the presence of M-CSF and mouse serum give rise to lipid laden cells with a fibroblast-like morphology (unpublished observation). Together, these studies support an HSC origin for both CAAs and CAFs, suggesting plasticity exists between adipocytes, CAAs, and CAFs. This plasticity may be one mechanism by which heterogeneity of the TME is generated.

## CONCLUSION

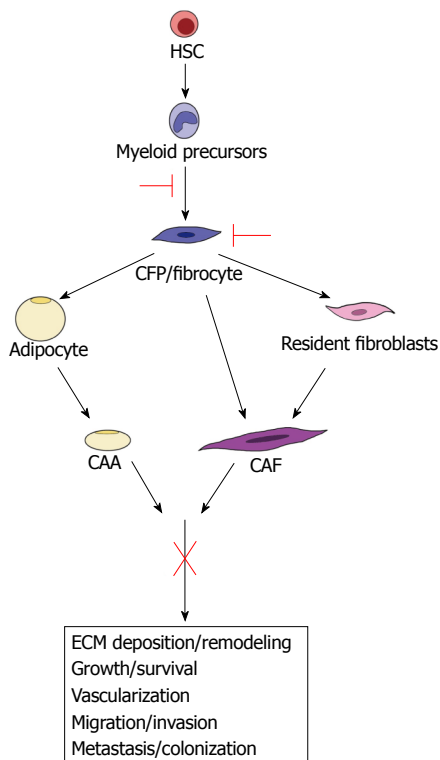
Evidence suggests that CAAs and CAFs play a critical role in tumor progression as well as patient prognosis and survival. It is thought that the metabolic changes associated with obesity underlie the increased risk of cancer and cancer-related mortalities. It has been estimated that excess weight and obesity were responsible for 20% of all cancer deaths in women in the United States<sup>[107]</sup>, consistent with poor outcome of cancer in overweight/obese patients (reviewed in<sup>[108]</sup>). While no direct comparisons of CAAs derived from distinct sources have been conducted, it is clear that HSC-derived adipocytes represent a subpopulation distinct from conventional white and brown adipocytes based on their low expression level of leptin, low mitochondrial/peroxisomal content and oxidative capacity, and elevated inflammatory cytokine production<sup>[96]</sup>. HSC-derived adipocytes also share numerous features with CAAs including low expression of terminal adipocyte markers and high expression levels of inflammatory cytokines, indicating that HSC-derived adipocytes may be considered "activated" contributors to the TME. Like CAAs, HSC-

derived adipocytes were found to be smaller in size than "resident" adipocytes (<sup>[81]</sup> and our unpublished observation), but whether, as with CAAs, this is related to a higher rate of lipolysis in these cells requires further exploration. It has been noted that HSC-derived adipocytes preferentially accumulate in visceral adipose tissue (VAT) rather than subcutaneous adipose tissue (SAT)<sup>[96]</sup>. Excess adiposity in VAT is specifically linked to type 2 diabetes and certain forms of cancer<sup>[76]</sup>. As compared to adipocytes from SAT, VAT adipocytes exhibited higher rates of fatty acid turnover and lipolysis<sup>[109]</sup> and produced more IL-6 and less adiponectin and leptin<sup>[110]</sup>. These data could indicate that VAT and SAT adipocytes are generated from different progenitors, or functional changes in different depots are due to differential accumulation of adipocytes arising from distinct progenitors. Furthermore, the accumulation of HSC-derived adipocytes was increased in female mice over males, which may have important inference in human biology<sup>[96]</sup>, as women generally possess a higher percentage of body fat and tend to disproportionally gain fat in VAT following menopause. Coupled with preferential accumulation of HSC-derived adipocytes in VAT, this pattern of adiposity represents a higher risk of adipose-related gynecological cancers for postmenopausal women and suggests HSC-derived CAAs may represent a novel target these patients.

Several studies suggest that CAFs and their unique phenotypes are associated with increased malignant potential. In the case of breast cancer, women with denser breast tissue have an increased tendency to develop cancer<sup>[15]</sup>. The presence of a fibrous stroma was found to be associated with poor prognosis in squamous cell carcinoma<sup>[18]</sup>. In non-small cell lung carcinoma, molecular analysis revealed a gene signature for CAFs that was associated with patient prognosis<sup>[16]</sup>. Interestingly, recent pre-clinical studies in pancreatic ductal adenocarcinoma have demonstrated a protective role for CAFs<sup>[111,112]</sup>, suggesting that the role of these cells is tumor-type dependent. CAFs are a heterogeneous population of cells that can differ based on both location within the tumor and between tumor types and demonstrate different phenotypes, activation states, and/or functions throughout tumor progression. This diversity may, in part, be due to the multiple proposed origins of CAFs, which have led to CAFs being referred to as a "cell state" rather than a specific cell type<sup>[113]</sup>. A more in depth understanding of the origins of CAFs may shed light on the array of markers expressed by these cells, help to better define the "CAF", and elucidate their roles based on origin and tumor type.

Given the essential roles of the TME in tumor development, progression and metastasis, it is clear that successful anti-tumor therapeutics should include those directed at the support cells of the TME. A key step towards this goal is the gaining knowledge of the role(s) of the different TME cell types (*e.g.*, CAAs





**Figure 3 Therapeutic implications.** We hypothesize that the identification of pathways from the hematopoietic stem cell (HSC) to the cancer-associated adipocyte (CAA) and cancer-associated fibroblast (CAF) provides a potential opportunity to target these pro-tumorigenic cells both early in their differentiation and at multiple points in their maturation, which may lead to a more encompassing downstream inhibition of their contributions to tumor progression and metastasis.

and CAFs), their origins, their temporal participation and mechanisms by which they influence tumor. However, the complexity of the crosstalk between the cells of the TME, the broad impact of CAAs/CAF on the reactive stroma and their contribution to the evolving TME has made therapeutically targeting individual stromal cell derived factors difficult. For example, targeting fibroblast activation protein alpha (FAP $\alpha$ ) produced by activated CAFs, while showing promise in preclinical studies<sup>[58,114,115]</sup>, was demonstrated to have no beneficial response in Phase II clinical trials for metastatic colorectal cancer and soft-tissue sarcoma patients<sup>[116]</sup> (and reviewed in<sup>[117]</sup>). However, we propose that targeting CAAs, CAFs and their precursors based on their origin may lead to significant advances in treatment by directly targeting the cells before their incorporation into tumor rather than targeting their varied products and multiple effects (Figure 3). We and others have shown that HSCs give rise to adipocytes *via* the myeloid lineage<sup>[88,96]</sup>. Likewise, our studies demonstrate an HSC origin for CAFs *via* the same myeloid lineage<sup>[91]</sup>. Studies have also demonstrated a myeloid lineage origin for the fibrocyte<sup>[118]</sup> that also gives rise to adipocytes<sup>[97]</sup> and fibroblasts<sup>[92,119]</sup>. These HSC-derived CAAs (unpublished observation) and CAFs<sup>[91,103]</sup> (and unpublished observation) contribute

to the TME and have a significant impact on tumor progression in mouse models. Identification of pathways from the HSC to the CAA or CAF provides a potential opportunity to target CAAs and CAFs both early in their differentiation and at multiple points in their maturation. For example, early inhibition of CFP/fibrocyte differentiation from the myeloid lineage would lead to fewer CAA and CAF precursors available for incorporation in the TME. Likewise, directly targeting CFPs/fibrocytes in circulation may prevent their incorporation into the local and metastatic TME as both CAAs and CAFs, essentially hitting two arms of pro-tumorigenic stromal cells. Given their ability to invade, circulate and extravasate, therapeutically targeting HSC-derived CFPs/fibrocytes may also directly affect the population of CAFs demonstrated to chaperone cancer cells to metastatic sites. Finally, the ability to isolate HSC-derived CAA/CAF progenitors in circulation, combined with their intrinsic ability to home to tumor<sup>[91]</sup>, may provide a novel modality for drug delivery vehicles for chemotherapy. Thus, targeting the precursors of CAAs and CAFs may lead to a more inclusive and encompassing downstream inhibition of their multiple contributions to tumor progression and metastasis. Taken together, these studies highlight the necessity of developing an understanding of the differences and similarities between TME cell types of multiple origins as well as research directed at elucidating the differentiation pathway of these populations for the ultimate goal of TME-based anti-tumor therapy.

## REFERENCES

- 1 **Paget J.** Lectures on surgical pathology. Philadelphia: Lindsay & Blakinston, 1860: 1-848
- 2 **Paget S.** The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev* 1989; **8**: 98-101 [PMID: 2673568]
- 3 **Fidler IJ.** The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 2003; **3**: 453-458 [PMID: 12778135 DOI: 10.1038/nrc1098]
- 4 **Weinberg RA.** Coevolution in the tumor microenvironment. *Nat Genet* 2008; **40**: 494-495 [PMID: 18443582 DOI: 10.1038/ng0508-494]
- 5 **Hanahan D, Weinberg RA.** The hallmarks of cancer. *Cell* 2000; **100**: 57-70 [PMID: 10647931]
- 6 **Hanahan D, Weinberg RA.** Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]
- 7 **Hanahan D, Coussens LM.** Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012; **21**: 309-322 [PMID: 22439926 DOI: 10.1016/j.ccr.2012.02.022]
- 8 **Nieman KM, Romero IL, Van Houten B, Lengyel E.** Adipose tissue and adipocytes support tumorigenesis and metastasis. *Biochim Biophys Acta* 2013; **1831**: 1533-1541 [PMID: 23500888 DOI: 10.1016/j.bbalip.2013.02.010]
- 9 **McMichael AJ.** Food, nutrition, physical activity and cancer prevention. Authoritative report from World Cancer Research Fund provides global update. *Public Health Nutr* 2008; **11**: 762-763 [PMID: 18462560 DOI: 10.1017/S1368980008002358]
- 10 **Reeves GK, Pirie K, Beral V, Green J, Spencer E, Bull D.** Cancer incidence and mortality in relation to body mass index in the



- Million Women Study: cohort study. *BMJ* 2007; **335**: 1134 [PMID: 17986716 DOI: 10.1136/bmj.39367.495995.AE]
- 11 **Rehnan AG**, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet* 2008; **371**: 569-578 [PMID: 18280327 DOI: 10.1016/S0140-6736(08)60269-X]
  - 12 **Kimijima I**, Ohtake T, Sagara H, Watanabe T, Takenoshita S. Scattered fat invasion: an indicator for poor prognosis in premenopausal, and for positive estrogen receptor in postmenopausal breast cancer patients. *Oncology* 2000; **59** Suppl 1: 25-30 [PMID: 11096353 DOI: 10.1159/000055284]
  - 13 **Yamaguchi J**, Ohtani H, Nakamura K, Shimokawa I, Kanematsu T. Prognostic impact of marginal adipose tissue invasion in ductal carcinoma of the breast. *Am J Clin Pathol* 2008; **130**: 382-388 [PMID: 18701411 DOI: 10.1309/MX6KKA1UNJ1YG8VN]
  - 14 **Tan J**, Buache E, Chenard MP, Dali-Youcef N, Rio MC. Adipocyte is a non-trivial, dynamic partner of breast cancer cells. *Int J Dev Biol* 2011; **55**: 851-859 [PMID: 21948738 DOI: 10.1387/ijdb.113365jt]
  - 15 **Boyd NF**, Guo H, Martin LJ, Sun L, Stone J, Fishell E, Jong RA, Hislop G, Chiarelli A, Minkin S, Yaffe MJ. Mammographic density and the risk and detection of breast cancer. *N Engl J Med* 2007; **356**: 227-236 [PMID: 17229950 DOI: 10.1056/NEJMoa062790]
  - 16 **Navab R**, Strumpf D, Bandarchi B, Zhu CQ, Pintilie M, Ramnarine VR, Ibrahimov E, Radulovich N, Leung L, Barczyk M, Panchal D, To C, Yun JJ, Der S, Shepherd FA, Jurisica I, Tsao MS. Prognostic gene-expression signature of carcinoma-associated fibroblasts in non-small cell lung cancer. *Proc Natl Acad Sci USA* 2011; **108**: 7160-7165 [PMID: 21474781 DOI: 10.1073/pnas.1014506108]
  - 17 **Franco OE**, Jiang M, Strand DW, Peacock J, Fernandez S, Jackson RS, Revelo MP, Bhowmick NA, Hayward SW. Altered TGF- $\beta$  signaling in a subpopulation of human stromal cells promotes prostatic carcinogenesis. *Cancer Res* 2011; **71**: 1272-1281 [PMID: 21303979 DOI: 10.1158/0008-5472.CAN-10-3142]
  - 18 **Takahashi Y**, Ishii G, Taira T, Fujii S, Yanagi S, Hishida T, Yoshida J, Nishimura M, Nomori H, Nagai K, Ochiai A. Fibrous stroma is associated with poorer prognosis in lung squamous cell carcinoma patients. *J Thorac Oncol* 2011; **6**: 1460-1467 [PMID: 21849853 DOI: 10.1097/JTO.0b013e318229189d]
  - 19 **Dvorak HF**. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986; **315**: 1650-1659 [PMID: 3537791 DOI: 10.1056/NEJM198612253152606]
  - 20 **Andarawewa KL**, Motrescu ER, Chenard MP, Gansmuller A, Stoll I, Tomasetto C, Rio MC. Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front. *Cancer Res* 2005; **65**: 10862-10871 [PMID: 16322233 DOI: 10.1158/0008-5472.CAN-05-1231]
  - 21 **Dirat B**, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, Wang YY, Meulle A, Salles B, Le Gonidec S, Garrido I, Escourrou G, Valet P, Muller C. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res* 2011; **71**: 2455-2465 [PMID: 21459803 DOI: 10.1158/0008-5472.CAN-10-3323]
  - 22 **Iyengar P**, Combs TP, Shah SJ, Gouon-Evans V, Pollard JW, Albanese C, Flanagan L, Tenniswood MP, Guha C, Lisanti MP, Pestell RG, Scherer PE. Adipocyte-secreted factors synergistically promote mammary tumorigenesis through induction of anti-apoptotic transcriptional programs and proto-oncogene stabilization. *Oncogene* 2003; **22**: 6408-6423 [PMID: 14508521 DOI: 10.1038/sj.onc.1206737]
  - 23 **Iyengar P**, Espina V, Williams TW, Lin Y, Berry D, Jelicks LA, Lee H, Temple K, Graves R, Pollard J, Chopra N, Russell RG, Sasisekharan R, Trock BJ, Lippman M, Calvert VS, Petricoin EF, Liotta L, Dadachova E, Pestell RG, Lisanti MP, Bonaldo P, Scherer PE. Adipocyte-derived collagen VI affects early mammary tumor progression in vivo, demonstrating a critical interaction in the tumor/stroma microenvironment. *J Clin Invest* 2005; **115**: 1163-1176 [PMID: 15841211 DOI: 10.1172/JCI23424]
  - 24 **Motrescu ER**, Blaise S, Etique N, Messaddeq N, Chenard MP, Stoll I, Tomasetto C, Rio MC. Matrix metalloproteinase-11/stromelysin-3 exhibits collagenolytic function against collagen VI under normal and malignant conditions. *Oncogene* 2008; **27**: 6347-6355 [PMID: 18622425 DOI: 10.1038/ncr.2008.218]
  - 25 **Rajala MW**, Scherer PE. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* 2003; **144**: 3765-3773 [PMID: 12933646 DOI: 10.1210/en.2003-0580]
  - 26 **Dirat B**, Bochet L, Escourrou G, Valet P, Muller C. Unraveling the obesity and breast cancer links: a role for cancer-associated adipocytes? *Endocr Dev* 2010; **19**: 45-52 [PMID: 20551667 DOI: 10.1159/000316896]
  - 27 **Tisdale MJ**. Cachexia in cancer patients. *Nat Rev Cancer* 2002; **2**: 862-871 [PMID: 12415256 DOI: 10.1038/nrc927]
  - 28 **Choy L**, Derynck R. Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. *J Biol Chem* 2003; **278**: 9609-9619 [PMID: 12524424 DOI: 10.1074/jbc.M212259200]
  - 29 **Bierie B**, Moses HL. Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat Rev Cancer* 2006; **6**: 506-520 [PMID: 16794634 DOI: 10.1038/nrc1926]
  - 30 **Rønnov-Jessen L**, Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. *Lab Invest* 1993; **68**: 696-707 [PMID: 8515656]
  - 31 **Desmoulière A**, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 1993; **122**: 103-111 [PMID: 8314838 DOI: 10.1083/jcb.122.1.103]
  - 32 **Chen H**, Yang WW, Wen QT, Xu L, Chen M. TGF-beta induces fibroblast activation protein expression; fibroblast activation protein expression increases the proliferation, adhesion, and migration of HO-8910PM [corrected]. *Exp Mol Pathol* 2009; **87**: 189-194 [PMID: 19747910 DOI: 10.1016/j.yexmp.2009.09.001]
  - 33 **Roberts AB**, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci USA* 1986; **83**: 4167-4171 [PMID: 2424019 DOI: 10.1073/pnas.83.12.4167]
  - 34 **Finak G**, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, Chen H, Omeroglu G, Meterissian S, Omeroglu A, Hallett M, Park M. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med* 2008; **14**: 518-527 [PMID: 18438415 DOI: 10.1038/nm1764]
  - 35 **Gaggioli C**. Collective invasion of carcinoma cells: when the fibroblasts take the lead. *Cell Adh Migr* 2008; **2**: 45-47 [PMID: 19262123 DOI: 10.4161/cam.2.1.5705]
  - 36 **Kessenbrock K**, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 2010; **141**: 52-67 [PMID: 20371345 DOI: 10.1016/j.cell.2010.03.015]
  - 37 **Noël A**, Jost M, Maquoi E. Matrix metalloproteinases at cancer tumor-host interface. *Semin Cell Dev Biol* 2008; **19**: 52-60 [PMID: 17625931 DOI: 10.1016/j.semedb.2007.05.011]
  - 38 **Barker HE**, Bird D, Lang G, Erler JT. Tumor-secreted LOXL2 activates fibroblasts through FAK signaling. *Mol Cancer Res* 2013; **11**: 1425-1436 [PMID: 24008674 DOI: 10.1158/1541-7786.MCR-13-0033-T]
  - 39 **Karvonen HM**, Lehtonen ST, Sormunen RT, Lappi-Blanco E, Sköld CM, Kaarteenaho RL. Lung cancer-associated myofibroblasts reveal distinctive ultrastructure and function. *J Thorac Oncol* 2014; **9**: 664-674 [PMID: 24662457 DOI: 10.1097/JTO.0000000000000149]
  - 40 **Horie M**, Saito A, Mikami Y, Ohshima M, Morishita Y, Nakajima J, Kohyama T, Nagase T. Characterization of human lung cancer-associated fibroblasts in three-dimensional in vitro co-culture model. *Biochem Biophys Res Commun* 2012; **423**: 158-163 [PMID: 22634307 DOI: 10.1016/j.bbrc.2012.05.104]



- 41 **Lee HO**, Mullins SR, Franco-Barraza J, Valianou M, Cukierman E, Cheng JD. FAP-overexpressing fibroblasts produce an extracellular matrix that enhances invasive velocity and directionality of pancreatic cancer cells. *BMC Cancer* 2011; **11**: 245 [PMID: 21668992 DOI: 10.1186/1471-2407-11-245]
- 42 **Liao D**, Luo Y, Markowitz D, Xiang R, Reisfeld RA. Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model. *PLoS One* 2009; **4**: e7965 [PMID: 19956757 DOI: 10.1371/journal.pone.0007965]
- 43 **Barnas JL**, Simpson-Abelson MR, Yokota SJ, Kelleher RJ, Bankert RB. T cells and stromal fibroblasts in human tumor microenvironments represent potential therapeutic targets. *Cancer Microenviron* 2010; **3**: 29-47 [PMID: 21209773 DOI: 10.1007/s12307-010-0044-5]
- 44 **Su X**, Ye J, Hsueh EC, Zhang Y, Hoft DF, Peng G. Tumor microenvironments direct the recruitment and expansion of human Th17 cells. *J Immunol* 2010; **184**: 1630-1641 [PMID: 20026736 DOI: 10.4049/jimmunol.0902813]
- 45 **Kobayashi N**, Miyoshi S, Mikami T, Koyama H, Kitazawa M, Takeoka M, Sano K, Amano J, Isogai Z, Niida S, Oguri K, Okayama M, McDonald JA, Kimata K, Taniguchi S, Itano N. Hyaluronan deficiency in tumor stroma impairs macrophage trafficking and tumor neovascularization. *Cancer Res* 2010; **70**: 7073-7083 [PMID: 20823158 DOI: 10.1158/0008-5472.CAN-09-4687]
- 46 **Nieman KM**, Kenny HA, Penicka CV, Ladanyi A, Buell-Gutbrod R, Zillhardt MR, Romero IL, Carey MS, Mills GB, Hotamisligil GS, Yamada SD, Peter ME, Gwin K, Lengyel E. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat Med* 2011; **17**: 1498-1503 [PMID: 22037646 DOI: 10.1038/nm.2492]
- 47 **Gazi E**, Gardner P, Lockyer NP, Hart CA, Brown MD, Clarke NW. Direct evidence of lipid translocation between adipocytes and prostate cancer cells with imaging FTIR microspectroscopy. *J Lipid Res* 2007; **48**: 1846-1856 [PMID: 17496269 DOI: 10.1194/jlr.M700131-JLR200]
- 48 **Kaneko A**, Satoh Y, Tokuda Y, Fujiyama C, Udo K, Uozumi J. Effects of adipocytes on the proliferation and differentiation of prostate cancer cells in a 3-D culture model. *Int J Urol* 2010; **17**: 369-376 [PMID: 20409231 DOI: 10.1111/j.1442-2042.2010.02472.x]
- 49 **Onuma M**, Bub JD, Rummel TL, Iwamoto Y. Prostate cancer cell-adipocyte interaction: leptin mediates androgen-independent prostate cancer cell proliferation through c-Jun NH2-terminal kinase. *J Biol Chem* 2003; **278**: 42660-42667 [PMID: 12902351 DOI: 10.1074/jbc.M304984200]
- 50 **Behan JW**, Yun JP, Proektor MP, Ehsanipour EA, Arutyunyan A, Moses AS, Avramis VI, Louie SG, Butturini A, Heisterkamp N, Mittelman SD. Adipocytes impair leukemia treatment in mice. *Cancer Res* 2009; **69**: 7867-7874 [PMID: 19773440 DOI: 10.1158/0008-5472.CAN-09-0800]
- 51 **Bochet L**, Meulle A, Imbert S, Salles B, Valet P, Muller C. Cancer-associated adipocytes promotes breast tumor radioresistance. *Biochem Biophys Res Commun* 2011; **411**: 102-106 [PMID: 21712027 DOI: 10.1016/j.bbrc.2011.06.101]
- 52 **Gao MQ**, Kim BG, Kang S, Choi YP, Yoon JH, Cho NH. Human breast cancer-associated fibroblasts enhance cancer cell proliferation through increased TGF- $\alpha$  cleavage by ADAM17. *Cancer Lett* 2013; **336**: 240-246 [PMID: 23684931 DOI: 10.1016/j.canlet.2013.05.011]
- 53 **Pardali K**, Moustakas A. Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. *Biochim Biophys Acta* 2007; **1775**: 21-62 [PMID: 16904831 DOI: 10.1016/j.bbcan.2006.06.004]
- 54 **Jia CC**, Wang TT, Liu W, Fu BS, Hua X, Wang GY, Li TJ, Li X, Wu XY, Tai Y, Zhou J, Chen GH, Zhang Q. Cancer-associated fibroblasts from hepatocellular carcinoma promote malignant cell proliferation by HGF secretion. *PLoS One* 2013; **8**: e63243 [PMID: 23667593 DOI: 10.1371/journal.pone.0063243]
- 55 **Cirri P**, Chiarugi P. Cancer associated fibroblasts: the dark side of the coin. *Am J Cancer Res* 2011; **1**: 482-497 [PMID: 21984967]
- 56 **Pavlidis S**, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, Casimiro MC, Wang C, Fortina P, Addya S, Pestell RG, Martinez-Outschoorn UE, Sotgia F, Lisanti MP. The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. *Cell Cycle* 2009; **8**: 3984-4001 [PMID: 19923890 DOI: 10.4161/cc.8.23.10238]
- 57 **Heldin CH**, Rubin K, Pietras K, Ostman A. High interstitial fluid pressure - an obstacle in cancer therapy. *Nat Rev Cancer* 2004; **4**: 806-813 [PMID: 15510161 DOI: 10.1038/nrc1456]
- 58 **Loeffler M**, Krüger JA, Niethammer AG, Reisfeld RA. Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake. *J Clin Invest* 2006; **116**: 1955-1962 [PMID: 16794736 DOI: 10.1172/JCI26532]
- 59 **Li G**, Satyamoorthy K, Herlyn M. N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. *Cancer Res* 2001; **61**: 3819-3825 [PMID: 11325858]
- 60 **Dudley AC**, Shih SC, Cliffe AR, Hida K, Klagsbrun M. Attenuated p53 activation in tumour-associated stromal cells accompanies decreased sensitivity to etoposide and vincristine. *Br J Cancer* 2008; **99**: 118-125 [PMID: 18594537 DOI: 10.1038/sj.bjc.6604465]
- 61 **Folkman J**. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971; **285**: 1182-1186 [PMID: 4938153 DOI: 10.1056/NEJM197111182852108]
- 62 **Cao Y**. Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases. *Nat Rev Drug Discov* 2010; **9**: 107-115 [PMID: 20118961 DOI: 10.1038/nrd3055]
- 63 **Orimo A**, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005; **121**: 335-348 [PMID: 15882617 DOI: 10.1016/j.cell.2005.02.034]
- 64 **Xu LN**, Xu BN, Cai J, Yang JB, Lin N. Tumor-associated fibroblast-conditioned medium promotes tumor cell proliferation and angiogenesis. *Genet Mol Res* 2013; **12**: 5863-5871 [PMID: 24301956 DOI: 10.4238/2013.November.22.14]
- 65 **Nagasaki T**, Hara M, Nakanishi H, Takahashi H, Sato M, Takeyama H. Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour-stroma interaction. *Br J Cancer* 2014; **110**: 469-478 [PMID: 24346288 DOI: 10.1038/bjc.2013.748]
- 66 **Al-Ansari MM**, Hendrayani SF, Tulbah A, Al-Tweigeri T, Shehata AI, Aboussekhra A. p16INK4A represses breast stromal fibroblasts migration/invasion and their VEGF-A-dependent promotion of angiogenesis through Akt inhibition. *Neoplasia* 2012; **14**: 1269-1277 [PMID: 23308058]
- 67 **Pinto MP**, Badtke MM, Dudevoir ML, Harrell JC, Jacobsen BM, Horwitz KB. Vascular endothelial growth factor secreted by activated stroma enhances angiogenesis and hormone-independent growth of estrogen receptor-positive breast cancer. *Cancer Res* 2010; **70**: 2655-2664 [PMID: 20332242 DOI: 10.1158/0008-5472.CAN-09-4373]
- 68 **Rio MC**. From a unique cell to metastasis is a long way to go: clues to stromelysin-3 participation. *Biochimie* 2005; **87**: 299-306 [PMID: 15781316 DOI: 10.1016/j.biochi.2004.11.016]
- 69 **Noël A**, Boulay A, Kebers F, Kannan R, Hajitou A, Calberg-Bacq CM, Basset P, Rio MC, Foidart JM. Demonstration in vivo that stromelysin-3 functions through its proteolytic activity. *Oncogene* 2000; **19**: 1605-1612 [PMID: 10734321 DOI: 10.1038/sj.onc.1203465]
- 70 **Noël AC**, Lefebvre O, Maquoi E, VanHoorde L, Chenard MP, Mareel M, Foidart JM, Basset P, Rio MC. Stromelysin-3 expression promotes tumor take in nude mice. *J Clin Invest* 1996; **97**: 1924-1930 [PMID: 8621777 DOI: 10.1172/JCI118624]
- 71 **Kushiro K**, Chu RA, Verma A, Núñez NP. Adipocytes Promote B16BL6 Melanoma Cell Invasion and the Epithelial-to-Mesenchymal Transition. *Cancer Microenviron* 2012; **5**: 73-82 [PMID: 21892698 DOI: 10.1007/s12307-011-0087-2]



- 72 **Hu M**, Peluffo G, Chen H, Gelman R, Schnitt S, Polyak K. Role of COX-2 in epithelial-stromal cell interactions and progression of ductal carcinoma in situ of the breast. *Proc Natl Acad Sci USA* 2009; **106**: 3372-3377 [PMID: 19218449 DOI: 10.1073/pnas.0813306106]
- 73 **Hu M**, Yao J, Carroll DK, Weremowicz S, Chen H, Carrasco D, Richardson A, Violette S, Nikolskaya T, Nikolsky Y, Bauerlein EL, Hahn WC, Gelman RS, Allred C, Bissell MJ, Schnitt S, Polyak K. Regulation of in situ to invasive breast carcinoma transition. *Cancer Cell* 2008; **13**: 394-406 [PMID: 18455123 DOI: 10.1016/j.ccr.2008.03.007]
- 74 **Raffi S**, Lyden D. S100 chemokines mediate bookmarking of premetastatic niches. *Nat Cell Biol* 2006; **8**: 1321-1323 [PMID: 17139281 DOI: 10.1038/ncb1206-1321]
- 75 **Duda DG**, Duyverman AM, Kohno M, Snuderl M, Steller EJ, Fukumura D, Jain RK. Malignant cells facilitate lung metastasis by bringing their own soil. *Proc Natl Acad Sci USA* 2010; **107**: 21677-21682 [PMID: 21098274 DOI: 10.1073/pnas.1016234107]
- 76 **Majka SM**, Barak Y, Klemm DJ. Concise review: adipocyte origins: weighing the possibilities. *Stem Cells* 2011; **29**: 1034-1040 [PMID: 21544899 DOI: 10.1002/stem.653]
- 77 **Sugimoto H**, Mundel TM, Kieran MW, Kalluri R. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol Ther* 2006; **5**: 1640-1646 [PMID: 17106243 DOI: 10.4161/cbt.5.12.3354]
- 78 **Orimo A**, Weinberg RA. Heterogeneity of stromal fibroblasts in tumors. *Cancer Biol Ther* 2007; **6**: 618-619 [PMID: 18027438 DOI: 10.4161/cbt.6.4.4255]
- 79 **Tang W**, Zeve D, Suh JM, Bosnakovski D, Kyba M, Hammer RE, Tallquist MD, Graff JM. White fat progenitor cells reside in the adipose vasculature. *Science* 2008; **322**: 583-586 [PMID: 18801968 DOI: 10.1126/science.1156232]
- 80 **Rodeheffer MS**, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells in vivo. *Cell* 2008; **135**: 240-249 [PMID: 18835024 DOI: 10.1016/j.cell.2008.09.036]
- 81 **Crossno JT**, Majka SM, Grazia T, Gill RG, Klemm DJ. Rosiglitazone promotes development of a novel adipocyte population from bone marrow-derived circulating progenitor cells. *J Clin Invest* 2006; **116**: 3220-3228 [PMID: 17143331 DOI: 10.1172/JCI28510]
- 82 **Tomiyama K**, Murase N, Stolz DB, Toyokawa H, O'Donnell DR, Smith DM, Dudas JR, Rubin JP, Marra KG. Characterization of transplanted green fluorescent protein+ bone marrow cells into adipose tissue. *Stem Cells* 2008; **26**: 330-338 [PMID: 17975222 DOI: 10.1634/stemcells.2007-0567]
- 83 **Koh YJ**, Kang S, Lee HJ, Choi TS, Lee HS, Cho CH, Koh GY. Bone marrow-derived circulating progenitor cells fail to transdifferentiate into adipocytes in adult adipose tissues in mice. *J Clin Invest* 2007; **117**: 3684-3695 [PMID: 18060029 DOI: 10.1172/JCI32504]
- 84 **Pittenger MF**, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-147 [PMID: 10102814 DOI: 10.1126/science.284.5411.143]
- 85 **Phinney DG**, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells* 2007; **25**: 2896-2902 [PMID: 17901396 DOI: 10.1634/stemcells.2007-0637]
- 86 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]
- 87 **Bianco P**, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008; **2**: 313-319 [PMID: 18397751 DOI: 10.1016/j.stem.2008.03.002]
- 88 **Sera Y**, LaRue AC, Moussa O, Mehrotra M, Duncan JD, Williams CR, Nishimoto E, Schulte BA, Watson PM, Watson DK, Ogawa M. Hematopoietic stem cell origin of adipocytes. *Exp Hematol* 2009; **37**: 1108-1120, 1120.e1-4 [PMID: 19576951 DOI: 10.1016/j.exphem.2009.06.008]
- 89 **Mehrotra M**, Williams CR, Ogawa M, LaRue AC. Hematopoietic stem cells give rise to osteo-chondrogenic cells. *Blood Cells Mol Dis* 2013; **50**: 41-49 [PMID: 22954476 DOI: 10.1016/j.bcmd.2012.08.003]
- 90 **Visconti RP**, Ebihara Y, LaRue AC, Fleming PA, McQuinn TC, Masuya M, Minamiguchi H, Markwald RR, Ogawa M, Drake CJ. An in vivo analysis of hematopoietic stem cell potential: hematopoietic origin of cardiac valve interstitial cells. *Circ Res* 2006; **98**: 690-696 [PMID: 16456103 DOI: 10.1161/01.RES.0000207384.81818.d4]
- 91 **Abangan RS**, Williams CR, Mehrotra M, Duncan JD, LaRue AC. MCP1 directs trafficking of hematopoietic stem cell-derived fibroblast precursors in solid tumor. *Am J Pathol* 2010; **176**: 1914-1926 [PMID: 20167869 DOI: 10.2353/ajpath.2010.080839]
- 92 **Ebihara Y**, Masuya M, LaRue AC, Fleming PA, Visconti RP, Minamiguchi H, Drake CJ, Ogawa M. Hematopoietic origins of fibroblasts: II. In vitro studies of fibroblasts, CFU-F, and fibrocytes. *Exp Hematol* 2006; **34**: 219-229 [PMID: 16459190 DOI: 10.1016/j.exphem.2005.10.008]
- 93 **Ogawa M**, LaRue AC, Drake CJ. Hematopoietic origin of fibroblasts/myofibroblasts: Its pathophysiologic implications. *Blood* 2006; **108**: 2893-2896 [PMID: 16840726 DOI: 10.1182/blood-2006-04-016600]
- 94 **Shirai K**, Sera Y, Bulkeley W, Mehrotra M, Moussa O, LaRue AC, Watson DK, Stuart RK, Lazarchick J, Ogawa M. Hematopoietic stem cell origin of human fibroblasts: cell culture studies of female recipients of gender-mismatched stem cell transplantation and patients with chronic myelogenous leukemia. *Exp Hematol* 2009; **37**: 1464-1471 [PMID: 19786066 DOI: 10.1016/j.exphem.2009.09.008]
- 95 **Ogawa M**, LaRue AC, Mehrotra M. Hematopoietic stem cells are pluripotent and not just "hematopoietic". *Blood Cells Mol Dis* 2013; **51**: 3-8 [PMID: 23453528 DOI: 10.1016/j.bcmd.2013.01.008]
- 96 **Majka SM**, Fox KE, Psilas JC, Helm KM, Childs CR, Acosta AS, Janssen RC, Friedman JE, Woessner BT, Shade TR, Varella-Garcia M, Klemm DJ. De novo generation of white adipocytes from the myeloid lineage via mesenchymal intermediates is age, adipose depot, and gender specific. *Proc Natl Acad Sci USA* 2010; **107**: 14781-14786 [PMID: 20679227 DOI: 10.1073/pnas.1003512107]
- 97 **Hong KM**, Burdick MD, Phillips RJ, Heber D, Strieter RM. Characterization of human fibrocytes as circulating adipocyte progenitors and the formation of human adipose tissue in SCID mice. *FASEB J* 2005; **19**: 2029-2031 [PMID: 16188961 DOI: 10.1096/fj.05-4295fje]
- 98 **Dunphy JE**. The fibroblast-a ubiquitous ally for the surgeon. *NEJM* 1963; **268**: 1367-1377 [DOI: 10.1056/nejm196306202682501]
- 99 **Quante M**, Tu SP, Tomita H, Gonda T, Wang SS, Takashi S, Baik GH, Shibata W, Diprete B, Betz KS, Friedman R, Varro A, Tycko B, Wang TC. Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. *Cancer Cell* 2011; **19**: 257-272 [PMID: 21316604 DOI: 10.1016/j.ccr.2011.01.020]
- 100 **Xing F**, Saidou J, Watabe K. Cancer associated fibroblasts (CAFs) in tumor microenvironment. *Front Biosci (Landmark Ed)* 2010; **15**: 166-179 [PMID: 20036813 DOI: 10.2741/3613]
- 101 **Gonda TA**, Varro A, Wang TC, Tycko B. Molecular biology of cancer-associated fibroblasts: can these cells be targeted in anti-cancer therapy? *Semin Cell Dev Biol* 2010; **21**: 2-10 [PMID: 19840860 DOI: 10.1016/j.semcdb.2009.10.001]
- 102 **McDonald LT**, LaRue AC. Hematopoietic stem cell derived carcinoma-associated fibroblasts: a novel origin. *Int J Clin Exp Pathol* 2012; **5**: 863-873 [PMID: 23119103]
- 103 **LaRue AC**, Masuya M, Ebihara Y, Fleming PA, Visconti RP, Minamiguchi H, Ogawa M, Drake CJ. Hematopoietic origins of fibroblasts: I. In vivo studies of fibroblasts associated with solid tumors. *Exp Hematol* 2006; **34**: 208-218 [PMID: 16459189 DOI: 10.1016/j.exphem.2005.10.009]
- 104 **Charrière G**, Cousin B, Arnaud E, André M, Bacou F, Penicaud



- L, Casteilla L. Preadipocyte conversion to macrophage. Evidence of plasticity. *J Biol Chem* 2003; **278**: 9850-9855 [PMID: 12519759 DOI: 10.1074/jbc.M210811200]
- 105 **Chazenbalk G**, Bertolotto C, Heneidi S, Jumabay M, Trivax B, Aronowitz J, Yoshimura K, Simmons CF, Dumesic DA, Azziz R. Novel pathway of adipogenesis through cross-talk between adipose tissue macrophages, adipose stem cells and adipocytes: evidence of cell plasticity. *PLoS One* 2011; **6**: e17834 [PMID: 21483855 DOI: 10.1371/journal.pone.0017834]
- 106 **Jotzu C**, Alt E, Welte G, Li J, Hennessy BT, Devarajan E, Krishnappa S, Pinilla S, Droll L, Song YH. Adipose tissue derived stem cells differentiate into carcinoma-associated fibroblast-like cells under the influence of tumor derived factors. *Cell Oncol (Dordr)* 2011; **34**: 55-67 [PMID: 21327615 DOI: 10.1007/s13402-011-0012-1]
- 107 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638 [PMID: 12711737 DOI: 10.1056/NEJMoa021423]
- 108 **van Kruijsdijk RC**, van der Wall E, Visseren FL. Obesity and cancer: the role of dysfunctional adipose tissue. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 2569-2578 [PMID: 19755644 DOI: 10.1158/1055-9965.EPI-09-0372]
- 109 **Engfeldt P**, Arner P. Lipolysis in human adipocytes, effects of cell size, age and of regional differences. *Horm Metab Res Suppl* 1988; **19**: 26-29 [PMID: 3069692]
- 110 **Fain JN**, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* 2004; **145**: 2273-2282 [PMID: 14726444 DOI: 10.1210/en.2003-1336]
- 111 **Rhim AD**, Oberstein PE, Thomas DH, Mirek ET, Palermo CF, Sastra SA, Dekleva EN, Saunders T, Becerra CP, Tattersall IW, Westphalen CB, Kitajewski J, Fernandez-Barrena MG, Fernandez-Zapico ME, Iacobuzio-Donahue C, Olive KP, Stanger BZ. Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. *Cancer Cell* 2014; **25**: 735-747 [PMID: 24856585 DOI: 10.1016/j.ccr.2014.04.021]
- 112 **Özdemir BC**, Pentcheva-Hoang T, Carstens JL, Zheng X, Wu CC, Simpson TR, Laklai H, Sugimoto H, Kahlert C, Novitskiy SV, De Jesus-Acosta A, Sharma P, Heidari P, Mahmood U, Chin L, Moses HL, Weaver VM, Maitra A, Allison JP, LeBleu VS, Kalluri R. Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer Cell* 2014; **25**: 719-734 [PMID: 24856586 DOI: 10.1016/j.ccr.2014.04.005]
- 113 **Madar S**, Goldstein I, Rotter V. 'Cancer associated fibroblasts' -- more than meets the eye. *Trends Mol Med* 2013; **19**: 447-453 [PMID: 23769623 DOI: 10.1016/j.molmed.2013.05.004]
- 114 **Brennen WN**, Rosen DM, Wang H, Isaacs JT, Denmeade SR. Targeting carcinoma-associated fibroblasts within the tumor stroma with a fibroblast activation protein-activated prodrug. *J Natl Cancer Inst* 2012; **104**: 1320-1334 [PMID: 22911669 DOI: 10.1093/jnci/djs336]
- 115 **Brennen WN**, Isaacs JT, Denmeade SR. Rationale behind targeting fibroblast activation protein-expressing carcinoma-associated fibroblasts as a novel chemotherapeutic strategy. *Mol Cancer Ther* 2012; **11**: 257-266 [PMID: 22323494 DOI: 10.1158/1535-7163.MCT-11-0340]
- 116 **Hofheinz RD**, al-Batran SE, Hartmann F, Hartung G, Jäger D, Renner C, Tanswell P, Kunz U, Amelsberg A, Kuthan H, Stehle G. Stromal antigen targeting by a humanised monoclonal antibody: an early phase II trial of sibtrotuzumab in patients with metastatic colorectal cancer. *Onkologie* 2003; **26**: 44-48 [PMID: 12624517 DOI: 10.1159/000069863]
- 117 **Liu R**, Li H, Liu L, Yu J, Ren X. Fibroblast activation protein: A potential therapeutic target in cancer. *Cancer Biol Ther* 2012; **13**: 123-129 [PMID: 22236832 DOI: 10.4161/cbt.13.3.18696]
- 118 **Pilling DP**, Gomer RH. Regulatory pathways for fibrocyte differentiation. In: Bucala R, editor. Fibrocytes: New insights into tissue repair and systemic fibroses. Yale University, USA: World Scientific, 2007: 37-60
- 119 **Bucala R**, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med* 1994; **1**: 71-81 [PMID: 8790603]

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## Role of nanotopography in the development of tissue engineered 3D organs and tissues using mesenchymal stem cells

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### Abstract

Recent regenerative medicine and tissue engineering strategies (using cells, scaffolds, medical devices and gene therapy) have led to fascinating progress of translation of basic research towards clinical applications. In the past decade, great deal of research has focused on developing various three dimensional (3D) organs, such as bone, skin, liver, kidney and ear, using such strategies in order to replace or regenerate damaged organs for the purpose of maintaining or restoring organs' functions that may have been lost due to aging, accident or disease. The surface properties of a material or a device are key aspects in determining the success of the implant in biomedicine, as the majority of biological reactions in human body occur on surfaces or interfaces. Furthermore, it has been established in the literature that cell adhesion and proliferation are, to a great extent, influenced by the micro- and nano-surface characteristics of biomaterials and devices. In addition, it has been shown that the functions of stem cells, mesenchymal stem cells in particular, could be regulated through physical interaction with specific nanotopographical cues. Therefore, guided stem cell proliferation, differentiation and function are of great importance in the regeneration of 3D tissues and organs using tissue engineering strategies. This review will provide an update on the impact of nanotopography on mesenchymal stem cells for the purpose of developing laboratory-based 3D organs and tissues, as well as the most recent research and case studies on this topic.

**Key words:** Nanotopography; Mesenchymal stem cells; Tissue engineering; Nanotechnology; Three dimensional organs/tissues; Scaffolds

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**Core tip:** Tissue engineering and nanotechnology are both exciting fields that have enormous potentials to revolutionise medicine as we know it today. Use of nanotechnology is an attractive and effective way to control and direct biological events at cellular levels. Nanoscale architecture plays a pivotal role directing cellular activities. Here, the use of nanotopography for the purpose of 3D organ/tissue regeneration using mesenchymal stem cells (*i.e.*, their proliferation, differentiation and function), is reviewed by investigating the most recent, innovative, and effective studies in this field.

Salmasi S, Kalaskar DM, Yoon WW, Blunn GW, Seifalian AM. Role of nanotopography in the development of tissue engineered 3D organs and tissues using mesenchymal stem cells. *World J Stem Cells* 2015; 7(2): 266-280 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i2/266.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i2.266>

## INTRODUCTION

It is becoming progressively evident that with an ever increasingly older population and high costs associated with meeting the healthcare demands<sup>[1]</sup> as well as the shortage of organs and effective therapeutic methods<sup>[2]</sup>, the field of medicine has to move towards cutting edge, laboratory engineered techniques and devices if, we are to avoid a drastic world-wide healthcare collapse in the near future. To this end, the fields of nanotechnology, regenerative medicine and tissue engineering (TE) are expanding at a rapid pace. Recent regenerative medicine and TE strategies (using cells, scaffolds, medical devices and gene therapy) have led to fascinating progress of translation of basic research towards clinical applications<sup>[1,3]</sup>. In the past decade, great deal of research has focused on developing various three dimensional (3D) organs, such as bone<sup>[4]</sup>, skin<sup>[5]</sup>, liver<sup>[6]</sup>, kidney<sup>[7]</sup>, and ear<sup>[8]</sup>, using such strategies in order to replace or regenerate damaged organs for the purpose of maintaining or restoring organs' functions.

Human organs are responsible for various important functions of the body including, but not limited to, digesting food, serving as a barrier against infections, recognising and coordinating the body's response to its internal and external environmental changes, and providing oxygen (to be used for cellular respiration) as well as removing excess carbon dioxide. They are also responsible for maintaining homeostasis, transmission of information and generating force<sup>[3]</sup>. In cases when one or a few of the organs are severely damaged, to an extent that they are no longer capable of reconstructing or regenerating themselves, tissue engineering and nanotechnology based strategies, using previously established knowledge on cellular behaviour<sup>[9,10]</sup>,

could be employed to develop and construct tailored therapies, devices, or even whole organs. To this end, nanostructured, bio-inspired, or biological materials have attracted a great deal of attention as they poses unique chemical, mechanical and surface characteristics that could prove useful for organ or tissue TE.

The surface properties of a material or a device are key aspects in determining the success of the implant in biomedicine, as the majority of biological reactions in human body occur on surfaces or interfaces<sup>[11]</sup>. Furthermore, it has been established in the literature that cell adhesion and proliferation are, to a great extent, influenced by the micro- and nano-surface characteristics of biomaterials and devices<sup>[12,13]</sup>. In addition, it has been shown that the stem cells', mesenchymal stem cells (MSCs) in particular, functions can be regulated through physical interaction with specific nanotopographical cues<sup>[3,14]</sup>, further indicating the importance of surface characteristics at nanometre length scale. Therefore, guided stem cell proliferation, differentiation and function are of great importance in the regeneration of 3D organs and tissues using TE strategies.

This review will provide an update on the impact of nanotopography on MSCs for the purpose of developing laboratory-based 3D organs, as well as the most recent research and case studies on this topic.

## NANOFABRICATION OF 3D SCAFFOLDS WITH STEM CELLS

Previously, most research and investigations focused on growing cells in a petri dish (2D). However, in nature cells use 3D template of extracellular matrix (ECM) to shape functional tissues<sup>[15]</sup>. Micro- and nano-scale chemical and physical cues from the ECM environment control and direct various key cell behaviours including their adhesion, proliferation, migration and differentiation<sup>[16-18]</sup>. Therefore, the construction of a synthetic system that mimics the natural ECM and its component has become a field of topical interest<sup>[19]</sup>.

Recent investigations have shown rapid success in TE of sophisticated and complex nanoenvironments suitable for 3D growth of stem cells for the purpose of organ and tissue regeneration<sup>[19-22]</sup>. So far, various biofabrication techniques have been developed and employed to design an ideal 3D synthetic ECM-mimetic system that resembles the architecture and mechanical properties of the natural ECM<sup>[3]</sup>. Natural or synthetic polymers are used as scaffold materials and, depending on their nature, suitable biofabrication techniques are used to create a 3D environment with nanotopographical cues that can lead to controlled and directed growth and differentiation of stem cells toward a specific tissue or organ regeneration. Numerous studies have covered the currently available fabrication techniques for natural or synthetic polymers<sup>[15,23-27]</sup>. In general, the available fabrication techniques can



**Table 1** Classification of various types of nanotopography (nanofabrication) methods

Energy source	Method	Mechanism and final outcome	Processable polymers
Thermal	Replica modelling	Creating negative shape of the mold by thermal cross-linking of cavity-filled pre-polymer	Thermocurable polymers, <i>e.g.</i> , poly(dimethyl siloxane)
	Nanoimprint lithography	Creating negative shape of the mold by plastic deformation of polymer above T <sub>g</sub>	Thermoplastic, <i>e.g.</i> , polystyrene, poly(lactic acid), and conductive polymers, <i>e.g.</i> , polyaniline and polypyrrole
Optical	Block copolymer lithography	Creating nanoscale hole, line and lamellar structures by microphase separation of two immiscible polymers	Block copolymer, <i>e.g.</i> , polystyrene-block-poly(methyl methacrylate), styrenebutadiene-styrene
	Photolithography	Depending on mask design and selective UV exposure, solubility is changed	Photo curable polymers, <i>e.g.</i> , photoresist, polyurethane-based
	E-beam lithography	Formation of arbitrary patterns using different electron beam pathways and selective irradiation of focused electron beams to change solubility	E-beam sensitive polymers, <i>e.g.</i> , polymethyl methacrylate
	Direct laser writing	Formation of arbitrary patterns by selective cross-linking of the polymer by laser irradiation	Photo-curable polymers
Chemical	Microcontact printing	Creating extruded patterns of elastomeric stamp using relative surface energy difference needed for transferring materials	Proteins and self-assembled monolayers
	Dip-pen lithography	Formation of arbitrary patterns by direct writing of molecules with a sharp tip	Self-assembled monolayers
	Salt leaching/gas foaming	Formation of a block of polymer with voids by dissolution of salt particles (salt leaching) and/or bubble formation in the polymer block (gas foaming)	Solvent soluble polymers, <i>e.g.</i> , thermoplastic and conductive ones
Electrical	Electrochemical deposition	Forming negatively shaped molds by electrochemical reduction of the polymer	Conductive polymers
	Electrospinning	Drawing a three dimensional nanofibrous mesh from the polymer solution using an electric field	Solvent soluble polymers
Physical	Capillary force lithography	Formation of partially filled negative shape of the mold by capillary rise of thermoplastic polymer above T <sub>g</sub>	Thermoplastic and solvent soluble polymers
	Micromolding in capillaries	Creating a negative shape of the mold by capillary-driven microchannel filling	Solvent soluble polymers
	Wrinkle	Formation of random or aligned micro- or nanolines using mechanical buckling Mechanical buckling between elastic substrate and rigid film	Elastomeric polymers, <i>e.g.</i> , polydimethylsiloxane
	Crack	Formation of aligned or inter-crossing line patterns by mechanical fracturing of the stiff film adhered onto elastic substrate	Elastomeric polymers

Adapted from Kim *et al.*<sup>[3]</sup>.

be classified into different categories based on their energy source, *i.e.*, thermal, optical, physical, chemical or electrical (Table 1). It is beyond the scope of this paper to review the different available techniques in each of these categories, however, a review conducted by Kim *et al.*<sup>[3]</sup> could be referred to for further and in detail information on this topic.

## CHARACTERISTICS OF MSCs AND THEIR APPLICATION IN TE OF 3D ORGANS

An extensive number of studies have demonstrated the great potentials of using MSCs for TE approaches<sup>[28-33]</sup>. Among many advantageous characteristics of MSCs the fact that these cells can be isolated from several tissues and that they have the potential to be expanded in culture and exhibit multilineage differentiation (Figure 1) make MSCs a highly interesting stem cell source for TE and regenerative medicine research<sup>[31]</sup>. Other interesting properties of MSCs include; their ability to self-renew, modulate immune responses, and their availability (they can be obtained from a small scale aspirate of bone marrow or adipose tissues)<sup>[34-36]</sup>. Furthermore,

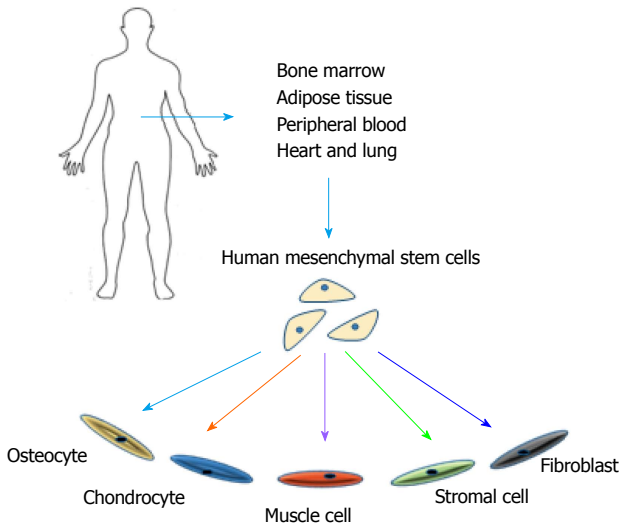
MSCs can be isolated from adults, therefore, allogeneic transplant of these cells would eliminate raising ethical issues in regards to their use in TE and regenerative medicine<sup>[36]</sup>.

Among most potential characteristics of MSCs, it is probably their ability of multilineage differentiation that is mostly exploited for TE and regenerative medicine purposes. The differentiation of MSCs is controlled by some regulatory genes and induction chemicals that lead to the specific differentiation of these progenitor cells<sup>[37,38]</sup>. In addition to growth factors and induction chemicals, various biomaterials (*i.e.*, natural and synthetic polymers) are used to provide appropriate scaffolding for the proliferation and differentiation of MSCs for the purpose of reconstruction of several hard and soft tissues and organs, such as bone, cartilage, tendons, and skin<sup>[39,40]</sup> (Figure 2).

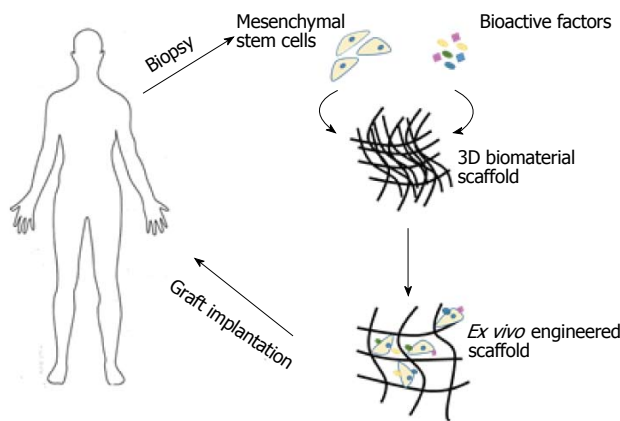
## THE ROLE OF NANOTOPOGRAPHY ON THE GROWTH AND PROLIFIRATION OF MSCS

As mentioned earlier, surface nanotopography of





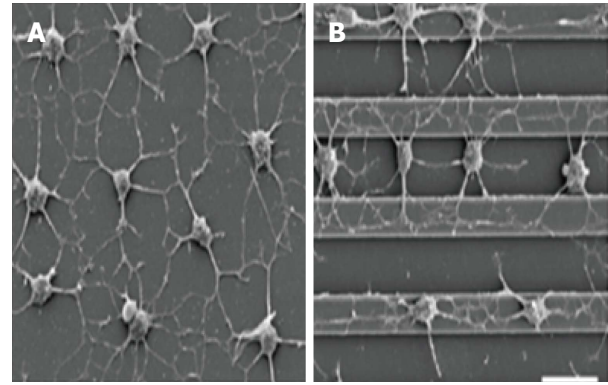
**Figure 1 Potentials and sources of mesenchymal stem cells.** Mesenchymal stem cells can be collected from various sources within human body and have the ability to differentiate into a variety of lineages.



**Figure 2 Overview of tissue engineering strategy of incorporating scaffolds with mesenchymal stem cells.** Mesenchymal stem cells (derived directly from the patient) are expanded in the laboratory, whereby the necessary environment for their growth has been prepared. These cells are then seeded onto a scaffold and either allowed to differentiate *ex vivo* pre-implantation or the scaffold is immediately implanted.

biomaterials can evoke specific cellular responses. Materials with unique nanotopographical characteristics offer properties, similar to growth factors, which can be used to induce specific biological performances of safe and cost effective manners in the human body<sup>[41]</sup>. Previous studies show that various nanotopographical cues can potentially impact the adhesion<sup>[42,43]</sup>, orientation<sup>[44]</sup>, and cytoskeletal organisation<sup>[45]</sup> of MSCs as well as their self-renewal<sup>[46]</sup>, proliferation and differentiation<sup>[41]</sup>. Furthermore, nanotopographical cues could influence morphology, migratory capacity, gene expression and subsequently the fate of MSCs<sup>[47,48]</sup>.

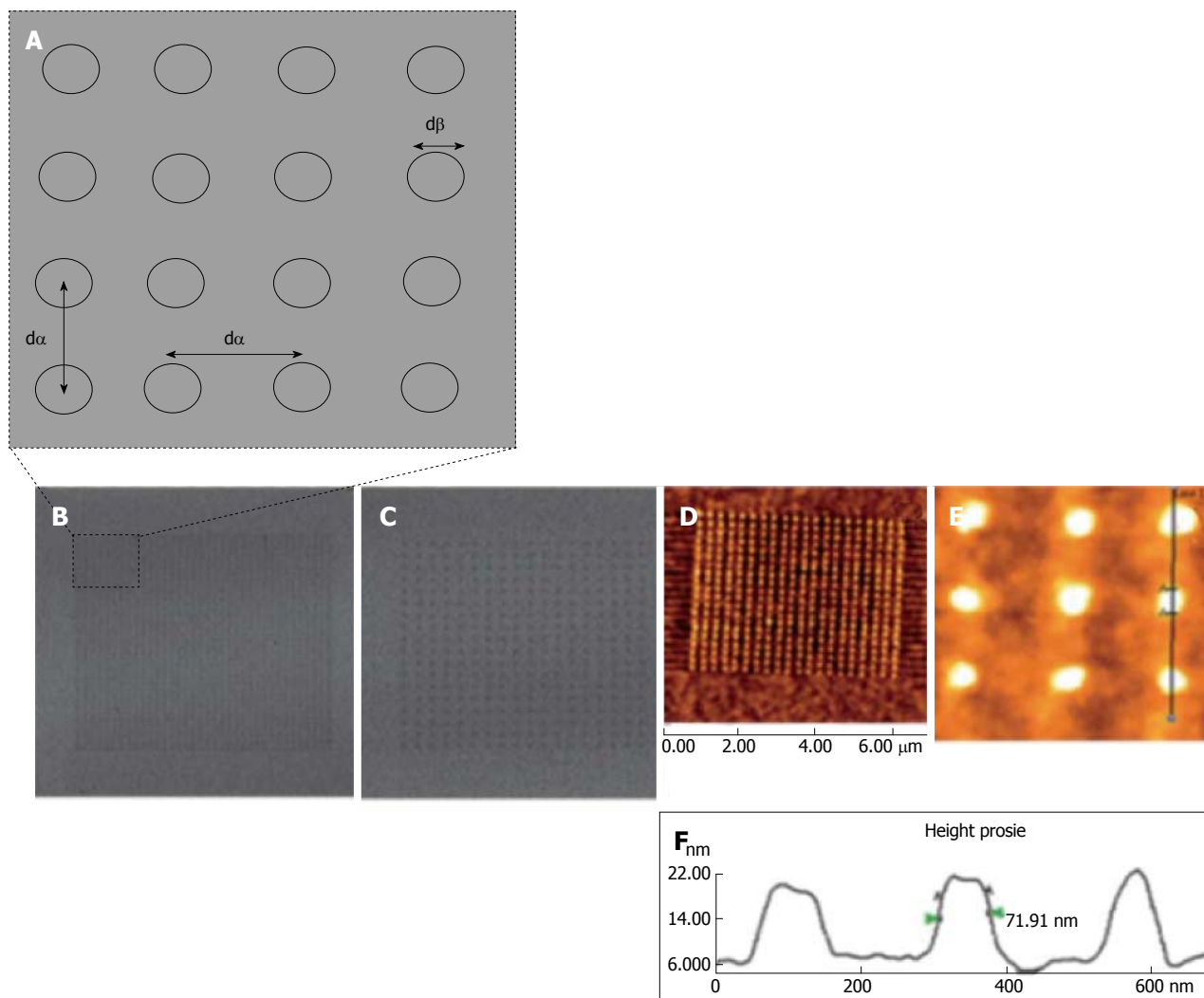
It has been shown that nanofeatures including nanopits, nanogratings and nanoprotusions have the potentials to influence the cell morphology, proliferation and differentiation of MSCs<sup>[49,50]</sup> (Figure



**Figure 3 Comparison of different topography strategies employed to investigate the effects of anisotropic vs isotropic cytoskeletal tension on cultured mesenchymal stem cells.** (A) nonpatterned substrates caused randomly oriented cell protrusions to be formed, while (B) alignment of elaborated processes in the direction of the grooves were induced by micropatterned surfaces, mimicking the native structure and orientation of the natural extracellular matrix proteins<sup>[52]</sup>.

3). For instance, it has been shown that homogeneously nanopatterned and chemically modified surfaces can have direct effect on cellular responses of MSCs, including their self-renewal abilities, control over their initial cell interactions and subsequently their cell phenotype, by creating arrays of nanodots using dip pen nanolithography<sup>[51]</sup> (Figure 4). Furthermore, differentiation and proliferation of human MSCs (hMSCs) were investigated on nanogratings of 350 nm width combined with biochemical cues such as retinoic acid, and it was shown that synthetic nanostructures can induce hMSCs to differentiate into neuronal lineage<sup>[52]</sup>. This study, conducted by Yim *et al.*<sup>[52]</sup>, also confirmed the significance of nanotopography as it revealed that retinoic acid alone on unpatterned surfaces did not lead to strong neuronal marker expression as it was shown on surfaces with nanogratings. Other nanopatterned structures, such as grooves, ridges, and pores as well as holes, nodes, or rods are of other commonly techniques currently employed to change unpatterned surfaces for MSCs to grow on and to direct their cellular responses<sup>[49]</sup>. Such nanostructures have great applications to all areas of TE. For instance, Andersen *et al.*<sup>[53]</sup> investigated adhering nanoparticles containing different small-interfering RNAs (siRNAs) into nanostructured scaffolds consisting of nanopores and reported of spatial retention of the RNAs within nanopores seeded with MSCs, which resulted in enhanced osteogenic and adipogenic differentiation of MSCs<sup>[53]</sup>. This is an exciting finding as the ability of directing a single type of differentiation plays a crucial role in developing specific 3D organs. In another study, Watari *et al.*<sup>[54]</sup> used topographically-patterned substrates containing anisotropically ordered ridges and grooves to modulate osteogenic differentiation in hMSCs<sup>[54]</sup>. They reported that hMSCs cultured on 1400 or 4000 nm pitches, compared to those seeded on 400 nm pitch or planer control, exhibit better elongation





**Figure 4** Nanopatterned gold surfaces examination for the effect of both the nanotopography and terminating chemical functionality. A: Nanopatterned surfaces used for mesenchymal stem cell control and differentiation exhibiting dot to dot pitch ( $d\alpha$ ) and dot diameter ( $d\beta$ ); B: Lateral Force Microscopy (LFM) image of small area 280 nm pitch array; C: LFM image of 140 nm pitch array; D-F: Atomic force microscopy topographical image of an alkanethiol resist array fabricated on gold surface following chemical etching. An average diameter feature ( $d\beta$ ) of 70 nm was shown on the cursor profile<sup>[49]</sup>.

and alignment, while they showed a significant decrease in Runt-related transcription factor 2 (RUNX2) and bone gamma-carboxyglutamic acid-containing protein (BGLAP) expression. Their data also revealed that 400 nm pitch increased extracellular calcium deposition. Watari *et al.*<sup>[54]</sup> concluded that specific size scale of topographic cues could directly influence the osteogenic differentiation of hMSCs both with and without osteogenic agents. This is another important finding that could enable one to manipulate and develop nanostructures that could lead to controlled and directed differentiation of stem cells for the purpose of TE of 3D organs. Very recently, the effect of topographical design, in the form of nano-pillar, nano-hole and nano-grill, on hMSCs were investigated by Wu *et al.*<sup>[55]</sup> in which these nanotopographies were applied onto a polycaprolactone surface using thermal nanoimprinting. Their findings revealed that nanotopographical patterns trigger changes in the morphology and cytoskeletal structure of hMSCs. They

also found that, compared to non-patterned surfaces, nano-pillar and nano-hole topography determined MSCs chondrogenesis, resulting in specific cartilage formation. Furthermore, Kilian *et al.*<sup>[56]</sup> showed that geometric nanotopography cues, that increase actomyosin contractility, could influence and direct the osteogenesis of bone marrow-derived hMSCs. Such geometric cues direct and control mechanochemical signals and paracrine/autocrine factors necessary for specific differentiation of MSCs, also observed during the *in vivo* investigation of the microenvironment of the differentiated cells.

## CASE STUDIES ON THE APPLICATION OF NANOTOPOGRAPHY GUIDED TE OF 3D ORGANS/TISSUES USING MSCS

### Bone

Reconstruction of large bone defects caused by



surgery, trauma or tumours are common deficiencies, which present a significant medical challenge<sup>[57]</sup>. Autologous bone grafting is the gold standard for treating bone defects, but obstacles such as limited sources of tissue, and bone resorption before bone healing, have raised interests in synthetic materials as potential bone substitutes<sup>[58]</sup>. Furthermore, bone grafting has proven challenging for large bone defects reconstruction<sup>[59]</sup>. This is mainly due to difficulties in harvesting enough bone grafts from a healthy bone, potential postoperative pain, risk of infection, risk of hypersensitivity, risk of paresthesia, and time constraints<sup>[60,61]</sup>. TE, using stem cells, provides the opportunity to avoid the established drawbacks of bone graft materials for the purpose of reconstructing or regenerating bone defects at variety of scales. MSCs, because of their interesting properties, have been demonstrated as an attractive cell source for bone TE applications<sup>[62]</sup>. Controlled and directed differentiation of MSCs into osteoblasts (bone cells) is therefore a key aspect of this process. As mentioned above, nanotopographical cues could be used to influence MSCs cell behaviour and differentiation toward specific lineages. For instance, in a very recent study, McCafferty *et al.*<sup>[63]</sup> demonstrated the use of nanotopography to induce osteogenic differentiation of human bone marrow derived MSCs. They sputter deposited thin films of bioactive calcium phosphate onto a polycrystalline titanium nanostructured surface. These sputter deposited surfaces supported high levels of bone marrow-derived hMSCs proliferation and adhesion, determined by DNA quantification. Moreover, they were also able to directly promote significant levels of osteogenic differentiation. In this study, gene expression, alkaline phosphatase activity and immunocytochemical localisation of key osteogenic markers showed that the nanostructured titanium surfaces and the bioactive calcium phosphate coatings could direct differentiation towards an osteogenic lineage. The addition of the calcium phosphate chemistry to the topographical profile of the titanium was found to induce increased bone marrow-derived hMSCs differentiation compared to that observed for either the titanium or calcium phosphate coating without an underlying nanostructure. Therefore, the results presented a clear benefit from a surface engineering strategy that combines a defined nanoscale surface topography with a conformal bioactive chemistry. In another study by de Peppo *et al.*<sup>[64]</sup> osteogenic response of hMSCs to titanium-coated hemisphere-like topographic nanostructures of 50, 100, and 200 nm was assessed. Their aim was to look at the influence of different sizes of nanoscale topographies on the morphology, proliferation, and osteogenic differentiation of hMSCs *in vitro*. Here, the nanostructuring was fabricated using colloidal lithography and the desired structure sizes were achieved by etching the original 200 nm polymeric

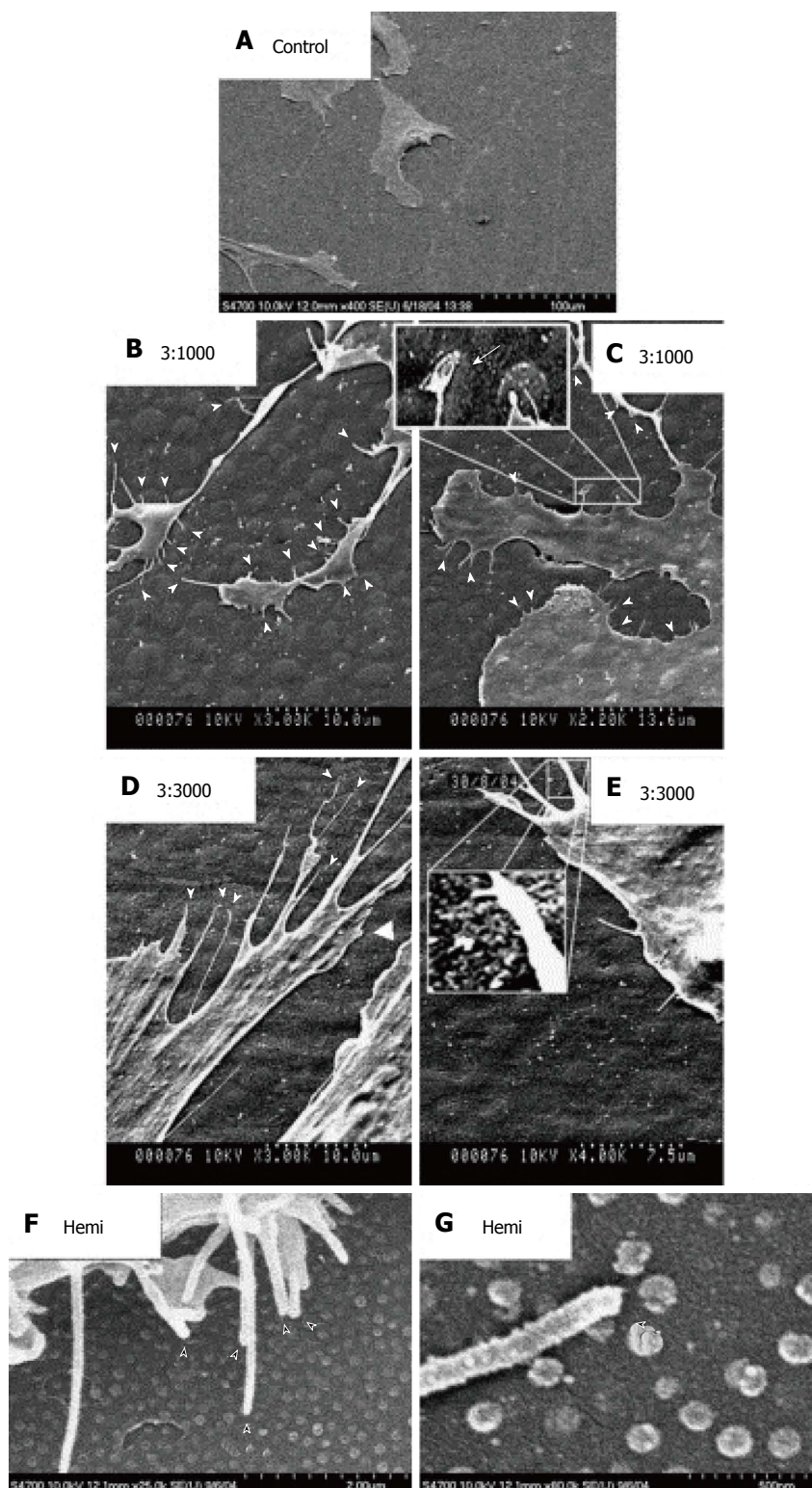
particles (polystyrene particles) and further heat-treating them above the transition temperature of polystyrene (118 °C), to create semispheroidal nanoparticles to increase their surface attachment abilities. Their results showed that there is a direct relationship between the proliferation and osteogenic differentiation of hMSCs and the size of the underlying structures, demonstrating that by varying the scale of the nanotopographic features at nanoscale, one can control the osteogenic behaviour of hMSCs. de Peppo *et al.*<sup>[64]</sup> concluded that colloidal lithography in combination with coating technologies can enable structuring well defined nanoscale topographies to control and direct hMSCs growth and differentiation. Dalby *et al.*<sup>[65]</sup> also investigated the osteogenic response of hMSCs to semi-ordered and random nanotopographies performed by colloidal lithography and polymer demixing on silicon and showed that hMSCs react robustly to nanotopographic features down to 10 nm in size with a low aspect ratio<sup>[65]</sup>. In this study, scanning electron microscopy of primary hMSCs on flat controls and scaffolds with nanotopographic structures showed that hMSCs exhibited strong reaction to nanofeatures as their filopodia extended and curled around these features (Figure 5). Dalby *et al.*<sup>[65]</sup> concluded that their recorded osteogenic response of hMSCs to nanotopographies could be employed to construct and design scaffolds with an appropriate osteogenic “environment” instead of planar control structures in order to direct and control MSCs growth and differentiation.

Furthermore, Rosa *et al.*<sup>[66]</sup> examined the osteoinductive potential of titanium (Ti) surfaces with nanotopographic features, yield by chemically treating polished Ti discs with H<sub>2</sub>SO<sub>4</sub>/H<sub>2</sub>O<sub>2</sub>, and cultured them with rat MSCs under osteogenic and non-osteogenic conditions. Untreated polished Ti surfaces were used as controls. Their findings revealed that Ti surfaces with nanotopography boosted cell proliferation and alkaline phosphatase (Alp) activity of rat MSCs under both osteogenic and non-osteogenic conditions (Figure 6). They also demonstrated that nanotopography upregulated the gene expression of major bone markers under both of the test conditions. Interestingly, they noticed that obtustatin, an  $\alpha$ 1 $\beta$ 1 integrin inhibitor, was able to reduce higher gene expression of key bone markers and Alkaline Phosphatase (ALP) activity on Ti Scaffolds with nanotopographic features. Therefore, suggesting that obtustatin signalling pathway plays a crucial role in determining the osteoinductive effect of nanotopography on MSCs, a finding that can be exploited as a novel mechanism of accelerating and/or enhancing MSCs osseointegration for the purpose of TE of complex organs or tissues in the future.

### Cartilage

Cartilage defects, caused by osteoarthritis, trauma or sport, are considered serious clinical problems. So far,





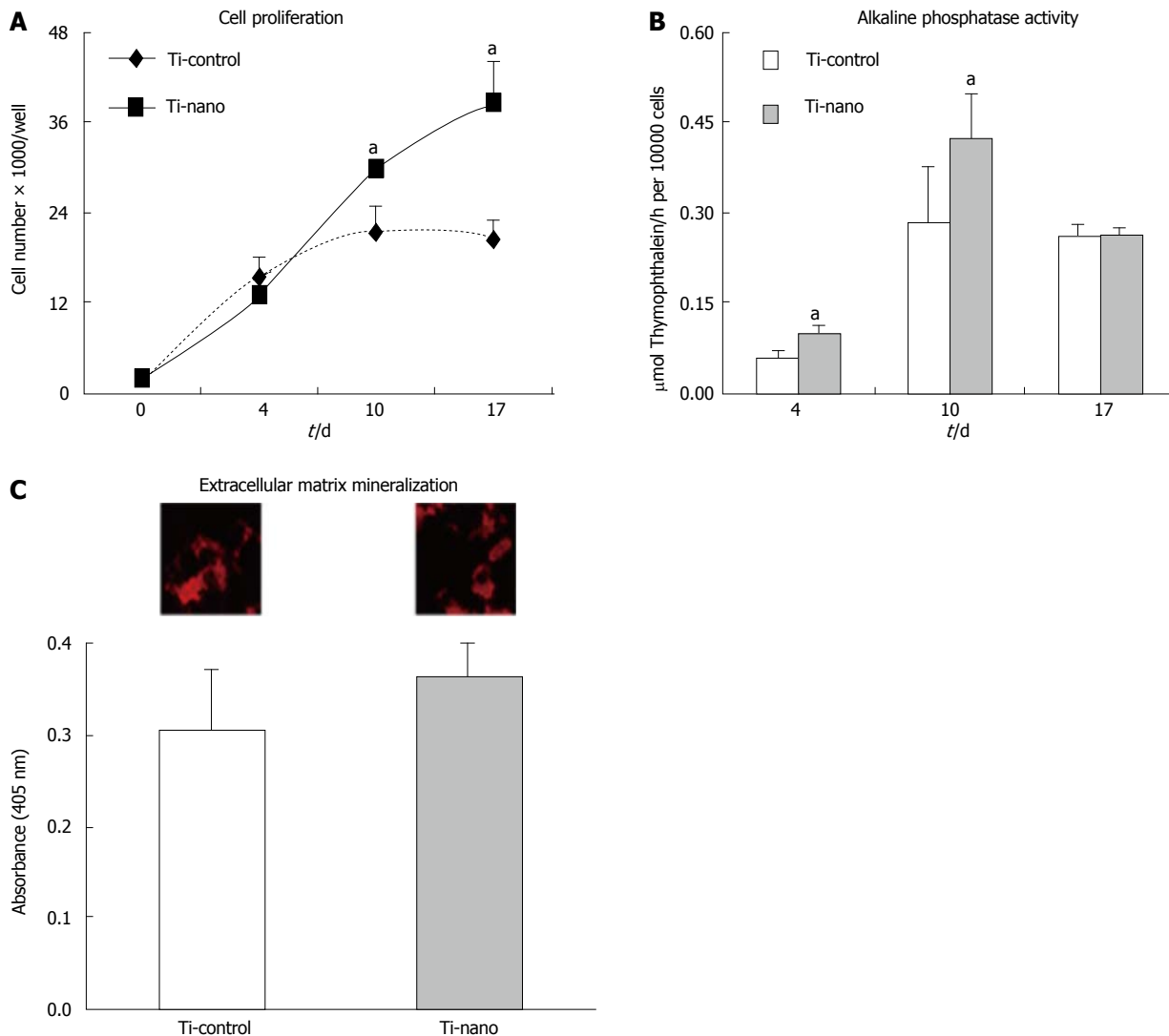
**Figure 5** Scanning electron micrographs of human mesenchymal stem cells cultured on control and test materials. A: On planar control materials cells showed normal morphologies; B, C: Filopodial of the human mesenchymal stem cells (hMSCs) interacts with the 3:1000 substrates (arrowheads); C: hMSC filopodia are curving around an island; D, E: Filopodial interactions with the 3:3000 substrates (arrowheads); E: A filopodia curving around an island is clearly observed; F: filopodial interactions with the hemi substrates (arrowheads); G: Filopodia curving around a hemisphere (arrowhead)<sup>[65]</sup>.

TE of cartilage has proven to be much more difficult than many other organs or tissues, due to cartilage's inherently poor regenerative ability<sup>[67]</sup>. Therefore, developing a functional TE system, capable of improving the regenerative ability of this tissue, would be of great interest. Most research on this field have been focused on using polymeric scaffolds with stem cells, in particular MSCs<sup>[8,67-69]</sup>. MSCs are considered the "gold standard" of stem cell source for cartilage TE as their

differentiation to chondrocytes can be easily controlled and directed using various techniques, in particular nanotopography<sup>[8,55]</sup>.

Previous studies have shown that a more rounded, spheroidal cell shape can enhance the rate of chondrogenesis, through increasing the expression of chondrocyte-related genes, markers, and proteins<sup>[70,71]</sup>. Based on this, Zhong *et al.*<sup>[72]</sup> attempted to create a microenvironment suitable for MSCs fibrochondrogenesis





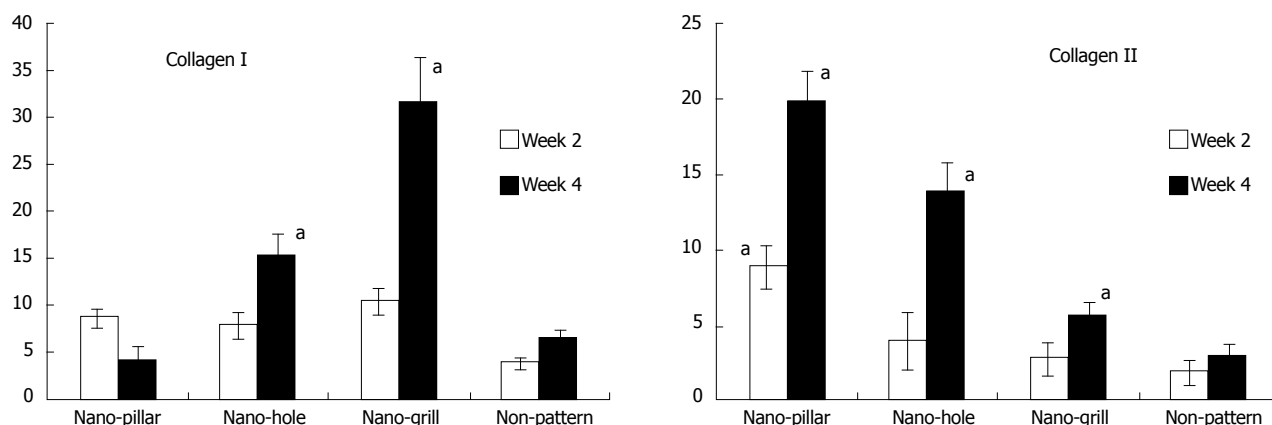
**Figure 6 Investigation of the effects of nanotopography on proliferation.** (A), alkaline phosphatase (Alp) activity (B), and extracellular matrix mineralization (C) of mesenchymal stem cells differentiated into osteoblasts and cultured on nanotopography in an osteogenic medium compared to control Ti surfaces. A: The number of cells was significantly increased on Ti with nanotopography on days 10 ( $P = 0.07$ ) and 17 ( $P = 0.03$ ); B: Higher Alp activity was supported by Ti surface with nanotopography supported on days 4 ( $P = 0.01$ ) and 10 ( $P = 0.04$ ); C: The difference in the level of calcium mineralisation in the matrix (insets) was not statistically significant ( $P = 0.13$ ) by comparing both surfaces. The data are presented as mean  $\pm$  standard deviation ( $n = 4$ ). <sup>a</sup>Indicates statistically significant difference<sup>[66]</sup>.

using simultaneously integrated nanotopography and flow stimulus. They developed a biomimetic microfluidic device consisting of aligned nanofibers of poly lactic-co-glycolic acid (PLGA), and micorchambers of different angles. The micorchambers were used to enable flow direction to create different angles with PLGA nanofibers. Their findings showed that the combination of fluid flow, nanotopography-induced cues, and the direction of flow in relation to the aligned nanofibers contributed towards the round shape morphology of MSCs, associated with fibrochondrogenesis during chondrogenic differentiation of these cells.

Although, most studies have shown positive contributions of various nanotopographical cues on MSCs growth and differentiation, a few studies have concluded that certain types of nanotopography could have adverse effect on the differentiation of MSCs into chondrocytes for the purpose of repairing

or regenerating cartilage. The study by Wu *et al.*<sup>[55]</sup> is a good example on this statement as their findings revealed that MSCs experienced delayed chondrogenesis on samples with nano-grill topography. They observed radically different morphological, proliferation and chondrogenesis changes as well as significantly higher upregulation of type II collagen on nano-pillar and nano-hole surfaces compared to nano-grill surfaces, where the expression of collagen I marker was drastically higher (Figure 7). Similarly, Trujillo *et al.*<sup>[73]</sup> reported of decreased chondrogenic differentiation of adipose-derived MSCs on nanowire surfaces. They used sintering and solvent-free nanotemplating to fabricate polycaprolactone (PCL, as control) and nanowire (NW) samples. After 4 and 7 d of culturing the samples with adipose-derived MSCs, both groups demonstrated positive support for cell attachment and proliferation. Once chondrogenic differentiation





**Figure 7** The effect of nano-patterned surfaces on the expression of cartilaginous genes. Real time polymerase chain reaction was used to analyse mRNA expression levels of cartilaginous genes at week 2, 4 or 6 of chondrogenic differentiation, which was normalised to their respective glyceraldehyde-3-phosphate dehydrogenase expression and expressed as fold changes relative to undifferentiated mesenchymal stem cells.  $n = 3$  per group, mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  which was considered statistically significant compared to non-patterned surface<sup>[55]</sup>.

media was supplemented to the cultures, alcian blue staining was used to confirm the presence of acidic polysaccharides, such as sulphated glycosaminoglycans, normally found in articular and hyaline cartilage tissue. At 3 wk, it was evident that there had been significantly higher production of polysaccharides on PCL compared to NW. The authors also investigated PCL and NW samples under adipogenic differentiation conditions and found the results to be reversed, *i.e.*, there was increased adipogenic differentiation of adipose-derived MSCs on the NW samples. These findings indicate that nanotopography can have bias and in some cases unexpected effects on the differentiation of MSCs towards a particular lineage. However, further research is required on this particular topic in the future, in order to better understand the underlying mechanism of such adverse events.

### Skin

Millions of burn injuries occur worldwide that cause serious harm to skin and subsequently to the general health of patients, as the first line of a patient's defence is compromised. In cases where the injuries are too severe for the natural repair process to take place, skin TE is considered. Skin, with a surface area of 1.8 m<sup>2</sup>, is the largest organ in the body, which consists of two layers: the outer protective epidermis and inner corium or dermis. Currently the main obstacle in front of skin TE is *in vitro* culture time required to grow epithelial sheets large enough to be used for severe cases. This is particularly dangerous as the longer the wound takes to heal, the patient is at higher risks of acquiring infection<sup>[74]</sup>. Another issue is that epithelial cells are very sensitive and adhering them to the burned surfaces is very difficult<sup>[75]</sup>.

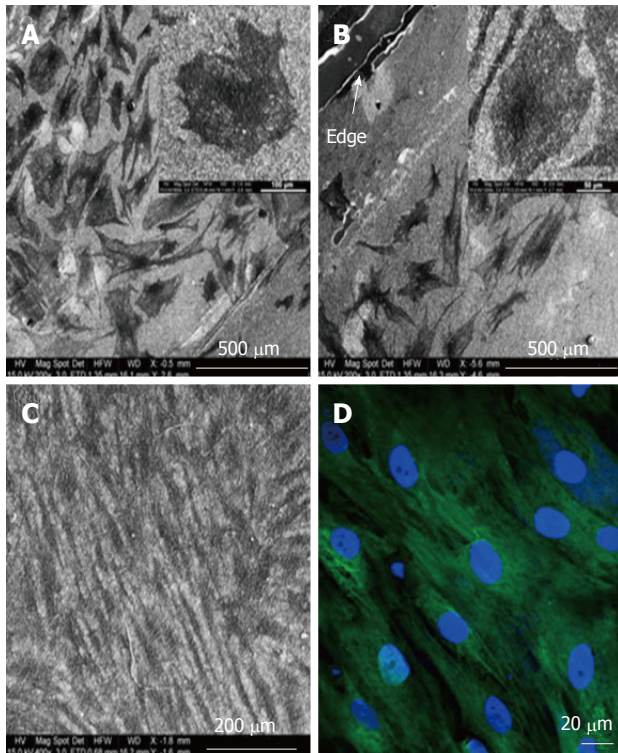
Like in other diseases, stem cells can help improve the healing and regenerating process of skin. Previously, bone marrow-derived MSCs have been shown to differentiate into epithelial cells of skin<sup>[76]</sup>, and promising results have been achieved in treating skin wounds,

especially chronic ones<sup>[77,78]</sup>. Wounds treated using bone marrow-derived MSCs have shown accelerated wound closure with rapid re-epithelisation, cellularity and angiogenesis<sup>[79]</sup>. Incorporation of these cells into a suitable scaffold, which closely mimics the native micro- and nanotopographical characteristics of the ECM of the skin, may offer improved opportunity to repair or regenerate skin<sup>[80]</sup>. For this to happen, two events should take place; (1) MSCs should be directed to the sites of injury; and (2) they should differentiate into cells of skin lineage. This is achievable by designing scaffolds with specific nanotopographical features<sup>[81]</sup>.

Based on the highly oriented nanogrooved structures of natural ECMs in human body, Kim *et al.*<sup>[82]</sup> designed nanotopographically variable grooved matrices, using UV assisted capillary force lithography, with curable polyurethane acrylate (PUA) polymer. The PUA nanogrooved matrices were then gelatine coated prior to cell culturing with hMSCs. In this study, the effect of nanotopographical density was investigated on hMSCs migration and proliferation for wound healing purposes. It was shown that hMSCs migrate into the target area (the wounded, cell free area) and that the hMSCs on nanogrooved matrices exhibited a significantly higher speed of cell migration than those on the flat controls. They also investigated various densities of nanogrooves and found that as the density of the nanogrooved matrices increases, the speed of hMSCs migration increases proportionally. Their analysis of hMSC proliferation on nanogrooved matrices, compared to flat ones, revealed no significant differences, hence concluding that proliferation of hMSCs may not be influenced by nanogrooves.

Recently, great deal of attention has been focused on electrospun nanofibrous for skin regeneration<sup>[74,80,83,84]</sup>. Using various nanotopographical designs, highly porous meshes of ultrafine fibers could be fabricated that closely resemble the nanotopography of the natural ECM of human skin<sup>[83]</sup>. Jin *et al.*<sup>[85]</sup> investigated the *in*





**Figure 8** Electron microscopy images of differentiated and undifferentiated mesenchymal stem cells on Coll/PLLCL nanostructured nanofibers. A: Mesenchymal stem cell (MSC) directed to differentiate along the epidermal lineage when cultured in epidermal induction medium; B: Epidermally differentiated MSC on the edge of a Coll/PLLCL scaffold (as shown by arrow); C: Electrospun Coll/PLLCL nanofibers seeded with undifferentiated MSC cultured in normal growth medium; D: MSC grown in normal growth medium on Coll/PLLCL nanofibers stained with Ker 10, after 15 d cell culture, imaged using laser scanning confocal microscope<sup>[85]</sup>.

*vitro* differentiation potentials of bone marrow-derived MSCs to epidermal cells on electrospun collagen/poly (L-lactic acid)-copoly (3-caprolactone) (Coll/PLLCL) nanofibrous scaffolds<sup>[85]</sup>. To further mimic the structure of the natural ECM of human skin, they incorporated Coll/PLLCL nanofibrous scaffolds with collagen, at a ratio of 30:70, respectively. Their findings demonstrated that electrospun Coll/PLLCL nanofibers enhanced the level of MSC and scaffold interaction and that the electrospun scaffolds could mimic the native skin ECM. Furthermore, their results showed controlled and directed differentiation of MSCs along the epidermal lineage on Coll/PLLCL nanofibrous scaffolds (Figure 8), suggesting their potential use in skin TE applications.

## POTENTIAL ADVERS EFFECTS OF ERODED NANOPARTICLES ON MSCS

Based on the evidence presented in this review, it is clear that nanotopography of a surface can have a great influence on the various cellular behaviours of MSCs including; their attachment, proliferation and most importantly their differentiation towards a specific lineage. However, based on the literature,

there have been various reports of the adverse effects of nanoparticles on the cellular behaviour of MSCs as the result of implant erosion over-time<sup>[86-88]</sup>. This is especially a concern of biodegradable materials as they have been specifically designed to be deteriorated once implanted in their host. In long-term clinical application, the physiochemical properties of implants are influenced by their constant chemical, mechanical, and biological interactions with their host tissue and its surrounding environment<sup>[89]</sup>. These factors cause the degradation of the implant and subsequently to the release microscale and/or nanoscale wear particles in their immediate vicinity. These released particles no longer exhibit any of the nanotopographical characteristic of the implant surface, prior to implantation.

Released nanoparticles, once exposed to tissues and bodily fluids, tend to absorb macromolecules in their vicinity and depending on the surface characteristics of the nanoparticles (e.g., their surface chemistry and surface energy), these macromolecules become attached onto the surface of the nanoparticles, leading to potential modification or functionalization of the surface of the nanoparticles<sup>[90,91]</sup>. Attachment of such macromolecules could change the affinity of a nanoparticle to bind with a specific protein, on the surface of a particular type of cells, which in return could have serious unaccounted for or adverse toxic effects on the proliferation and/or differentiation of cells<sup>[92]</sup>. For instance, there has been a report on serious DNA damage caused to MSCs when cultured with metallic silver nanoparticles, even at lower concentrations<sup>[87]</sup>. Calcium phosphate nanoparticles, very commonly used for bone tissue engineering applications, have also shown to affect MSC proliferation in a size-dependent manner, with larger particles causing more serious harm<sup>[86]</sup>. Furthermore, studies conducted by Hu *et al.*<sup>[93]</sup> and Liu *et al.*<sup>[88]</sup> showed that calcium phosphate nanoparticles could also affect MSC differentiation depending on their concentration and form of appearance. They reported that increasing the concentration of calcium phosphate nanoparticles, especially in the form of amorphous particles, rather than crystals, could negatively affect the osteogenic cell differentiation and matrix mineralisation of MSCs.

Other types of nanoparticles such as metallic ones are also of great concerns, as their release could lead to serious nanotoxicity in biomaterials and different cell lines. Various groups have investigated adverse systematic effects of titanium nanoparticles on different cell lines such as endothelial<sup>[94]</sup>, lymphoblastoid<sup>[95]</sup>, and fibroblasts<sup>[96]</sup> cells and reported of inflammatory reactions, DNA damage, and induction of apoptosis, respectively. However, until a year ago, there was no concise report on the adverse effect of titanium nanoparticles on the cellular behaviour of bone marrow-derived MSCs, despite titanium implants majorly being used for medical applications, such as in bone TE. As the result, very recently, Hou *et al.*<sup>[97]</sup> investigated



**Table 2 Significant studies on nanotopography and mesenchymal stem cells for developing 3D bone, cartilage and skin**

Tissue/organ	Nanotopographical cues	Description	Outcome	Ref.
Bone	Nano-ridges, and nanogrooves nanotopography surfaces	The effect of nanotopographic ridges and grooves on MSCs morphology, proliferation and differentiation to osteoblast cells were investigated	Osteogenic differentiation can be controlled and directed using specific size scale of topographic cues with or without osteogenic agents	Watari <i>et al</i> <sup>[54]</sup>
	Implementing nanostructures of different sizes	The effect of titanium-coated hemisphere-like topographic nanostructures of various sizes (50, 100, and 200 nm) on hMSCs cellular behaviour towards osteoblast lineage was investigated	Osteogenic differentiation of hMSCs is dependent on the size of the underlying nanotopographical structures. Colloidal lithography combined with coating technologies can have great potentials for fabricating nanoscale topography on scaffolds	de Peppo <i>et al</i> <sup>[64]</sup>
	Bioactive calcium phosphate thin films sputter deposited onto a polycrystalline titanium nanostructured surface	Calcium phosphate thin films were used to study the cellular response of hMSCs to nanostructured titanium surfaces with the aim of directing them towards osteogenic differentiation	Various <i>in vitro</i> studies revealed that the use of nanostructured titanium surfaces and the bioactive calcium phosphate coatings could allow for directed and controlled differentiation of hMSCs towards osteogenic lineage. The combination of the two materials together showed higher rate of osteogenic differentiation compared to that of each of these materials on their own	McCafferty <i>et al</i> <sup>[63]</sup>
	Polished Ti surfaces chemically treated with H <sub>2</sub> SO <sub>4</sub> /H <sub>2</sub> O <sub>2</sub> to create nanotopography	Chemically treated Ti surfaces with nanotopography and seeded with rat MSCs were used to investigate their osteoinductive potentials compared to untreated surfaces. Signalling pathways responsible for osteoinductive effect of nanotopography on MSCs were also investigated	Ti surfaces with nanotopography exhibited increased cell proliferation and alkaline phosphate activity. Gene expression of key bone markers was upregulated on nanotopography surfaces, under non-osteogenic conditions, compared to control	Rosa <i>et al</i> <sup>[66]</sup>
Cartilage	A biomimetic microfluidic device embedded with aligned nanofibers consisting of microchambers of different angle	A device was developed to create a microenvironment that integrates nanotopography and flow stimulus of the ECM of natural cartilage for the purpose of investigating the effect of microfluidic and nanotopography on the cellular behaviour and fibrochondrogenesis of MSCs	The angle of flow direction, in relation to the aligned nanofibers, affects MSCs behaviour. Fibrochondrogenesis of MSCs was evident when the flow direction was perpendicular to the aligned nanofibers	Zhang <i>et al</i> <sup>[75]</sup>
	Nanowire nanotopographic surfaces	Polycaprolactone nanowires surfaces were fabricated using a solvent-free gravimetric template technique to investigate their nanotopographical effects on the adhesion, proliferation, differentiation and ECM synthesis of adipose-derived MSCs	The results demonstrated that adhesion and proliferation of adipose-derived MSCs were enhanced on nanowire surfaces compared to the control. Nanowires also effected the morphology of these cells Interestingly, it was shown that nanowires supported adipogenic differentiation of these cells rather than chondrogenic differentiation	Trujillo <i>et al</i> <sup>[73]</sup>
	Nano-pillar, nano-hole and nano-grill nanotopography surfaces	Nano-pillar, nano-hole and nano-grill structures were formed on polycaprolactone surface using thermal nanoimprinting to investigate their effect on chondrogenic differentiation of hMSCs	Nanotopographical patterns have the ability to induce changes in MSC morphology and cytoskeletal structure towards a specific lineage, in this case chondrocyte cells. Delayed chondrogenesis was observed on nanogrill topography compared to nano-pillar and nano-hole topography, which enhance MSC chondrogenesis	Wu <i>et al</i> <sup>[55]</sup>
Skin	Electrospun nanofibrous scaffolds	Electrospun Coll/PLLCL and PLLCL nanofibrous scaffolds were prepared to investigate the proliferation and differentiation of MSCs to epidermal lineages	Cell proliferation was significantly higher on Coll/PLLCL nanofibrous scaffolds compared to PLLCL scaffolds. MSC morphology was also different on Coll/PLLCL nanofibrous scaffolds compared to control. Electrospun Coll/PLLCL exhibited similar properties to the native skin ECM	Jin <i>et al</i> <sup>[85]</sup>
	Nanotopographically variable grooved matrices	Nanotopographically variable grooved matrices, using UV assisted capillary force lithography, with curable PUA polymer were fabricated and then coated with gelatine to investigate the effect of nanotopographical density on hMSC migration and proliferation for wound healing purposes	As the density of the nanogrooved matrices increased, the speed of hMSCs migration increased proportionally. It was shown that hMSC proliferation was not significantly different on nanogrooved matrices, compared to flat control. Therefore, suggesting that proliferation of hMSCs may not be influenced by the nanogrooves	Kim <i>et al</i> <sup>[82]</sup>

PUA: Polyurethane acrylate; Coll/PLLCL: Collagen/poly(lactic acid)-co-poly(3-caprolactone); Ti: Titanium; hMSCs: Human mesenchymal stem cells.

the effects of titanium nanoparticles on adhesion, migration, proliferation and differentiation of MSCs and

reported of serious negative effects of the nanoparticles on MSC migration as particle size increased. They



also demonstrated that exposure of MSCs to titanium nanoparticles negatively affected their osteogenic differentiation<sup>[97]</sup>.

## DISCUSSION AND FUTURE PROSPECTIVE

In this review, the role of nanotopography on controlling and directing cellular behaviour of various types of MSCs, with respect to specific tissues and organs, has been described. Based on the studies presented here, it can be established that various nanofabrication methods can be employed to design and fabricate nanostructured scaffolds with distinct nanotopographical cues to control and direct various cellular behaviours of MSCs including; their attachment, proliferation and most importantly their differentiation towards a specific lineage (Table 2). Therefore, incorporating nanotopography on the design of scaffolds would open doors to new generation of TE strategies for the development of functional organs and tissues.

The review of the literature demonstrated that most studies on this topic have been focused on bone TE applications. In most of these studies, such strategy seemed to improve the proliferation and differentiation of MSCs for repair or regeneration of bone. In most cases, authors have reported of controlled and directed osteogenesis of these cells on various polymeric based composite or nanocomposite scaffolds. However, there seems to be a clear lack of investigation into the potentials of using nanotopography for the development of more complex organs or tissues such as heart, kidney, or bladder. This is despite of the fact that some studies have confirmed the abilities of MSCs to differentiate into various other cell types; including muscle cells, stromal cells and fibroblast cells. Therefore, in the future, TE and regenerative medicine could greatly benefit from research focused on developing more complex organs or tissues using nanotopography guided differentiation of MSCs, while at the same time, there needs to be a more comprehensive investigation on the potential adverse effect of various types of nanoparticles, released from eroded nanotopographical surfaces, on the cellular behaviour of MSCs.

## REFERENCES

- Messenger MP, Tomlins PE. Regenerative medicine: a snapshot of the current regulatory environment and standards. *Adv Mater* 2011; **23**: H10-H17 [PMID: 21433095 DOI: 10.1002/adma.201100254]
- Hopley EL, Salmasi S, Kalaskar DM, Seifalian AM. Carbon nanotubes leading the way forward in new generation 3D tissue engineering. *Biotechnol Adv* 2014; **32**: 1000-1014 [PMID: 24858314 DOI: 10.1016/j.biotechadv]
- Kim HN, Jiao A, Hwang NS, Kim MS, Kang do H, Kim DH, Suh KY. Nanotopography-guided tissue engineering and regenerative medicine. *Adv Drug Deliv Rev* 2013; **65**: 536-558 [PMID: 22921841 DOI: 10.1016/j.addr.2012.07.014]
- Oryan A, Alidadi S, Moshiri A, Maffulli N. Bone regenerative medicine: classic options, novel strategies, and future directions. *J Orthop Surg Res* 2014; **9**: 18 [PMID: 24628910 DOI: 10.1186/1749-799X9-18]
- Catalano E, Cochis A, Varoni E, Rimondini L, Azzimonti B. Tissue-engineered skin substitutes: an overview. *J Artif Organs* 2013; **16**: 397-403 [PMID: 24096542 DOI: 10.1007/s10047-013-0734-0]
- Li YS, Harn HJ, Hsieh DK, Wen TC, Subeq YM, Sun LY, Lin SZ, Chiou TW. Cells and materials for liver tissue engineering. *Cell Transplant* 2013; **22**: 685-700 [PMID: 23127824 DOI: 10.3727/096368912X655163]
- Martovetsky G, Nigam SK. Cellular and developmental strategies aimed at kidney tissue engineering. *Nephron Exp Nephrol* 2014; **126**: 101 [PMID: 24854650 DOI: 10.1159/000360680]
- Nayyer L, Birchall M, Seifalian AM, Jell G. Design and development of nanocomposite scaffolds for auricular reconstruction. *Nanomedicine* 2014; **10**: 235-246 [PMID: 23792331 DOI: 10.1016/j.nano.2013.06.006]
- Zhao M, Song B, Pu J, Wada T, Reid B, Tai G, Wang F, Guo A, Walczysko P, Gu Y, Sasaki T, Suzuki A, Forrester JV, Bourne HR, Devreotes PN, McCaig CD, Penninger JM. Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-gamma and PTEN. *Nature* 2006; **442**: 457-460 [PMID: 16871217 DOI: 10.1038/nature04925]
- Petrie RJ, Doyle AD, Yamada KM. Random versus directionally persistent cell migration. *Nat Rev Mol Cell Biol* 2009; **10**: 538-549 [PMID: 19603038 DOI: 10.1038/nrm2729]
- Stylianou A, Yova D, and Alexandratou E. Nanotopography of collagen thin films in correlation with fibroblast response. *J of Nanophotonics* 2013; **7**: 073590 [DOI: 10.1117/1.JNP.7.073590]
- Tay CY, Irvine SA, Boey FY, Tan LP, Venkatraman S. Micro-/nano-engineered cellular responses for soft tissue engineering and biomedical applications. *Small* 2011; **7**: 1361-1378 [PMID: 21538867 DOI: 10.1002/sml.201100046]
- Phong HQ, Wang SL, Wang MJ. Cell behaviors on micro-patterned porous thin films. *Mat Sci & Eng: B* 2010; **169**: 94-100 [DOI: 10.1016/j.mseb.2010.01.009]
- Kim J, Kim HN, Lim KT, Kim Y, Pandey S, Garg P, Choung YH, Choung PH, Suh KY, Chung JH. Synergistic effects of nanotopography and co-culture with endothelial cells on osteogenesis of mesenchymal stem cells. *Biomaterials* 2013; **34**: 7257-7268 [PMID: 23834896 DOI: 10.1016/j.biomaterials.2013.06.029]
- Shapira A, Kim DH, Dvir T. Advanced micro- and nanofabrication technologies for tissue engineering. *Biofabrication* 2014; **6**: 020301 [PMID: 24876336 DOI: 10.1088/1758-5082/6/2/020301]
- Guvendiren M, Burdick JA. The control of stem cell morphology and differentiation by hydrogel surface wrinkles. *Biomaterials* 2010; **31**: 6511-6518 [PMID: 20541257 DOI: 10.1016/j.biomaterials.2010.05.037]
- Ayala R, Zhang C, Yang D, Hwang Y, Aung A, Shroff SS, Arce FT, Lal R, Arya G, Varghese S. Engineering the cell-material interface for controlling stem cell adhesion, migration, and differentiation. *Biomaterials* 2011; **32**: 3700-3711 [PMID: 21396708 DOI: 10.1016/j.biomaterials.2011.02.004]
- Zouani OF, Chanseau C, Brouillaud B, Bareille R, Deliane F, Foulc MP, Mehdi A, Durrieu MC. Altered nanofeature size dictates stem cell differentiation. *J Cell Sci* 2012; **125**: 1217-1224 [PMID: 22302989 DOI: 10.1242/jcs.093229]
- Das RK, Zouani OF. A review of the effects of the cell environment physicochemical nanoarchitecture on stem cell commitment. *Biomaterials* 2014; **35**: 5278-5293 [PMID: 24720880 DOI: 10.1016/j.biomaterials.2014.03.044]
- Discher DE, Janmey P, Wang YL. Tissue cells feel and respond to the stiffness of their substrate. *Science* 2005; **310**: 1139-1143 [PMID: 16293750 DOI: 10.1126/science.1116995]
- Discher DE, Mooney DJ, Zandstra PW. Growth factors, matrices, and forces combine and control stem cells. *Science* 2009; **324**: 1673-1677 [PMID: 19556500 DOI: 10.1126/science.1171643]
- Baker BM, Chen CS. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. *J Cell Sci* 2012; **125**: 3015-3024 [PMID: 22797912 DOI: 10.1242/jcs.079509]



- 23 **Liang L**, Liu J, Windisch Jr CF, Exarhos GJ, Lin Y. Direct assembly of large arrays of oriented conducting polymer nanowires. *Angew Chem Int Ed Engl* 2002; **41**: 3665-3668, 3520 [PMID: 12370924]
- 24 **Tsai IY**, Kimura M, Stockton R, Green JA, Puig R, Jacobson B, Russell TP. Fibroblast adhesion to micro- and nano-heterogeneous topography using diblock copolymers and homopolymers. *J Biomed Mater Res A* 2004; **71**: 462-469 [PMID: 15484209 DOI: 10.1002/jbm.a.30183]
- 25 **Tsang VL**, Bhatia SN. Three-dimensional tissue fabrication. *Adv Drug Deliv Rev* 2004; **56**: 1635-1647 [PMID: 15350293 DOI: 10.1016/j.addr.2004.05.001]
- 26 **Zhu X**, Mills KL, Peters PR, Bahng JH, Liu EH, Shim J, Naruse K, Csete ME, Thouless MD, Takayama S. Fabrication of reconfigurable protein matrices by cracking. *Nat Mater* 2005; **4**: 403-406 [PMID: 15834415 DOI: 10.1038/nmat1365]
- 27 **Guo LJ**. Nanoimprint lithography: methods and material requirements. *Adv Mat* 2007; **19**: 495-513 [DOI: 10.1002/adma.200600882]
- 28 **Bianco P**, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008; **2**: 313-319 [PMID: 18397751 DOI: 10.1016/j.stem.2008.03.002]
- 29 **Prockop DJ**. Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. *Mol Ther* 2009; **17**: 939-946 [PMID: 19337235 DOI: 10.1038/mt.2009.62]
- 30 **Prockop DJ**, Kota DJ, Bazhanov N, Reger RL. Evolving paradigms for repair of tissues by adult stem/progenitor cells (MSCs). *J Cell Mol Med* 2010; **14**: 2190-2199 [PMID: 20716123 DOI: 10.1111/j.15824934.2010.01151.x]
- 31 **Santos JL**, Pandita D, Rodrigues J, Pêgo AP, Granja PL, Tomás H. Non-viral gene delivery to mesenchymal stem cells: methods, strategies and application in bone tissue engineering and regeneration. *Curr Gene Ther* 2011; **11**: 46-57 [PMID: 21182464 DOI: 10.2174/156652311794520102]
- 32 **Ahmed TA**, Hincke MT. Mesenchymal stem cell-based tissue engineering strategies for repair of articular cartilage. *Histol Histopathol* 2014; **29**: 669-689 [PMID: 24452855]
- 33 **Serakinci N**, Fahrioglu U, Christensen R. Mesenchymal stem cells, cancer challenges and new directions. *Eur J Cancer* 2014; **50**: 1522-1530 [PMID: 24613620 DOI: 10.1016/j.ejca.2014.02.011]
- 34 **Porada CD**, Zanjani ED, Almeida-Porad G. Adult mesenchymal stem cells: a pluripotent population with multiple applications. *Curr Stem Cell Res Ther* 2006; **1**: 365-369 [PMID: 18220880 DOI: 10.2174/157488806778226821]
- 35 **Parekkadan B**, Milwid JM. Mesenchymal stem cells as therapeutics. *Annu Rev Biomed Eng* 2010; **12**: 87-117 [PMID: 20415588 DOI: 10.1146/annurev-bioeng-070909-105309]
- 36 **Patel DM**, Shah J, Srivastava AS. Therapeutic potential of mesenchymal stem cells in regenerative medicine. *Stem Cells Int* 2013; **2013**: 496218 [DOI: 10.1155/2013/496218]
- 37 **Hoogduijn MJ**, Dor FJ. Mesenchymal stem cells in transplantation and tissue regeneration. *Front Immunol* 2011; **2**: 84
- 38 **Yokoo T**, Matsumoto K, Yokote S. Potential use of stem cells for kidney regeneration. *Int J of Nephrology* 2011; **2011**: 9 [DOI: 10.4061/2011/591731]
- 39 **Zippel N**, Schulze M, Tobiasch E. Biomaterials and mesenchymal stem cells for regenerative medicine. *Recent Pat Biotechnol* 2010; **4**: 1-22 [PMID: 20201799 DOI: 10.2174/187220810790069497]
- 40 **Ding DC**, Shyu WC, Lin SZ. Mesenchymal stem cells. *Cell Transplant* 2011; **20**: 5-14 [PMID: 21396235 DOI: 10.3727/096368910X]
- 41 **Unadkat HV**, Hulsman M, Cornelissen K, Papenburg BJ, Truckenmüller RK, Carpenter AE, Wessling M, Post GF, Uetz M, Reinders MJ, Stamatialis D, van Blitterswijk CA, de Boer J. An algorithm-based topographical biomaterials library to instruct cell fate. *Proc Natl Acad Sci USA* 2011; **108**: 16565-16570 [PMID: 21949368 DOI: 10.1073/pnas.1109861108]
- 42 **Biggs MJ**, Richards RG, Gadegaard N, Wilkinson CD, Oreffo RO, Dalby MJ. The use of nanoscale topography to modulate the dynamics of adhesion formation in primary osteoblasts and ERK/MAPK signalling in STRO-1+ enriched skeletal stem cells. *Biomaterials* 2009; **30**: 5094-5103 [PMID: 19539986 DOI: 10.1016/j.biomaterials.2009.05.049]
- 43 **Altrock E**, Muth CA, Klein G, Spatz JP, Lee-Thedieck C. The significance of integrin ligand nanopatterning on lipid raft clustering in hematopoietic stem cells. *Biomaterials* 2012; **33**: 3107-3118 [PMID: 22269650 DOI: 10.1016/j.biomaterials.2012.01.002]
- 44 **Charest JL**, García AJ, King WP. Myoblast alignment and differentiation on cell culture substrates with microscale topography and model chemistries. *Biomaterials* 2007; **28**: 2202-2210 [PMID: 17267031 DOI: 10.1016/j.biomaterials.2007.01.020]
- 45 **Dalby MJ**, Childs S, Riehle MO, Johnstone HJ, Affrossman S, Curtis AS. Fibroblast reaction to island topography: changes in cytoskeleton and morphology with time. *Biomaterials* 2003; **24**: 927-935 [PMID: 12504513 DOI: 10.1016/S0142-9612(02)00427-1]
- 46 **McMurray RJ**, Gadegaard N, Tsimbouri PM, Burgess KV, McNamara LE, Tare R, Murawski K, Kingham E, Oreffo RO, Dalby MJ. Nanoscale surfaces for the long-term maintenance of mesenchymal stem cell phenotype and multipotency. *Nat Mater* 2011; **10**: 637-644 [PMID: 21765399 DOI: 10.1038/nmat3058]
- 47 **Curtis A**, Wilkinson C. Topographical control of cells. *Biomaterials* 1997; **18**: 1573-1583 [PMID: 9613804 DOI: 10.1016/S0142-9612(97)00144-0]
- 48 **Stevens MM**, George JH. Exploring and engineering the cell surface interface. *Science* 2005; **310**: 1135-1138 [PMID: 16293749 DOI: 10.1126/science.1106587]
- 49 **Curran JM**, Stokes R, Irvine E, Graham D, Amro NA, Sanedrin RG, Jamil H, Hunt JA. Introducing dip pen nanolithography as a tool for controlling stem cell behaviour: unlocking the potential of the next generation of smart materials in regenerative medicine. *Lab Chip* 2010; **10**: 1662-1670 [PMID: 20390207 DOI: 10.1039/c004149a]
- 50 **Yim EK**, Pang SW, Leong KW. Synthetic nanostructures inducing differentiation of human mesenchymal stem cells into neuronal lineage. *Exp Cell Res* 2007; **313**: 1820-1829 [PMID: 17428465 DOI: 10.1016/j.yexcr.2007.02.031]
- 51 **Guduru D**, Niepel M, Vogel J, Groth T. Nanostructured material surfaces--preparation, effect on cellular behavior, and potential biomedical applications: a review. *Int J Artif Organs* 2011; **34**: 963-985 [PMID: 22161281 DOI: 10.5301/IJAO.5000012]
- 52 **Nava MM**, Raimondi MT, Pietrabissa R. Controlling self-renewal and differentiation of stem cells via mechanical cues. *J Biomed Biotechnol* 2012; **2012**: 797410 [PMID: 23091358]
- 53 **Andersen MØ**, Nygaard JV, Burns JS, Raarup MK, Nyengaard JR, Bünger C, Besenbacher F, Howard KA, Kassem M, Kjems J. siRNA nanoparticle functionalization of nanostructured scaffolds enables controlled multilineage differentiation of stem cells. *Mol Ther* 2010; **18**: 2018-2027 [PMID: 20808289 DOI: 10.1038/mt.2010.166]
- 54 **Watari S**, Hayashi K, Wood JA, Russell P, Nealey PF, Murphy CJ, Genetos DC. Modulation of osteogenic differentiation in hMSCs cells by submicron topographically-patterned ridges and grooves. *Biomaterials* 2012; **33**: 128-136 [PMID: 21982295 DOI: 10.1016/j.biomaterials.2011.09.058]
- 55 **Wu YN**, Law JB, He AY, Low HY, Hui JH, Lim CT, Yang Z, Lee EH. Substrate topography determines the fate of chondrogenesis from human mesenchymal stem cells resulting in specific cartilage phenotype formation. *Nanomedicine* 2014; **10**: 1507-1516 [PMID: 24768908 DOI: 10.1016/j.nano.2014.04.002]
- 56 **Kilian KA**, Bugarija B, Lahn BT, Mrksich M. Geometric cues for directing the differentiation of mesenchymal stem cells. *Proc Natl Acad Sci USA* 2010; **107**: 4872-4877 [PMID: 20194780 DOI: 10.1073/pnas.0903269107]
- 57 **Hu J**, Zhou Y, Huang L, Liu J, Lu H. Effect of nano-hydroxyapatite coating on the osteoinductivity of porous biphasic calcium phosphate ceramics. *BMC Musculoskelet Disord* 2014; **15**: 114 [PMID: 24690170 DOI: 10.1186/1471-2474-15-114]
- 58 **Deng M**, James R, Laurencin CT, Kumbar SG. Nanostructured polymeric scaffolds for orthopaedic regenerative engineering. *IEEE Trans Nanobioscience* 2012; **11**: 3-14 [PMID: 22275722 DOI: 10.1109/TNB.2011.2179554]
- 59 **Yamada Y**, Nakamura S, Ito K, Umemura E, Hara K, Nagasaka



- T, Abe A, Baba S, Furuichi Y, Izumi Y, Klein OD, Wakabayashi T. Injectable bone tissue engineering using expanded mesenchymal stem cells. *Stem Cells* 2013; **31**: 572-580 [PMID: 23225744 DOI: 10.1002/stem.1300]
- 60 **Joshi A**, Kostakis GC. An investigation of post-operative morbidity following iliac crest graft harvesting. *Br Dent J* 2004; **196**: 167-171; discussion 155 [PMID: 14963443 DOI: 10.1038/sj.bdj.4810945]
- 61 **Chatterjea A**, Meijer G, van Blitterswijk C, de Boer J. Clinical application of human mesenchymal stromal cells for bone tissue engineering. *Stem Cells Int* 2010; **2010**: 215625-37 [DOI: 10.4061/2010/215625]
- 62 **Mauney JR**, Volloch V, Kaplan DL. Role of adult mesenchymal stem cells in bone tissue engineering applications: current status and future prospects. *Tissue Eng* 2005; **11**: 787-802 [PMID: 15998219 DOI: 10.1089/ten.2005.11.787]
- 63 **McCafferty MM**, Burke GA, Meenan BJ. Calcium phosphate thin films enhance the response of human mesenchymal stem cells to nanostructured titanium surfaces. *J Tissue Eng* 2014; **5**: 2041731414537513 [PMID: 24904730 DOI: 10.1177/2041731414537513]
- 64 **de Peppo GM**, Agheli H, Karlsson C, Ekström K, Brisby H, Lennerås M, Gustafsson S, Sjövall P, Johansson A, Olsson E, Lausmaa J, Thomsen P, Petronis S. Osteogenic response of human mesenchymal stem cells to well-defined nanoscale topography in vitro. *Int J Nanomedicine* 2014; **9**: 2499-2515 [PMID: 24904210 DOI: 10.2147/IJN.S58805]
- 65 **Dalby MJ**, McCloy D, Robertson M, Agheli H, Sutherland D, Affrossman S, Oreffo RO. Osteoprogenitor response to semi-ordered and random nanotopographies. *Biomaterials* 2006; **27**: 2980-2987 [PMID: 16443268 DOI: 10.1016/j.biomaterials.2006.01.010]
- 66 **Rosa AL**, Kato RB, Castro Raucci LM, Teixeira LN, de Oliveira FS, Bellesini LS, de Oliveira PT, Hassan MQ, Beloti MM. Nanotopography drives stem cell fate toward osteoblast differentiation through  $\alpha 1 \beta 1$  integrin signaling pathway. *J Cell Biochem* 2014; **115**: 540-548 [PMID: 24122940 DOI: 10.1002/jcb.24688]
- 67 **Childs A**, Hemraz UD, Castro NJ, Fenniri H, Zhang LG. Novel biologically-inspired rosette nanotube PLLA scaffolds for improving human mesenchymal stem cell chondrogenic differentiation. *Biomed Mater* 2013; **8**: 065003 [PMID: 24225196 DOI: 10.1088/1748-6041/8/6/065003]
- 68 **Patel KH**, Nayyer L, Seifalian AM. Chondrogenic potential of bone marrow-derived mesenchymal stem cells on a novel, auricular-shaped, nanocomposite scaffold. *J Tissue Eng* 2013; **4**: 2041731413516782 [PMID: 24555012 DOI: 10.1177/2041731413516782]
- 69 **Jung H**, Park JS, Yeom J, Selvapalam N, Park KM, Oh K, Yang JA, Park KH, Hahn SK, Kim K. 3D tissue engineered supramolecular hydrogels for controlled chondrogenesis of human mesenchymal stem cells. *Biomacromolecules* 2014; **15**: 707-714 [PMID: 24605794 DOI: 10.1021/bm401123m]
- 70 **McBride SH**, Knothe Tate ML. Modulation of stem cell shape and fate A: the role of density and seeding protocol on nucleus shape and gene expression. *Tissue Eng Part A* 2008; **14**: 1561-1572 [PMID: 18774910 DOI: 10.1089/ten.tea.2008.0112]
- 71 **Gao L**, McBeath R, Chen CS. Stem cell shape regulates a chondrogenic versus myogenic fate through Rac1 and N-cadherin. *Stem Cells* 2010; **28**: 564-572 [PMID: 20082286 DOI: 10.1002/stem.308]
- 72 **Zhong W**, Zhang W, Wang S, Qin J. Regulation of fibrochondrogenesis of mesenchymal stem cells in an integrated microfluidic platform embedded with biomimetic nanofibrous scaffolds. *PLoS One* 2013; **8**: e61283 [PMID: 23637803 DOI: 10.1371/journal.pone.0061283]
- 73 **Trujillo NA**, Popat KC. Increased adipogenic and decreased chondrogenic differentiation of adipose derived stem cells on nanowire surfaces. *Materials* 2014; **7**: 2605-2630 [DOI: 10.3390/ma7042605]
- 74 **Prabhakaran MP**, Venugopal J, Ghasemi-Mobarakeh L, Kai D, Jin G, Ramakrishna S. Stem cells and nanostructures for advanced tissue regeneration. *Biomed App of Polymeric Nanofibers* 2012; **246**: 21-62 [DOI: 10.1007/12\_2011\_113]
- 75 **Zhang CP**, Fu XB. Therapeutic potential of stem cells in skin repair and regeneration. *Chin J Traumatol* 2008; **11**: 209-221 [PMID: 18667118 DOI: 10.1016/S1008-1275(08)60045-0]
- 76 **Krause DS**, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* 2001; **105**: 369-377 [PMID: 11348593 DOI: http://dx.doi.org/10.1016/S0092-8674(01)00328-2]
- 77 **Badiavas EV**, Falanga V. Treatment of chronic wounds with bone marrow-derived cells. *Arch Dermatol* 2003; **139**: 510-516 [PMID: 12707099 DOI: doi: 10.1001/archderm.139.4.510]
- 78 **Babiri G**, Heiner D, Falanga V. The emerging use of bone marrow-derived mesenchymal stem cells in the treatment of human chronic wounds. *Expert Opin Emerg Drugs* 2013; **18**: 405-419 [PMID: 24004161 DOI: 10.1517/14728214.2013.833184]
- 79 **Wu Y**, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 2007; **25**: 2648-2659 [PMID: 17615264 DOI: 10.1634/stemcells.2007-0226]
- 80 **Sundaramurthi D**, Krishnan UM, Sethuraman S. Electrospun nanofibers as scaffolds for Skin tissue engineering. *Polymer Reviews* 2014; **54**: 348-376 [DOI: 10.1080/15583724.2014.881374]
- 81 **Ma K**, Liao S, He L, Lu J, Ramakrishna S, Chan CK. Effects of nanofiber/stem cell composite on wound healing in acute full-thickness skin wounds. *Tissue Eng Part A* 2011; **17**: 1413-1424 [PMID: 21247260 DOI: 10.1089/ten.TEA.2010.0373]
- 82 **Kim J**, Kim HN, Lim KT, Kim Y, Seonwoo H, Park SH, Lim HJ, Kim DH, Suh KY, Choung PH, Choung YH, Chung JH. Designing nanotopographical density of extracellular matrix for controlled morphology and function of human mesenchymal stem cells. *Sci Rep* 2013; **3**: 3552 [PMID: 24352057 DOI: 10.1038/srep03552]
- 83 **Lim SH**, Liu XY, Song H, Yarema KJ, Mao HQ. The effect of nanofiber-guided cell alignment on the preferential differentiation of neural stem cells. *Biomaterials* 2010; **31**: 9031-9039 [PMID: 20797783 DOI: 10.1016/j.biomaterials]
- 84 **Subramanian A**, Krishnan UM, Sethuraman S. 14 - Skin tissue regeneration. Electrospinning for Tissue Regeneration. In: Bosworth LA, Downes S, editors. UK: Woodhead Publishing, 2011: 298-316
- 85 **Jin G**, Prabhakaran MP, Ramakrishna S. Stem cell differentiation to epidermal lineages on electrospun nanofibrous substrates for skin tissue engineering. *Acta Biomater* 2011; **7**: 3113-3122 [PMID: 21550425 DOI: 10.1016/j.actbio.2011.04.017]
- 86 **Cai Y**, Liu Y, Yan W, Hu Q, Tao J, Zhang M, Shi Z, Tang R. Role of hydroxyapatite nanoparticle size in bone cell proliferation. *J Mater Chem* 2007; **17**: 3780-3787 [DOI: 10.1039/B705129H]
- 87 **Hutmacher DW**, Schantz T, Zein I, Ng KW, Teoh SH, Tan KC. Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling. *J Biomed Mater Res* 2001; **55**: 203-216 [PMID: 11255172 DOI: 10.1002/1097-4636(200105)55:2<203::AID-JBM1007>3.0.CO;2-7]
- 88 **Liu Y**, Wang G, Cai Y, Ji H, Zhou G, Zhao X, Tang R, Zhang M. In vitro effects of nanophase hydroxyapatite particles on proliferation and osteogenic differentiation of bone marrow-derived mesenchymal stem cells. *J Biomed Mater Res A* 2009; **90**: 1083-1091 [PMID: 18671263 DOI: 10.1002/jbm.a.32192]
- 89 **Azevedo HS**, Reis RL. Understanding the enzymatic degradation of biodegradable polymers and strategies to control their degradation rate. Biodegradable systems in tissue engineering and regenerative medicine. Boca Raton, FL: CRC Press, 2005: 177-201 [DOI: 10.1201/9780203491232.ch12]
- 90 **Schellenberger EA**, Reynolds F, Weissleder R, Josephson L. Surface-functionalized nanoparticle library yields probes for apoptotic cells. *ChemBiochem* 2004; **5**: 275-279 [PMID: 14997519 DOI: 10.1002/cbic.200300713]
- 91 **Sperling RA**, Parak WJ. Surface modification, functionalization and bioconjugation of colloidal inorganic nanoparticles. *Philos Trans A Math Phys Eng Sci* 2010; **368**: 1333-1383 [PMID: 20156828 DOI: 10.1098/rsta.2009.0273]



- 92 **Tautzenberger A**, Kovtun A, Ignatius A. Nanoparticles and their potential for application in bone. *Int J Nanomedicine* 2012; **7**: 4545-4557 [PMID: 22923992 DOI: 10.2147/IJN.S34127]
- 93 **Hu Q**, Tan Z, Liu Y, Tao J, Cai Y, Zhang M, Pan H, Xu X, Tang R. Effect of crystallinity of calcium phosphate nanoparticles on adhesion, proliferation, and differentiation of bone marrow mesenchymal stem cells. *J Mater Chem* 2007; **17**: 4690-4698 [DOI: 10.1039/B710936A]
- 94 **Peters K**, Unger RE, Kirkpatrick CJ, Gatti AM, Monari E. Effects of nano-scaled particles on endothelial cell function in vitro: studies on viability, proliferation and inflammation. *J Mater Sci Mater Med* 2004; **15**: 321-325 [PMID: 15332593 DOI: 10.1023/B: JMSM.000 0021095.36878.1b]
- 95 **Wang JJ**, Sanderson BJ, Wang H. Cyto- and genotoxicity of ultrafine TiO<sub>2</sub> particles in cultured human lymphoblastoid cells. *Mutat Res* 2007; **628**: 99-106 [PMID: 17223607 DOI: 10.1016/j.mrgentox.2006.12.003]
- 96 **Rahman Q**, Lohani M, Dopp E, Pemsel H, Jonas L, Weiss DG, Schiffmann D. Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. *Environ Health Perspect* 2002; **110**: 797
- 97 **Hou Y**, Cai K, Li J, Chen X, Lai M, Hu Y, Luo Z, Ding X, Xu D. Effects of titanium nanoparticles on adhesion, migration, proliferation, and differentiation of mesenchymal stem cells. *Int J Nanomedicine* 2013; **8**: 3619-3630 [PMID: 24101871 DOI: 10.2147/IJN.S38992]

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## Evaluating alternative stem cell hypotheses for adult corneal epithelial maintenance

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hypothesis is most widely accepted. This proposes that stem cells in the basal layer of the limbal epithelium, at the periphery of the cornea, maintain themselves and also produce transient (or transit) amplifying cells (TACs). TACs then move centripetally to the centre of the cornea in the basal layer of the corneal epithelium and also replenish cells in the overlying suprabasal layers. The LESC hypothesis maintains the corneal epithelium during normal homeostasis and become more active to repair significant wounds. Second, the corneal epithelial stem cell (CESC) hypothesis postulates that, during normal homeostasis, stem cells distributed throughout the basal corneal epithelium, maintain the tissue. According to this hypothesis, LESC are present in the limbus but are only active during wound healing. We also consider a third possibility, that the corneal epithelium is maintained during normal homeostasis by proliferation of basal corneal epithelial cells without any input from stem cells. After reviewing the published evidence, we conclude that the LESC and CESC hypotheses are consistent with more of the evidence than the third hypothesis, so we do not consider this further. The LESC and CESC hypotheses each have difficulty accounting for one main type of evidence so we evaluate the two key lines of evidence that discriminate between them. Finally, we discuss how lineage-tracing experiments have begun to resolve the debate in favour of the LESC hypothesis. Nevertheless, it also seems likely that some basal corneal epithelial cells can act as long-term progenitors if limbal stem cell function is compromised. Thus, this aspect of the CESC hypothesis may have a lasting impact on our understanding of corneal epithelial maintenance, even if it is eventually shown that stem cells are restricted to the limbus as proposed by the LESC hypothesis.

**Key words:** Eye; Cornea; Corneal epithelium; Limbal epithelium; Stem cell; Lineage tracing

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### Abstract

In this review we evaluate evidence for three different hypotheses that explain how the corneal epithelium is maintained. The limbal epithelial stem cell (LESC)



**Core tip:** This review article evaluates the evidence for different hypotheses that have been proposed to explain how the corneal epithelium is maintained. It identifies core observations in favour of the conventional limbal epithelial stem cell (LESC) hypothesis and an alternative corneal epithelial stem cell hypothesis and describes how lineage-tracing experiments are helping to reconcile the two sets of conflicting evidence in favour of the LES C hypothesis.

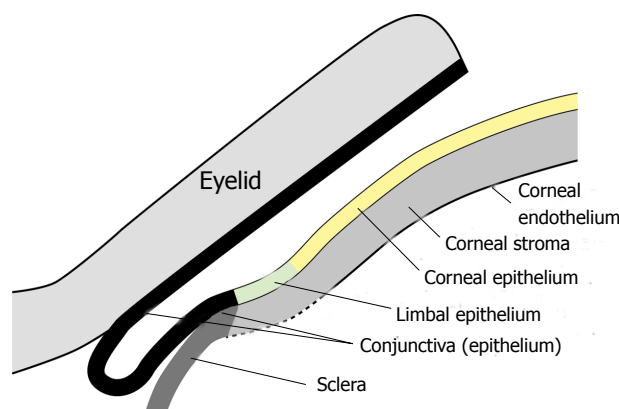
West JD, Dorà NJ, Collinson JM. Evaluating alternative stem cell hypotheses for adult corneal epithelial maintenance. *World J Stem Cells* 2015; 7(2): 281-299 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i2/281.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i2.281>

## INTRODUCTION

It is widely accepted that adult corneal epithelium is maintained by stem cells located in a region called the limbus, at the corneal periphery. However, this limbal epithelial stem cell (LESC) hypothesis has been challenged by an alternative corneal epithelial stem cell (CESC) hypothesis, based on experimental studies with mice<sup>[1]</sup>. This accepts that LESCs exist but proposes that they only contribute to corneal epithelial repair in response to wounding and that, during normal homeostasis, the corneal epithelium is maintained solely by stem cells scattered throughout the corneal epithelium itself. It has also been proposed that, in the absence of a wound, the corneal epithelium is maintained entirely by proliferation of its own basal cells without any involvement of stem cells<sup>[2]</sup>. The main purpose of this review is to compare the evidence for the alternative LES C and CESC hypotheses in order to identify where there is common ground and where differences need further experimental investigation. However, we also consider whether the experimental evidence is consistent with the possibility that the corneal epithelium is maintained without stem cells.

## THE CORNEAL AND LIMBAL EPITHELIA

The cornea is the specialised, avascular, transparent, dome-shaped region of the anterior ocular surface, which refracts light through the pupil to the lens and provides a protective, impermeable barrier. It consists of three cellular layers: (1) the inner corneal endothelium, which, despite its name, is a type of epithelium; (2) the middle corneal stroma, comprising specialised fibroblasts, called keratocytes, embedded in a collagen and proteoglycan matrix; and (3) the outer, non-keratinised, stratified squamous epithelium, comprising 5-6 layers of keratinocytes, which is kept moist by the tear film. The corneal epithelium is a very dynamic tissue. Differentiated cells are continuously shed from the outer layer and replaced by cells



**Figure 1** Diagrammatic representation of the tissues of the mouse ocular surface and eyelid.

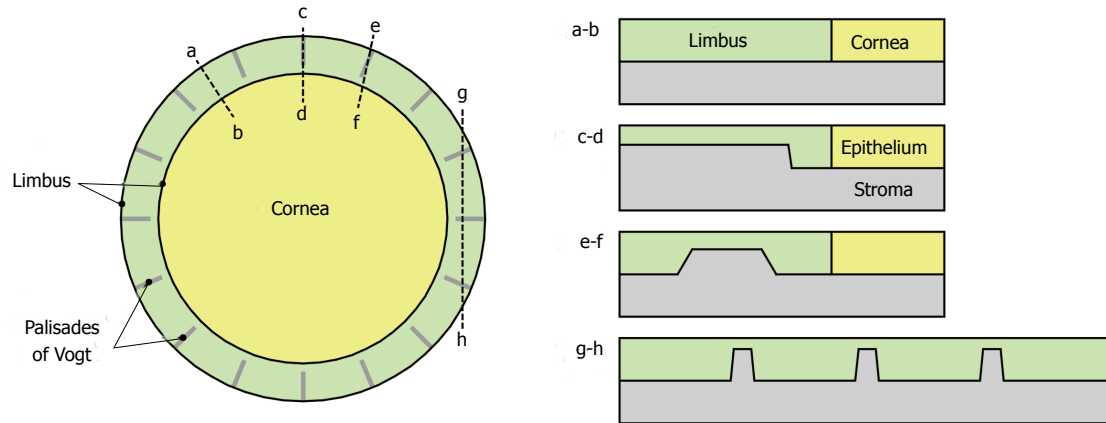
produced in the proliferative basal layer. According to the conventional stem cell paradigm these proliferative basal cells are considered to be transient (or transit) amplifying cells (TACs) and are replenished by stem cells. The early generation TACs are sometimes referred to as progenitor cells.

The limbus is a narrow transition zone, which encircles the cornea (Figures 1 and 2). The stroma and epithelial layers of the cornea extend into the limbus where they become the limbal stroma and limbal epithelium. However, the corneal endothelium does not extend into the limbus and is replaced by the drainage channels of the trabecular meshwork. On the other side of the limbus, the stroma merges with the sclera, which forms most of the ocular surface (the white part of the human eye) and the limbal epithelium becomes the conjunctiva. The conjunctiva is an epithelium, which covers the anterior sclera, folds back to form the conjunctival sac and lines the inner surface of the eyelids (Figure 1). Thus, the conjunctiva attaches the eyeball to the eyelids and orbit and permits some rotation of the eyeball in the orbit. Unlike the transparent cornea, both the limbus and the conjunctiva are vascularised.

The human limbus contains radial fibrovascular ridges, called the palisades of Vogt, which project upwards from the stroma deep into the epithelium (Figure 2) but many species, including mice, do not have limbal palisade structures. Another anatomical difference between these species is that, in the mouse, the corneal epithelium is thickest in the centre of the cornea and has fewer cell layers in the peripheral cornea and limbus whereas, in humans, the limbal epithelium is thicker (about 8-10 cell layers) than the corneal epithelium (5-6 layers)<sup>[3]</sup>.

It is widely accepted that some basal limbal epithelial cells are stem cells<sup>[4,5]</sup> and that the limbal stroma, vasculature and other associated cell types provides a suitable stem cell niche microenvironment, which is required to maintain the LESCs in a relatively undifferentiated state<sup>[6,7]</sup>. The limbal palisades (Figure 2) increase the area of interface between the limbal





**Figure 2 Palisades of Vogt.** Diagram showing the arrangement of the palisades of Vogt (upward projections of limbal stroma into the limbal epithelium) in the human limbus in plan view (left) and how they might appear in differently orientated sections through the limbus (right). Only 16 palisades are shown but in reality there are many more. For anatomy see references<sup>[114,115]</sup>.

epithelium and stroma, so increasing the size of the region that is likely to harbour the LESC niches. Furthermore, it has been suggested that LESC niches may be particularly enriched in two types of epithelial crypts associated with the palisades. One type of crypt is formed by the regions of limbal epithelium between the upward-projecting stroma of the limbal palisades and these have been named "limbal crypts" (LCs)<sup>[8,9]</sup>. The other type of crypt (named "limbal epithelial crypts"; LECs) are more sparsely distributed (only 6-7 per eye) and are formed from epithelial projections from the periphery of the limbal palisades, which extend either radially from the limbus into the conjunctival stroma or circumferentially within the limbus (perpendicular to the palisades)<sup>[10,11]</sup>. However, many species do not have limbal palisades and associated crypts, which could, therefore, be considered to be species-specific adaptations, possibly related to eye size. Thus, if the limbus provides a niche microenvironment, presumably it is either not dependent on these structures or differs among species. Maintenance of the niche microenvironment is more likely to depend on the presence of the vasculature and other cell types that are present in the limbus in all species.

## ALTERNATIVE HYPOTHESES OF CORNEAL EPITHELIAL MAINTENANCE

We consider two hypotheses, which propose alternative ways that stem cells may maintain the corneal epithelium, and a third hypothesis, which does not include stem cells. For the present purposes, we define a stem cell as an undifferentiated cell with high proliferative potential that is capable of renewing itself and also producing one or more differentiated cell types with lower proliferative potential. While most adult stem cells are multipotent, generating multiple cell types, stem cells that maintain the corneal epithelium are generally presumed to be unipotent,

only producing the corneal epithelial cells. Although one report shows that they may also produce the goblet cells that enter the corneal epithelium in response to large wounds<sup>[12]</sup>, we have not considered this possible additional role of the stem cells in this review.

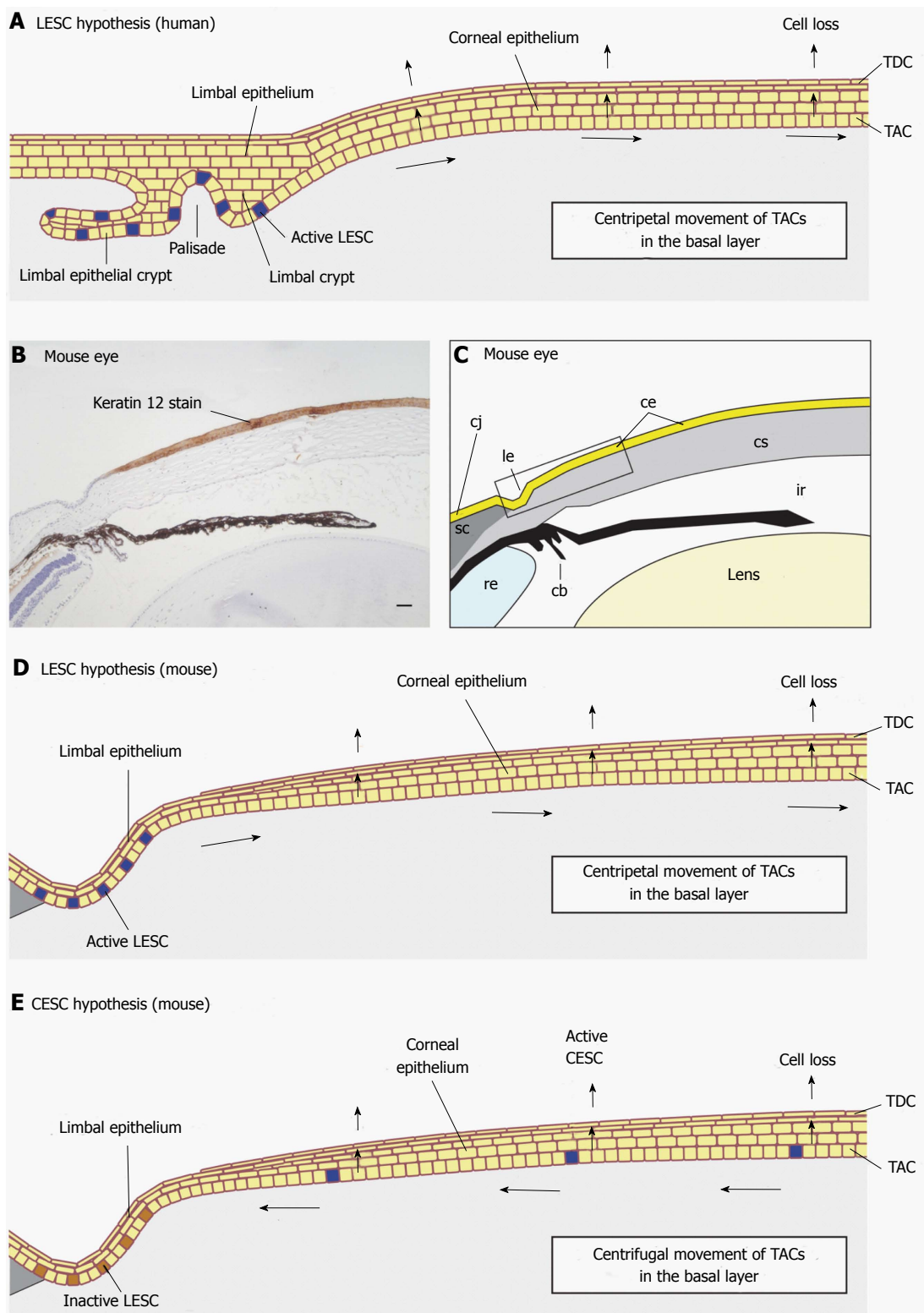
### Limbal epithelial stem cell hypothesis

According to the conventional LESC hypothesis (Figure 3A and D), LESC act as a source of new basal corneal epithelial cells (the TACs) during normal corneal epithelial homeostasis and become more active during episodes of significant wound healing<sup>[13]</sup>, although small wounds may be healed without upregulating stem cells. In this scheme LESC remain in the limbus where they maintain themselves and also generate the first generation of TACs. Some of these early TACs move to the overlying, non-mitotic suprabasal epithelial layers, and become terminally differentiated. Other early generation TACs continue to divide and move centripetally in the basal layer to maintain the corneal epithelium. Once cells leave the basal layer they differentiate and move rapidly through the suprabasal layers to the superficial layer from where they are shed. It seems that both daughter cells of a dividing basal cell usually share the same fate<sup>[14]</sup> so that they either both remain in the proliferative basal layer or both move suprabasally. It is not known what determines whether cells leave the basal layer. For example, it could be a combination of declining suprabasal cell numbers, caused by cell loss, and overcrowding in the basal layer, caused by cell proliferation, as described for the *Drosophila notum*<sup>[15]</sup>.

### Corneal epithelial stem cell hypothesis

The corneal epithelial stem cell (CESC) hypothesis accepts that there are stem cells in the limbus but proposes that these are only activated for repairing wounds and that during normal homeostasis the corneal epithelium is maintained by stem cells





**Figure 3 Limbal epithelial stem cell vs corneal epithelial stem cell hypotheses.** A: Diagram of human corneal epithelial maintenance according to the limbal epithelial stem cell (LESC) hypothesis showing active LESC in the limbal epithelium in both a limbal crypt and a limbal epithelial crypt. The LESC divide slowly replacing themselves and producing daughter transient (or transit) amplifying cells (TACs), which divide more quickly and move centripetally from the basal layer of the limbal epithelium to the basal layer of the corneal epithelium. After a final cell division TACs leave the basal layer, move through the suprabasal layers and are shed from the surface as terminally differentiated cells (TDCs); B: Histological section showing mouse cornea, limbus and part of the conjunctiva immunohistochemically stained for keratin 12 (K12; dark brown staining) to show the border between the corneal epithelium (K12 positive) and limbal epithelium (K12 negative); C: Drawing of photograph shown in (B) with different tissues labelled. The boxed area shows part of the limbal and corneal epithelia, equivalent to that represented in (D) and (E); D: Diagram of mouse corneal epithelial maintenance according to the LESC hypothesis. The principles are the same as described for (A); E: Diagram of mouse corneal epithelial maintenance according to the corneal epithelial stem cell (CESC) hypothesis. The CESC divide slowly replacing themselves and producing daughter TACs, which divide more quickly and move centrifugally as originally proposed<sup>[1]</sup>. After a final cell division TACs leave the basal layer, move through the suprabasal layers and are shed from the surface. cb: Ciliary body; ce: Corneal epithelium; cj: Conjunctiva; cs: Corneal stroma; ir: Iris; le: Limbal epithelium; re: Retina; sc: Sclera. Photograph (B) is reproduced from Mort *et al.*<sup>[18]</sup> with kind permission of Springer Science + Business Media.



**Table 1 Evidence discriminating between alternative hypotheses**

Observations	Species	Ref.	Consistent with hypothesis?		
			LESC	CESC	GBL
Holoclone-producing cells are present in limbus but not cornea	Human	[25]	++	±	±
Holoclone-producing cells are present in limbus and cornea	Pig	[1]	±	++	±
Production of clonogenic spheres from limbus and central cornea	Human	[29]	±	++	±
Limbal epithelial cells are superior to corneal epithelial cells for corneal repair	Human	[32,33]	++	+	±
The central corneal epithelium can maintain itself when isolated from the limbus	Rabbit, mouse, human	[1,39-42]	+ <sup>a</sup>	++	++
Label-retaining cells are present in the limbus but not the corneal epithelium during normal homeostasis	Mouse, rabbit	[13,52-54,57]	++	+	0
After a 40-d chase <sup>3</sup> H-TdR-labelled cells are present in both the limbus and the corneal epithelium during normal homeostasis	Rabbit	[2]	+ <sup>b</sup>	+ <sup>b</sup>	+ <sup>b</sup>
Cells move centripetally from the periphery of the corneal epithelium during normal homeostasis	Human, mouse	[58,61-63]	++	± <sup>c</sup>	± <sup>c</sup>
Mosaic patterns change after birth and clones of labelled cells emerge from the limbus as radial stripes	Mouse	[27,72,77-79]	++	0	0
Distribution of rare stripes in corneas of <i>KRT5-LacZ</i> <sup>+/+</sup> transgenic mice	Mouse	[17]	++	±	±
Transplanted limbal tissue contributes to replacing experimentally debrided corneal epithelium	Rabbit, mouse	[1,76]	++	++	+
Transplanted limbal tissue does not contribute to the unwounded corneal epithelium	Mouse	[1]	0	++	++
More tumours arise from the limbal epithelium than corneal epithelium	Human	[80]	++ <sup>d</sup>	+	+
Diverse cell types and blood supply makes limbus a likely stem cell niche	All species	[6]	++ <sup>d</sup>	+	+
Distribution of markers associated with undifferentiated or stem cell phenotype	Human, mouse, rabbit	See Table 2	++	±	±
Lineage tracing studies show that limbal cells contribute to the unwounded corneal epithelium during normal homeostasis	Mouse	[64]	++	0	0

++: Expected for hypothesis; +: Consistent with hypothesis; ±: Not consistent with hypothesis unless specific assumptions are made or technical issues compromise the interpretation of the experiment; 0: Not consistent with hypothesis. <sup>a</sup>This is compatible with the LESChypothesis if self-maintenance of the central corneal epithelium is a back-up mechanism that is used when homeostasis is compromised and LESCs are unable to maintain the corneal epithelium. <sup>b</sup>The chase period may not have been sufficient to identify label-retaining cells (see text). <sup>c</sup>The CESC hypothesis, as originally stated<sup>[1]</sup>, proposed that corneal epithelial cell movement was centrifugal but this assumption is not necessary. However, it requires *ad hoc* assumptions to account for centripetal movement across the full radius. <sup>d</sup>Evidence is circumstantial. LESCh: Limbal epithelial stem cell.

scattered throughout the corneal epithelium itself<sup>[1]</sup>. This hypothesis is based largely on surgical transplantation experiments in mice. These experiments showed that labelled limbal epithelial tissue, transplanted to the limbus of immunologically compromised mice, only produced labelled clones in the cornea if the host corneal epithelium was subsequently removed. The authors reasoned that if LESCs were active during normal homeostasis, as the LESChypothesizes, the donor limbal tissue should colonise the cornea without being stimulated to do so by wounding. However, others have pointed out that the CESC hypothesis is not consistent with some of the earlier experimental evidence<sup>[16-18]</sup>.

### **Germinative basal layer hypothesis**

A third possibility harks back to earlier explanations before the importance of tissue stem cells was recognised. Like the CESC hypothesis, this proposes that the corneal epithelium is normally maintained entirely from cells in the basal layer of the corneal epithelium but unlike the other two hypotheses it proposes there are no stem cells in either the limbal or corneal epithelia. Haddad *et al.*<sup>[2]</sup> referred to the basal layer as the “germinative basal layer” and proposed this alternative mechanism for corneal epithelial maintenance to explain the results of their label-retaining cell experiment with rabbits. These

results are inconsistent with other label-retaining cell experiments, as discussed below. However, we have considered this hypothesis because there is evidence that some other adult tissues are maintained during normal homeostasis by proliferation of more differentiated cell types. In such cases, stem cells are either absent or only active during wound repair. For example, this type of tissue maintenance has been proposed for pancreas  $\beta$ -cells<sup>[19]</sup>, epidermis<sup>[20]</sup>, lung<sup>[21]</sup> and liver hepatocytes<sup>[22,23]</sup>.

## **EXPERIMENTAL EVIDENCE AND EVALUATION OF ALTERNATIVE HYPOTHESES**

The three alternative hypotheses are discussed below with respect to the available experimental evidence and evaluations are summarised in Table 1.

### **Cells with high proliferative potential**

One of the hallmarks of stem cells is that they have a greater proliferative potential than TACs and this can be identified using *in vitro* colony-forming assays with cultured cells. The proliferation characteristics of cultures of explanted epithelial cells can be investigated in culture and clones derived from single cells can be classified as holoclones, meroiclones and



paraclones. These are thought to represent *in vitro* descendants of stem cells, TACs and differentiated cells, respectively<sup>[24]</sup>. (On indicator dishes in culture, holoclones form large, smooth-edged, fast-growing colonies with large numbers of small tightly packed cells. Meroclones form smaller colonies that are irregular in outline and include a mixture of small tightly packed cells and larger more loosely packed cells, which are predominantly at the edge. Paraclones form small, diffuse colonies and most cells are large, flattened and loosely packed). Clonal analysis of cells from the human ocular surface epithelia identified holoclone-producing cells in the limbal epithelium but not the corneal epithelium from a 54-year-old individual<sup>[25]</sup>, suggesting that stem cells are present in the limbal epithelium.

However, there is also evidence that some cells of the central cornea are self-sustaining and have high proliferative potential. Majo *et al.*<sup>[1]</sup> showed that cultured corneal epithelial cells of many species produced colonies of cells *in vitro* although there were significant species differences. Pig corneal epithelial cells grew particularly well and clonal analysis of cultured pig cells identified holoclone-producing cells in the central corneal epithelium as well as the limbus. This suggests that stem cells are present in the central corneal epithelium of pigs as well as the limbus but this result cannot be evaluated fully as the age of pigs was not given and fetal cells with stem cell characteristics may persist in younger individuals<sup>[26-28]</sup>.

The production of clonogenic spheres of cells in culture has also been associated with the presence of stem cells and these have been isolated from the human limbus and central cornea, although isolation is most efficient from the limbus and from younger individuals<sup>[29]</sup>. These culture experiments imply that both the limbal epithelium and the central corneal epithelium have cells that are able to behave like stem cells in clonogenic assays *in vitro* which argues against the LESC and germinative basal layer (GBL) hypotheses. However, if culture conditions unmasked proliferative potential of corneal epithelial cells, which is not expressed during normal homeostasis *in vivo*, this result would be compatible with all three hypotheses.

Despite their slow proliferation *in vivo* (see next section and reference<sup>[30]</sup>), human limbal epithelial cells grow well in culture and have a higher mitotic rate than corneal epithelial cells<sup>[31]</sup>. Furthermore, clinical observations indicate that human limbal tissue is superior to central corneal tissue for treating patients with severely wounded corneal epithelia, which is likely to reflect a greater proliferative potential. The corneal epithelium can be restored using grafts of human limbal epithelial tissue<sup>[32,33]</sup> or cells cultured from explanted limbal tissue<sup>[34-38]</sup>. Although the limbus is the preferred source of cells for clinical therapeutic use, this does not help determine whether LSCs are active during normal tissue homeostasis (LESC hypothesis)

or only during wound healing (CESC hypothesis).

There is also evidence that the central cornea of several species contains highly proliferative cells. For example, rabbit central cornea is able to survive for months after the limbus is removed or separated from the cornea<sup>[39,40]</sup>, although corneal integrity slowly degenerates and it does not heal properly after corneal wounding. Similarly, the mouse corneal epithelium was able to sustain itself for four months after the limbus was cauterised to destroy the limbal epithelium<sup>[1]</sup>. It has also been reported that some patients with symptoms of total LESC deficiency retain central islands of normal corneal epithelium for several years<sup>[41]</sup> and in one case this appeared to be sufficient to restore the corneal epithelium<sup>[42]</sup>. These studies show that the central cornea can maintain itself to some extent when the limbus is eliminated or disconnected. This implies that the central corneal epithelium has cells that are able to act as progenitors, if LSCs are unable to maintain the corneal epithelium. However, again this does not show whether these cells act as progenitors during normal homeostasis so it does not provide conclusive evidence against the LESC hypothesis.

### **Cell division characteristics and identification of slow-cycling label-retaining cells**

Stem cell populations maintain themselves and produce more differentiated cells throughout the lifetime of the organism. This is sometimes interpreted as requiring stem cells to divide asymmetrically (producing one stem cell and one TAC) but this does not mean that each division of every stem cell has to be asymmetric as long as the population average achieves this. There have been few attempts to identify asymmetrically dividing cells in the ocular surface<sup>[43]</sup> and results are insufficient to discriminate among the three hypotheses.

A more widely studied characteristic of many stem cells is that they are relatively quiescent so divide infrequently. A slow cell division rate is not an obligatory phenotype of stem cells but it has been used to try to locate the stem cells that maintain the corneal epithelium. Slow-cycling cells (including putative stem cells) are usually identified as "label-retaining cells". These are cells that retain a DNA-label such as BrdU or <sup>3</sup>H-TdR, or a chromatin label such as GFP-tagged histone-2B, after prolonged labelling and a chase period to dilute the label from more rapidly dividing cells. The prolonged period of labelling is to label as many cells as possible including relatively quiescent stem cells that divide infrequently. The chase period is calibrated to dilute label from most cells in the tissue but not any slow-cycling cells (which include putative quiescent stem cells). This method is useful for identifying the location of putative stem cells but is not specific and will also identify other slow-cycling cell types and cells that divide during the labelling



period and then stop dividing when they terminally differentiate.

The ocular surface is a suitable tissue for label-retaining cell experiments because the corneal epithelial TACs divide quite frequently so will readily dilute the label. (The average mitotic rate has been estimated for rats as 14.5% per day for the whole corneal epithelium<sup>[44]</sup> and this equates to approximately 37% for just the mitotic basal layer, based on the relative basal and suprabasal cell numbers in mouse corneas<sup>[45]</sup>). Similarly, BrdU experiments indicate that almost 50% of basal corneal epithelial cells are in S-phase of the cell cycle, during a 24-h labelling period<sup>[46]</sup>. The effectiveness of the chase period is also helped by the constant loss of cells from the superficial layer, as stem cells will not be lost in this way. It has been estimated that once cells leave the basal corneal epithelial layer the time to cell loss (turnover time) is only about 7 d (range  $3\frac{1}{2}$  to 14 d) for mice, rats and humans<sup>[45,47-50]</sup> but a longer turnover time of between 14 and 21 d has been estimated for rabbits<sup>[51]</sup>.

Most investigations have identified label-retaining cells in the basal limbal epithelium but not in the corneal epithelium either after wounding<sup>[5,13]</sup> or during normal homeostasis in mice<sup>[13,45,52-54]</sup>, rats<sup>[55,56]</sup> and rabbits<sup>[57]</sup>. Two caveats about the exclusive location of label-retaining cells to the limbus in these experiments should be mentioned: (1) species differences in cell cycle kinetics and technical differences between studies may affect the number of cells that remain labelled so the chase period needs to be optimised for each species (for example, in these studies, chase periods for treatments without wounding varied from 4 to 11 wk); (2) using a relatively short chase period of 4 wk, Chen *et al.*<sup>[56]</sup> showed that approximately 20% of the label-retaining cells were slow-cycling Langerhans cells rather than putative slow-cycling stem cells. (These Langerhans cells also shared two other characteristics of putative stem cells, discussed below, as they were positive for the marker ABCG2 and had a high nucleus to cytoplasm ratio). Nevertheless, the results of all these studies consistently identified label-retaining cells in the basal limbal epithelium but not in the basal corneal epithelium and it is likely that most of these will be stem cells. Thus, these studies favour the LESC hypothesis, unless there is an additional stem cell population in the corneal epithelium, which is not slow cycling. They also argue against the GBL hypothesis unless none of the limbal label-retaining cells are stem cells.

A completely different result was found for one study with rabbits<sup>[2]</sup>, which prompted the authors to conclude that the corneal epithelium is not maintained by LSCs but by virtually all the cells of the basal corneal epithelium (referred to here as the GBL hypothesis). Rabbits were given 3 intravitreal injections of <sup>3</sup>H-TdR at intervals of 4 d. After a 41-d chase (49 d after the first injection) the labelling index was higher in the corneal epithelium (17.8%) than the limbal

epithelium (3.8%)<sup>[2]</sup>. However, the high labelling index suggests that many of the labelled cells were TACs, rather than slow-cycling stem cells, and the chase period was insufficient to detect label-retaining cells. Paradoxically, the chase period was comparable to that used in an earlier experiment, which identified label-retaining cells in the limbus but not corneal epithelium of rabbits<sup>[57]</sup>. In this experiment, BrdU was infused from an osmotic mini-pump for 14 d, the pump was removed at 17 d, and the rabbits were left for a further 38-d chase period (*i.e.*, until 55 d after the beginning of labelling). In the <sup>3</sup>H-TdR study<sup>[2]</sup>, autoradiography was used to detect label in high quality semi-thin sections and it is possible that this is more sensitive than the BrdU immunofluorescence used earlier<sup>[57]</sup>. If so, more cell divisions and a longer chase period would be required to dilute the <sup>3</sup>H-TdR below detectable levels in the majority of cells in order to identify any label-retaining cells. It would, therefore, be worth repeating the <sup>3</sup>H-TdR experiment with a longer chase period before drawing conclusions that contradict the other label-retaining cell studies.

#### **Movement of corneal epithelial cells**

Early experimental evidence showed that cells moved from the limbus to the cornea to repair a corneal wound in guinea pigs<sup>[58]</sup>. The observation that donor corneal epithelial cells, transplanted to the centre of rabbit corneas, were replaced by host cells more quickly at the periphery of the transplant also suggested that new host cells were moving centripetally from the periphery of the cornea to replace the older donor cells<sup>[59]</sup>. Other indirect evidence that cells move centripetally from the limbus during homeostasis of unwounded corneas is reviewed elsewhere<sup>[60]</sup>. More importantly, direct observations of radial epithelial movement during normal corneal homeostasis have consistently demonstrated that cells move centripetally from the periphery to the centre of the cornea. This supports the LESC hypothesis, which proposes that LSCs remain in their limbal niche but TACs move centripetally to maintain the corneal epithelium.

These experiments also provided estimates of the rate of centripetal movement of corneal epithelial cells for the unwounded cornea. This was estimated to be 28  $\mu$ m/d from observations of one human subject over 24 h using *in vivo* confocal microscopy<sup>[61]</sup>. For mouse corneas, the rate of centripetal movement has been estimated as 11-26  $\mu$ m/d using three different approaches involving direct observations of labelled cells. Corneal epithelial cells labelled with India ink moved 17  $\mu$ m/d over 7 d<sup>[62]</sup>, patches of brightly fluorescent cells moved 26  $\mu$ m/d over 7 wk in mosaic GFP transgenic mice<sup>[63]</sup> and fluorescent clones of cells extended 11  $\mu$ m/d over 12 wk in K14-CreERT2;R26R-confetti transgenic mice (from 9 to 21 wk after tamoxifen-activation of the reporter transgene)<sup>[64]</sup>. Furthermore, the evidence from the mosaic GFP



transgenic mice<sup>[63]</sup> and tamoxifen-activated reporter transgenic mice<sup>[64]</sup> demonstrated that the same clonal lineage of cells moved across the full radius from the periphery to the centre. This is in contrast to cells in the conjunctiva, which do not move significantly at all<sup>[62,65]</sup>.

When Majo *et al.*<sup>[1]</sup> proposed the CESC cell hypothesis they also proposed that the corneal and conjunctival epithelia continuously expand towards the limbus, which they described as a zone of equilibrium, so any movement in the corneal epithelium was predicted to be centrifugal. This is inconsistent both with the absence of movement in the conjunctiva<sup>[62,65]</sup> and the convincing, direct evidence that movement of corneal epithelial cells is centripetal not centrifugal<sup>[62-64]</sup>. The evidence for centripetal cell movement in the corneal epithelium is inconsistent with the CESC hypothesis as originally proposed<sup>[1]</sup> but there is no need to link the stem cell location and movement aspects of the original CESC hypothesis. In principle, it would be possible for the corneal epithelium to be maintained by stem cells, scattered throughout the tissue, without invoking centrifugal movement. It is likely that TACs produced by CESC would only move radially because evidence from various mosaics and chimaeras implies that lateral movement is constrained (discussed in the next section). In theory, radial movement of TACs could be either centripetal or centrifugal but, as noted above, evidence for centripetal movement is compelling.

The causes of centripetal movement are not known and suggestions include: (1) population pressure from the periphery due to production of new TACs by LESC<sup>[66-68]</sup>; (2) preferential loss of epithelial cells from the central cornea<sup>[30,60]</sup>; (3) differential stiffness of cornea and limbus<sup>[69]</sup>; (4) chemotaxis<sup>[62]</sup>; (5) stimulation by corneal nerves<sup>[70]</sup>; and (6) response to endogenous electric currents<sup>[71]</sup>.

If the LESC hypothesis was incorrect, centripetal movement could still be explained by a mechanism other than population pressure from the limbus. However, if LESC were absent or only active during wound healing, a peripheral source of cells would be required to replace peripheral cells that move centripetally, during normal homeostasis. This might be provided by limbal TACs for the GBL hypothesis or CESC in or near the limbus for the CESC hypothesis. Thus, both the CESC and GBL hypotheses could account for centripetal movement of cells in separately maintained regions on the same radius. However, evidence from transgenic mice shows that a single clone of cells moves across the full radius<sup>[63,64]</sup>, implying that there is a single source of cells in the limbus or peripheral cornea, rather than multiple sources throughout the cornea. This is more difficult for the CESC and GBL hypotheses to explain unless it is argued that not all radial regions are maintained by a single CESC or progenitor TAC. Another problem for the CESC hypothesis is that the CESC would tend to

move centripetally with the TACs, and so accumulate in the centre, unless the CESC were somehow stabilised in unidentified niches and the TACs could move past them. Overall, centripetal movement strongly favours the LESC hypothesis and it is difficult to reconcile this with the other two hypotheses without *ad hoc* assumptions.

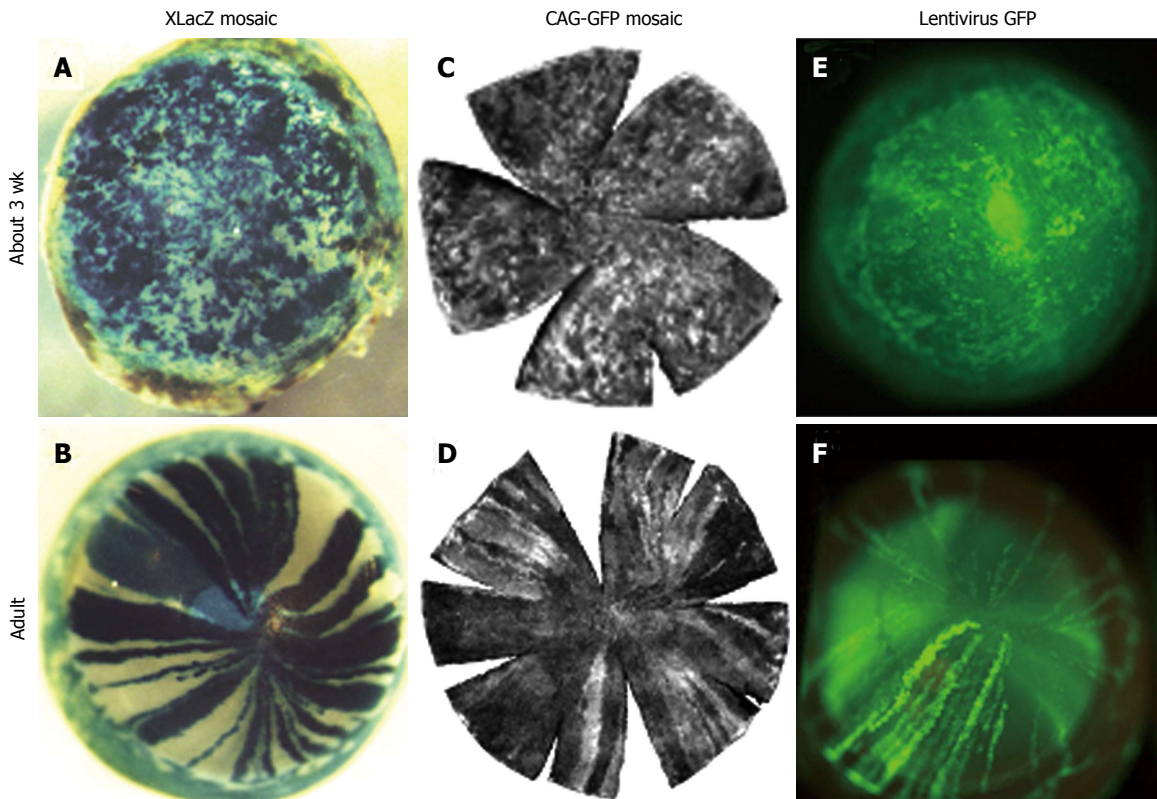
### **Change in mosaic patterns after birth**

In addition to direct studies of cell movement in real time, changes in patterns in several types of mosaic mice have provided additional evidence that cells emerge from the limbus at the periphery of the corneal epithelium and continue to move centripetally across the cornea. Mosaic patterns in adult mouse and rat chimaeras and mouse X-inactivation mosaics (*XLacZ* mosaics) are arranged as radial stripes in the corneal epithelium<sup>[27,72,73]</sup>, which is consistent with either centripetal or centrifugal movement, without significant lateral dispersion. Similar radial stripes, have been observed with various endogenous markers in human corneas, including traces of pigment<sup>[58]</sup> and various opacities, cell inclusions or drug-induced lipidosis associated with vortex keratopathy (cornea verticillata) or hurricane keratopathy<sup>[60,66,74,75]</sup>. In many cases the stripes form a spiral-pattern in the centre, which fits well with the more direct evidence for centripetal movement, discussed above, because centripetal movement of labelled cells transplanted to the rabbit limbus sometimes formed a similar spiral<sup>[76]</sup>.

Before about 5 wk of age, the pattern in X-inactivation mosaics is completely different from the adult radial stripes and the  $\beta$ -gal-positive and  $\beta$ -gal-negative cell populations initially form randomly orientated patches<sup>[27,72]</sup>. Groups of  $\beta$ -gal-positive and  $\beta$ -gal-negative cells emerge from the periphery by about 5 wk and extend as radial stripes across the cornea. The simplest interpretation is that the formation of stripes coincides with the onset of activation of stem cells in the limbus that generate new cells, which replace those produced during development<sup>[27]</sup>. This is supported by similar observations with mosaic transgenic mice<sup>[77,78]</sup> and lineage tracing with a GFP-tagged lentiviral marker<sup>[79]</sup>, as illustrated in Figure 4, and is consistent with the LESC hypothesis but not with the CESC or GBL hypotheses.

One problem with these mosaic systems is that similar proportions of labelled and unlabelled cells were present so many of the radial stripes may comprise more than one adjacent clone that are similarly marked. Observations on *KRT5-LacZ*<sup>+/-</sup> transgenic mice showed that they had rare  $\beta$ -gal-positive stripes in a predominantly  $\beta$ -gal-negative corneal epithelium, so largely avoiding the problem of multiple adjacent clones<sup>[17]</sup>. The distribution of  $\beta$ -gal-positive stripes was not consistent with predictions of centrifugal extension of clones of labelled cells from  $\beta$ -gal-positive CESC distributed randomly in the corneal epithelium and the simplest interpretation is that the stripes represent clonal lineages derived from LESC located





**Figure 4** Transition from randomly orientated patches to radial stripes in corneal epithelia of different types of mosaic mice between 3 wk and adulthood. A and B:  $\beta$ -gal staining in XLacZ X-inactivation mosaics<sup>[27]</sup>; C and D: Green fluorescent protein (GFP) fluorescence in CAG-GFP transgenic mosaics<sup>[77]</sup>; E and F: GFP fluorescence in corneal epithelium after transfecting conceptuses with lentiviral vectors encoding GFP at embryonic day 9 or 10<sup>[79]</sup>. Photographs (A and D) are reproduced from *Developmental Dynamics*<sup>[27]</sup> with kind permission of John Wiley and Sons, (C and D) are reproduced from *Molecular Vision*<sup>[77]</sup> with kind permission of the authors and editors, and photographs (E and F) are reproduced from *Molecular Therapy*<sup>[79]</sup> with kind permission of the authors and the Nature Publishing Group. This combination of photographs was first published by Mort *et al*<sup>[16]</sup>.

in the limbus. However, analysis of striped patterns in *KRT5-LacZ*<sup>+/-</sup> corneas is not unequivocal and similar analyses with inducible lineage markers are required, as discussed below.

#### Transplantation experiments

Bradshaw *et al*<sup>[76]</sup> labelled rabbit limbal tissue *ex vivo* and transplanted it back to the limbus of the donor rabbits after first debriding the corneal epithelium across the full diameter. The labelled cells quickly colonised the corneal epithelium but, as the corneal epithelium was completely removed, this is equivalent to wound healing rather than normal corneal homeostasis. Majo *et al*<sup>[11]</sup> transplanted either  $\beta$ -gal-positive limbal or central corneal tissue from transgenic mice into the limbus of  $\beta$ -gal-negative, immunocompromised mice and both sources of tissue produced similar results. Consistent with the earlier experiment with rabbits<sup>[76]</sup>, labelled clones of donor cells moved centripetally into the corneal epithelium if the host corneal epithelium was removed but it failed to contribute to the corneal epithelium if the host cornea was left intact. Thus, although the transplanted limbal tissue contributed to corneal repair, it did not contribute to steady state corneal maintenance during normal tissue homeostasis, as predicted by the LESC

hypothesis. This was a key result, which prompted Majo *et al*<sup>[11]</sup> to propose the CESC hypothesis.

#### Circumstantial evidence

In addition to the specific investigations discussed so far, there are two circumstantial observations that favour the limbus as a site for stem cells. First, tumours of the ocular surface commonly involve the limbus<sup>[80]</sup> and for other systems it has been suggested that tumour cells may preferentially arise from stem cells<sup>[81,82]</sup>. This provides only weak, circumstantial evidence in favour of the LESC hypothesis.

Second, it is generally agreed that stem cells need a specialised niche environment to maintain the stem cell phenotype and this is likely to involve interactions with several cell types<sup>[6,83]</sup>. For example, signalling from the microvasculature plays an important role in the mouse neural stem cell niche<sup>[84]</sup>. Undeniably, the limbus provides a more diverse population of cell types than cornea and this is enriched further by its blood supply and for this reason it seems arguably a more likely location for a stem cell niche than the cornea. As already mentioned, an additional issue is that a stem cell niche in the basal corneal epithelium might be unstable because of the continuous centripetal movement of TACs. These considerations also make



**Table 2** Examples of marker gene expression differences between the basal limbal and corneal epithelia during normal homeostasis identified by immunostaining

Positive markers			Negative markers		
Marker	Species	Ref.	Marker	Species	Ref.
Integrin $\alpha 9$	Mouse, human	[3,105,106]	Keratin 3	Rabbit	[4]
$\Delta Np63^1$	Human	[3,107,108]	Keratin 3/Keratin 12	Human	[3,108]
$\Delta Np63\alpha$	Human	[96]	NGF receptor (p75NTR)	Human	[3]
ABCG2	Human, rat, rabbit	[3,57,91-93,108]	Involucrin	Human	[3]
Vimentin	Human	[108]	Connexin 43	Human	[3,108]
Keratin 19	Human, mouse	[108,109]	E-cadherin	Human	[3]
Keratin 15	Human, mouse	[109]	Nestin	Human	[108]
N-cadherin	Human	[110]			
Bmi1	Human	[111]			
C/EBP $\delta$	Human	[111]			
ABCB5	Human, mouse	[54]			

<sup>1</sup> $\Delta Np63$  is not expressed in the human basal corneal epithelium<sup>[107]</sup> but it is expressed in mouse and rat corneal epithelia<sup>[27,112,113]</sup>. Positive markers are expressed in basal limbal but not basal corneal epithelium. Negative markers are expressed in basal corneal but not basal limbal epithelium.

it more likely that stem cell niches would be located preferentially in the limbus rather than the cornea.

Although some tissues are maintained during normal homeostasis by stem cells in the main body of the tissue, the limbus is not the only putative stem cell niche with a more peripheral location. For example, there are two types of stem cells that maintain the epithelium that lines the intestinal crypts and villi: crypt base columnar cells and position +4 reserve stem cells. These are both located near the base of the intestinal crypts, from where they produce TACs, which move up the crypt and generate the different functional cell types of the villus epithelium<sup>[85]</sup>. Maintenance of the corneal epithelium by stem cells located in the limbal epithelium, as proposed by the LESC hypothesis, is essentially analogous to the way the intestinal epithelium is maintained. The circumstantial evidence that the limbus is a likely location for a stem cell niche supports the LESC hypothesis. However, it does not provide strong evidence against the CESC hypothesis, which accepts that LSCs exist, or the GBL hypothesis, which predicts there are no stem cells and so no niches.

### Stem cell markers and phenotype

The Holy Grail of stem cell research is to find a phenotype or cell marker that allows the stem cells to be unequivocally distinguished from all neighbouring cells, including early generation TACs, and isolated for further study. This has not yet proved possible for the putative stem cell population(s) that maintain the corneal epithelium. Early evidence that the basal limbal epithelium contained stem cells was produced by an immunohistochemical study of keratin 3 (K3), which is considered to be a corneal differentiation marker<sup>[4]</sup>. K3 is expressed in the basal and suprabasal layers of the rabbit corneal epithelium but only the suprabasal layers of the limbal epithelium, leading to the conclusion the basal limbal epithelium was less differentiated than the other epithelial layers. The

mouse has no K3<sup>[86]</sup> but K12, which normally pairs with K3, is present and expression is restricted to the cornea<sup>[87]</sup>, as shown in Figure 3B. Several authors have also noted that cell morphology of cells in the basal limbal epithelium was more characteristic of stem cells (smaller, euchromatin-rich, high nucleus to cytoplasm ratio) than the corneal epithelium<sup>[3,88]</sup> but, as already noted, Langerhans cells in the limbus also share this phenotype<sup>[56]</sup>. These observations are consistent with the hypothesis that the limbus contains stem cells but no more than that.

The discovery of the K3 difference between basal limbal and corneal epithelia, as a whole, was followed by a quest for a specific cell marker to identify the LSCs within the limbal epithelium. Many candidate markers have been proposed based on differential expression studies (reviewed in reference<sup>[18]</sup>) or conventional immunostaining (Table 2) but no definitive marker has been found, that is known to be expressed in putative stem cells in the limbus but not in neighbouring early generation TACs.

Some of the markers expressed in the limbal but not the corneal epithelium have been identified as putative stem cell markers in other tissues. ATP-binding cassette transporters (ABC transporters) are a family of transmembrane proteins whose functions include the transport of (potentially harmful) metabolic products out of the cells<sup>[89]</sup>. Conceptually, they may form a component of the molecular mechanisms by which long-lived stem cells reduce the potential for genomic damage over their extended lives, and their expression has been correlated with stem cell activity<sup>[90]</sup>. ABCG2 expression in the limbus is one such example and cells expressing this marker can be isolated as a "side population" by fluorescence-activated cell sorting (FACS)<sup>[3,57,91-95]</sup>. However, as noted above, some of the ABCG2-positive, label-retaining cells with a high nucleus to cytoplasm ratio cells in the rat limbus have been identified as Langerhans cells rather than epithelial stem cells<sup>[56]</sup>.



It has recently been shown that ABCB5 appears to be a promising new marker for LESC and early TACs in both mice and humans, which should also allow enrichment by FACS sorting<sup>[54]</sup>.

Despite the absence of a marker that is only expressed in the stem cells,  $\Delta$ Np63a has proved useful for identifying cultures of human limbal cells with sufficient LESC and early TACs for clinical transplantation<sup>[37,96]</sup>.

## RESOLVING THE LESC VS CESC DEBATE

Some of the evidence discussed so far (summarised in rows 1-15 of Table 1) is inconclusive. Evidence from holoclone experiments with pig and human tissues is inconsistent and, in any case, the critical thing is to understand how the corneal epithelium is maintained *in vivo* during normal homeostasis. Various studies have shown that some cells in the central corneal epithelium are capable of acting as long-term progenitor cells. On the face of it, this favours the CESC and GBL hypotheses. However, these observations are also consistent with the LESC hypothesis if some basal corneal epithelial TACs have a latent proliferative potential that is only used if LESC function is compromised so homeostasis is disrupted. Drawing attention to this latent proliferative potential is an important outcome of Majo *et al*'s<sup>[1]</sup> investigations even if the CESC hypothesis ultimately proves to be incorrect.

Other evidence provides better discrimination. There are several strong arguments against the GBL hypothesis. It is inconsistent with the consensus of results from label-retaining cell experiments and the evidence that mosaic patterns in the mouse cornea change after birth, when clones of cells emerge from the peripheral cornea and form radial stripes. The GBL hypothesis also has difficulty accounting for the convincing evidence that corneal epithelial cells move centripetally across the full radius. In our view, this evidence (summarised in Table 1) is sufficient to exclude the GBL hypothesis and it is, therefore, not considered further.

These same observations also argue against the CESC hypothesis. However, results of the label-retaining cell experiments could be accommodated by the CESC hypothesis if CESC were not slow cycling. Nevertheless, the CESC hypothesis is inconsistent with the developmental switch from randomly orientated patches to stripes that emerge from the periphery in various types of genetic mosaics in mice and then extend across the full radius to the centre. By the same token, the CESC hypothesis requires *ad hoc* assumptions to account for centripetal movement, if the same clone of cells moves across the full radius.

Conversely, the LESC hypothesis is inconsistent with the observation that when genetically marked limbal tissue was surgically transplanted to the limbus of immunocompromised mice, donor cells failed to

move into the cornea unless the corneal epithelium was removed<sup>[1]</sup>. Thus, the LESC and CESC hypotheses each have difficulty accounting for one type of evidence. In each case, critical evidence is based on experiments with mice so there are no grounds for suggesting that maintenance of the corneal epithelium differs between mice and humans or other species.

Clinically, it may not matter whether the LESC or CESC hypothesis is correct as both agree that the limbus is a suitable source of stem cells for therapeutic use. However, we need to know where the stem cells that maintain the corneal epithelium during normal homeostasis are located in order to understand the biology of this process. The key issue that needs to be resolved is why evidence from mouse mosaics and transfection with lentiviral markers conflict with results of surgical transplantation experiments. Analysis of mosaics show that, during normal homeostasis, clones of cells appear at the corneal periphery at around 5 wk after birth and extend centripetally across the corneal radius, consistent with activation of LESC. In contrast, surgical studies in mice show that transplantation of labelled cells to the limbus fail to colonise the cornea in a similar way.

One possible explanation is that the transplanted limbal tissue failed to colonise the corneal epithelium because the surgical manipulation or other aspects of the experimental procedure somehow perturbed normal homeostasis and affected the outcome. In principle, one way of testing this would be to label individual cells in the limbus of adult mice, using a genetic switch rather than surgical transplantation and test whether any of the labelled cells produce long-lived clones of cells that colonise the corneal epithelium. Similar genetic labelling of cells in the adult basal corneal epithelium would also allow investigation of whether these produce long-lived clones of cells in the corneal epithelium. This requires lineage tracing experiments and some possibilities are discussed below.

It should be borne in mind that some of the apparent contradictions between the different hypotheses may be more imagined than real, and arise as a result of different research groups attempting to subdivide and label what is, perhaps, a continuum of biological situations. It seems certain that limbal stem cells exist, that they can contribute to regeneration of the cornea, and yet that the basal corneal epithelial cells themselves also have massive regenerative potential. There is no reason why the balance between limbal-mediated and corneal-mediated corneal regeneration should not shift over the lifetime of the animal, with age, disease and injury, and no reason why the balance should necessarily be the same in different species.

Although we talk about wounded and unwounded corneas as if they are separate entities, in fact the corneal epithelium is, of necessity constantly regenerating, because of normal desquamation of cells. Desquamation rate is modulated by rate of blinking,



tear film composition, irritants and abrasive dust in the environment, chronic abrasion caused by *e.g.*, contact lens wear and diseases such as trachoma, acute minor scratches, ranging through to significant physical or chemical injuries and acute infections such as Herpes simplex keratitis. This continuum of insults to the corneal surface may require different levels of limbal response to support the regenerative potential of the corneal epithelial cells. It may be that the genuinely uninjured cornea does not require limbal input, but the genuinely uninjured corneal epithelium does not exist. Experimentally, factors such as the abrasive nature of bedding and dust from food that laboratory mice are exposed to, may modulate corneal regeneration and be a source of variation between research institutes. Furthermore, the large circular central corneal wounds that are so widely used as models of induced regeneration do not really recapitulate any of the most common injuries that happen in life.

There may also be problems with the practical definition of stem cells, particularly with regard to their property of "immortality". We accept that TACs do not renew indefinitely and therefore have a finite lifespan, whereas stem cells do not have this constraint. However, outside the laboratory, a stem cell cannot outlive the individual. We do not know the maximum lifespan of a TAC but this is likely to vary stochastically. If the longer-lived TACs survive for 6 mo or more, this will encompass the effective lifespan of most laboratory mice used experimentally. At that stage, the difference between a long-lived TAC and a stem cell becomes blurred. On the other hand, in larger animals such as humans, a lifespan of 6 mo or a year for a TAC is utterly insignificant in terms of the lifespan of the individual. Differences such as this may start to explain why stem-like regenerative ability may be assigned to the corneal epithelium on experimental small animals, while at the same time the data do not reflect clinical experience in humans.

### Lineage tracing experiments

To test whether any cells in the limbal and/or corneal epithelia can generate long-lived clones of cells that colonise the corneal epithelium, an inducible lineage tracing method is required that can label some of the putative stem cells in the adult at a chosen time without surgical intervention or disturbing homeostasis. Sophisticated, inducible lineage tracing methods using Cre/*loxP* transgenic mice are now available that can be used to throw a genetic switch to label a chosen cell population with a fluorescent or histochemical marker that will identify them and all their mitotic progeny. Such methods have been used to trace stem cell lineages in other tissues, including the hair follicle<sup>[97]</sup>, intestinal epithelium<sup>[98-100]</sup> and ovarian surface epithelium<sup>[101]</sup>, and this approach could be used to help resolve the LESC vs CESC debate. A similar approach has already been used, in conjunction with a multi-coloured reporter construct, to trace clonal lineages in

the ocular surface of zebrafish and demonstrate that, as in mice, the initial patchwork of cells established in the embryo is replaced by a radial pattern of clones that extend from the limbus<sup>[102]</sup>.

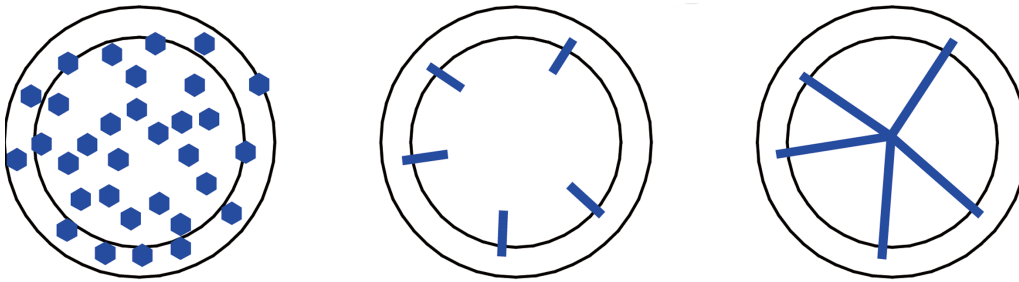
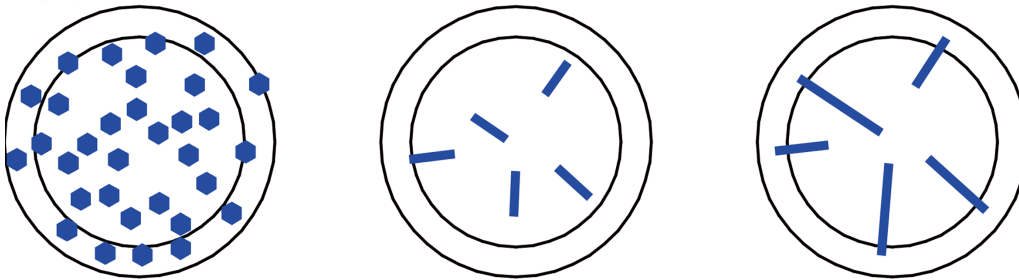
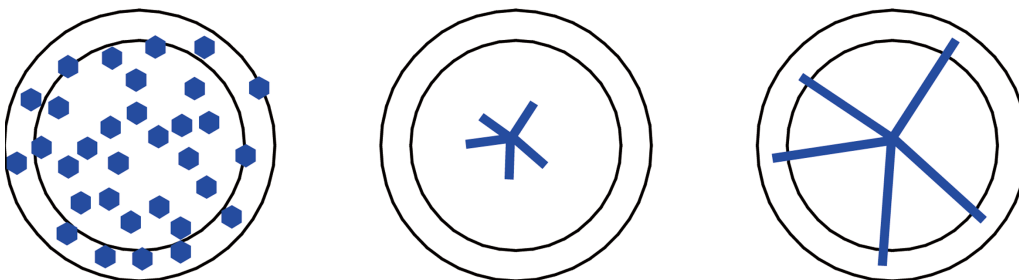
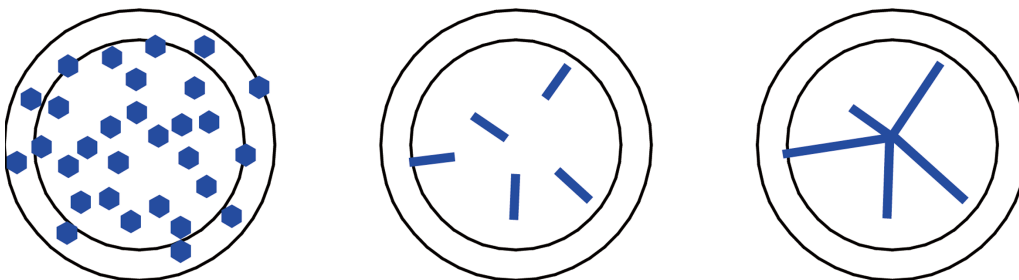
We have begun to explore this Cre/*loxP* lineage tracing approach using transgenic mice in which a reporter transgene is ubiquitously expressed once the flanking *loxP* sites are recombined by active Cre recombinase to remove an upstream stop sequence. Cre recombinase is provided in the form of a CreER fusion protein, which is produced by another transgene under the control of a ubiquitous promoter. The CreER fusion protein is normally sequestered in the cytoplasm unless the mouse is treated with tamoxifen. This binds to the modified oestrogen receptor (ER) and translocates CreER to the nucleus, where it can recombine the *loxP* sites, so removing the stop sequence and activating the reporter transgene. Use of a ubiquitous promoter to drive expression of CreER provides an unbiased approach, which allows putative stem cells in the limbus or cornea (and any other tissue) to be labelled. However it is not specific for stem cells so initially most of the labelled cells will not be stem cells but, by including a chase period, short-lived clones founded by labelled TACs will be shed, leaving long-lived clones founded by labelled stem cells.

The genetic switch is activated when the mouse is injected with tamoxifen. Delaying tamoxifen treatment, until well after the adult stem cells are activated, should ensure that the genetic switch is thrown to label individual adult stem cells, during normal homeostasis. This avoids labelling ancestral cells, which could each generate multiple labelled stem cells. Furthermore, by titrating the dose of tamoxifen it should be possible to label a relatively small proportion of cells so only a few stem cells will be labelled per eye. Together, this will ensure that most of the clones of labelled cells that remain as stripes or patches after the chase period are individual clones produced by single stem cells. The predicted results for such an experiment are shown, for the LESC hypothesis and three versions of the CESC hypothesis, in Figure 5.

Tamoxifen-inducible labelling of a low proportion of stem cells in adults is a significant advantage over analysis of other types of mosaics, where the cells are labelled early in development and many patches and stripes of labelled TACs are likely to be derived from multiple adjacent labelled stem cells. This is because labelling a cell in the embryo will produce a large clone of labelled cells, some of which will remain close together throughout development. Later, when adult stem cells are specified, some adjacent stem cells will probably be clonally related making it difficult to identify TACs descended from a single stem cell in these mosaic systems.

Examples of results of a preliminary experiment of this type are shown in Figure 6 and indicate that the strategy suggested in Figure 5 is feasible. The cornea



**A** LESC hypothesis (centripetal movement)**B** Original CESC hypothesis (centrifugal movement)**C** Central CESC and centrifugal movement**D** CESC throughout cornea and centripetal movement

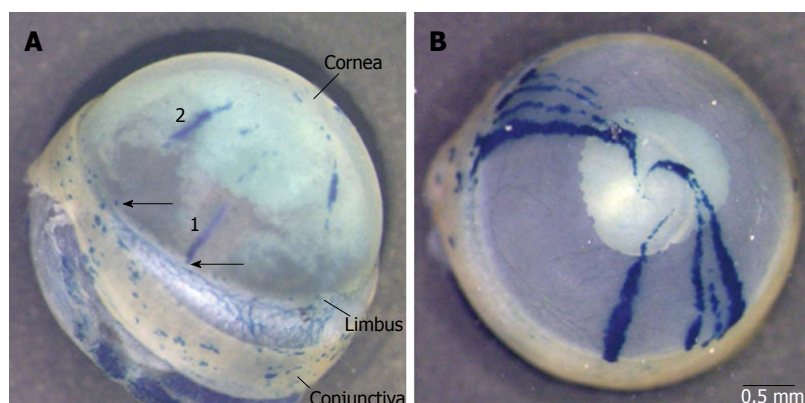
Time 1

Time 2

Time 3

**Figure 5 Hypothetical results from a lineage tracing experiment to distinguish between the limbal epithelial stem cell and corneal epithelial stem cell hypotheses.** In each figure the inner disc represents the corneal epithelium and the outer ring represents the limbal epithelium. If a reporter transgene is driven by a tamoxifen-inducible, ubiquitous promoter, a proportion of all the cell types in the ocular surface (and other tissues) will be labelled shortly after tamoxifen treatment. The frequency of labelled cells will depend partly on the dose of tamoxifen, which could be titrated to ensure only a few stem cells are labelled per eye. Time 1 is shortly after tamoxifen-treatment and the labelled cells may have divided to produce a small clone of labelled cells. By Time 2, the short-lived labelled clones produced by labelling transient (or transit) amplifying cells should have been shed from the corneal epithelium but long-lived labelled clones produced by long-lived labelled stem cells will remain. Expectations for distributions of labelled cells at Times 2 and 3 vary for the different hypotheses. A: The limbal epithelial stem cell hypothesis predicts clones of labelled cells will extend radially from the limbus and, by Time 3, clones of labelled cells will span the full radius; B: The original corneal epithelial stem cell (CESC) hypothesis predicts clones of labelled cells produced by labelled stem cells may also extend radially but will extend centrifugally from stem cells located throughout the corneal epithelium itself. Clones of labelled cells that do not arise from the centre of the cornea will not span the full radius; C: If the CESC hypothesis is modified so that all the CESC are at the very centre of the cornea, centrifugal movement will produce clones of labelled cells that span the full radius by Time 3 but at Time 2 there should be no labelled peripheral cells produced by stem cells; D: If the CESC hypothesis is modified so that the CESC are located throughout the corneal epithelium but movement is centripetal, clones of labelled cells that do not arise from the periphery of the cornea will not span the full radius. To distinguish between the various hypotheses it will be necessary to compare patterns of labelled clones at different times after tamoxifen treatment.





**Figure 6 Preliminary results from a lineage tracing experiment to distinguish between the limbal epithelial stem cell and corneal epithelial stem cell hypotheses.** Eyes from CAGG-CreER; R26R-LacZ mice that were injected with tamoxifen to induce LacZ reporter gene expression and stained for  $\beta$ -galactosidase ( $\beta$ -gal) activity after different chase periods. The pigmented iris is visible through the cornea and appears grey, whereas the  $\beta$ -gal staining is blue. A: Side view of a  $\beta$ -gal-stained eye, after a chase period of 9 wk and 4 d, with several radial  $\beta$ -gal-positive stripes and small patches in the cornea and numerous  $\beta$ -gal-positive patches in the conjunctiva. (The conjunctiva is torn near the limbus and hangs down at the bottom right of the photograph, so the sclera is visible between the limbus and conjunctiva.) Stripe 1 is a limbus-cornea (LC) stripe, with its more peripheral end in the limbus (arrow), consistent with expectations of the limbal epithelial stem cell (LESC) hypothesis (see Figure 5). It also appears to be aligned with other  $\beta$ -gal-positive patches towards the centre of the cornea so it may be part of a longer discontinuous stripe. Stripe 2 is a cornea-cornea stripe with both ends in the cornea, consistent with the corneal epithelial stem cell hypothesis. However, it is radially aligned with a small  $\beta$ -gal-positive patch in the limbus (arrow), which could be the location of a  $\beta$ -gal-positive LESCC. If so, stripe 2 might be a discontinuous stripe, which extended from a LESCC that was not continuously active (consistent with the LESCC hypothesis); B: Anterior (frontal) view of a  $\beta$ -gal-stained eye, after a 14-wk chase period, with eight radial  $\beta$ -gal-positive stripes. All eight stripes are LC stripes with one end at the limbus and many extend the full radius and have a curved end, consistent with a central spiral pattern, as reported for other chimaeric and mosaic eyes<sup>[27,72,73]</sup>. CAGG-CreER; R26R-LacZ mice were produced by crossing CAGG-CreER and R26R-LacZ mice [full names Tg(CAG-cre/Esr1\*)5Amc and B6.129S4-Gt(ROSA)26Sortm1Sor/J; references<sup>[116,117]</sup>]. LacZ reporter gene expression was induced at 12 wk by 3 injections of tamoxifen (100  $\mu$ g/g body weight per injection).

illustrated in Figure 6A was stained for  $\beta$ -gal activity after chase period of 9<sup>1</sup>/<sub>2</sub> weeks. Stripe 1 has its peripheral end in the limbus and its more central end in the cornea so, using the stripe classification system described for mosaic *KRT5<sup>LacZ</sup>* transgenic mice<sup>[17]</sup>, it is classified as a limbus-cornea (LC) stripe. This is consistent with expectations of the LESCC hypothesis (Time 2 in Figure 5A), particularly as the stripe does seem to extend into the limbus itself (arrow in Figure 6A). A peripheral stripe could also be consistent with the original CESC hypothesis (Figure 5B) or the centripetal version shown in Figure 5D, although, in the latter case, peripheral stripes would be expected to be entirely within the cornea (CC stripes). This LC stripe fits less well with the hypothesis that all the CESC are located in the very centre of the cornea (Figure 5C) unless additional assumptions are made (e.g., stripes have already extended to the periphery by 9<sup>1</sup>/<sub>2</sub> weeks).

Stripe 1 also appears to be radially aligned with other  $\beta$ -gal-stained tissue located more centrally so it may be a longer discontinuous stripe. Discontinuous stripes have been discussed elsewhere for *KRT5<sup>LacZ</sup>* transgenic mosaics<sup>[17]</sup> and also occur in mouse chimaeras and X-inactivation mosaics<sup>[27,72,103]</sup>. The discontinuities in  $\beta$ -gal staining shown in Figure 6A could reflect: (1) separate clones of cells derived from  $\beta$ -gal-positive and  $\beta$ -gal-negative CESC or TACs that are radially aligned; (2) dispersal of a  $\beta$ -gal-positive clones by incursions from laterally adjacent  $\beta$ -gal-negative clones; or (3) alternating contributions of more than one stem cell to a single radial stripe if individual stem cells cycle through phases of activity

and quiescence.

Stripe 2 in Figure 6A is a cornea-cornea (CC) stripe with both ends in the cornea, consistent with the predictions of the original CESC hypothesis at Time 2 (Figure 5B) or the centripetal version shown in Figure 5D. However, it might also be consistent with the LESCC hypothesis (Figure 5A) because this stripe is radially aligned with a small  $\beta$ -gal-stained patch in the limbus, which could mark the location of a  $\beta$ -gal-positive LESCC. If so, stripe 2 might be a discontinuous stripe formed in two phases. During the first phase a  $\beta$ -gal-positive region might have extended from a  $\beta$ -gal-positive LESCC, which was active during the early part of the chase period. This could have been followed by extension of a  $\beta$ -gal-negative region generated by an adjacent active  $\beta$ -gal-negative LESCC, if the  $\beta$ -gal-positive LESCC became inactive during the later part of the chase period.

The eye shown in Figure 6B was stained after a 14-wk chase period and has eight  $\beta$ -gal-positive stripes, all of which are LC stripes, consistent with the LESCC hypothesis (Time 3 in Figure 5A) and both the original CESC hypothesis (Figure 5B) and the variant shown in Figure 5C. However, the variant CESC hypothesis shown in Figure 5D predicts that only a small proportion of stripes would extend right to the limbus. Some of these stripes span the full radius, consistent with the LESCC hypothesis (Figure 5A) and the variant CESC hypothesis shown in Figure 5C. It could also be consistent with the original CESC hypothesis (Figure 5B) and the centripetal version, shown in Figure 5D, if full-radius stripes comprised several shorter stripes produced by  $\beta$ -gal-positive



CESCs aligned on the same radius. However, most of the cornea is  $\beta$ -gal-negative so it seems unlikely that the *LacZ* reporter would be activated in radially aligned CESCOs but not in many of the other CESCOs, located elsewhere in the corneal epithelium.

The preliminary results shown in Figure 6 and the alternative explanations of discontinuous stripes indicate that the interpretation of the stripe patterns may be more complicated than predicted in Figure 5, so detailed analysis of many more eyes and different chase periods will be required to resolve the LESC vs CESC debate. Nevertheless, this lineage-tracing approach appears to be a promising way of resolving the conflicting evidence from transplantation experiments and mosaics. Although important, the evidence from conventional mosaics is limited because the time of stem cell labelling cannot be controlled and occurs early in development. Even lineage tracing with a GFP-tagged lentiviral marker (Figure 4E and F) relied on marking one cell population before birth<sup>[79]</sup>. In contrast, a tamoxifen-inducible transgenic reporter system enables cells to be labelled at a specific time in the adult without the risk of disturbing homeostasis with surgical intervention.

While this review was in preparation, a similar lineage tracing study was published online, in a preliminary form, and this has already been mentioned in the section on cell movement<sup>[64]</sup>. This used K14-CreER<sup>T2</sup>; R26R-confetti mice, where the keratin 14 (K14 or *Krt14*) promoter, rather than a ubiquitous promoter, was used to drive tamoxifen-inducible CreER<sup>T2</sup>. The multi-colour "R26R-confetti" fluorescent reporter<sup>[100]</sup> was used to identify labelled cells. This reporter is based on an earlier "brainbow" construct<sup>[104]</sup> and is also similar to that used for the zebrafish cornea<sup>[102]</sup>. According to the authors, immunofluorescence showed that K14 protein was present in basal epithelial cells in the mouse limbus but the central corneal epithelium had much lower levels. Thus, limbal epithelial cells will be preferentially targeted for labelling, making it difficult to test whether CESCOs exist. On the other hand, a chase period to remove short-lived clones formed by TACs rather than stem cells is less important than for the experimental design shown in Figure 5, where CreER is expressed ubiquitously.

Mice were treated with tamoxifen at 6 wk and corneas of the same mice were imaged repeatedly to allow changes in size and position of different clones to be tracked in real time. This elegant time-lapse study showed that labelled cells began to emerge from the limbus 5 wk after tamoxifen treatment and the same stripes were tracked at 9, 13, 17 and 21 wk, as they extended into the centre of the unlabelled cornea without overlapping. The frequency of labelled clones was higher than for the preliminary results illustrated for tamoxifen-treated CAGG-CreER; R26R-*LacZ* mice, shown in Figure 6. However, because the multi-coloured R26R-confetti reporter randomly labelled cells with one of up to ten different colours, individual

adjacent clones could be distinguished easily. In addition to the radial stripes, the authors reported the presence of some rare, small patches of labelled cells in the cornea, which they suggested might have arisen in the cornea itself.

This is an important study as it is the first lineage tracing investigation of the mouse cornea with an inducible marker that was activated in adults. In particular, the informative, real-time study with live mice proved that individual cells labelled in the adult limbal epithelium could form long-lived clones that extend centripetally across the complete radius of the cornea during normal homeostasis. This effectively provides a non-surgical equivalent of the limbal transplantation experiment described by Majo *et al.*<sup>[11]</sup> and supports the interpretation that the outcome of the transplantation was adversely affected by the surgical procedures and homeostasis was perturbed. If so, this would undermine the evidence for the CESC hypothesis and reconcile the conflicting evidence from surgical transplantation experiments and conventional mosaics. In effect, all the available evidence would then be consistent with the LESC hypothesis. To further clarify the situation, additional investigations, driving CreER from a promoter expressed in the corneal epithelium, are now required to investigate whether long-lived clones can arise in the adult cornea as well as the limbus.

## CONCLUSION

There is strong evidence that the corneal epithelium is maintained by stem cells rather than solely by proliferation of more differentiated cells in the basal corneal epithelium. Most evidence also favours the conventional LESC hypothesis, which proposes that limbal epithelial stem cells maintain the corneal epithelium during normal homeostasis. Although limbal transplantation experiments favour the alternative CESC hypothesis, this result could be reconciled with the LESC hypothesis if surgical transplantation perturbed normal homeostasis and affected the outcome. This possibility is supported by a recent non-surgical, lineage-tracing experiment, which demonstrated that clonal derivatives of cells in the limbal epithelium move into the corneal epithelium during normal homeostasis. Thus, the available evidence supports the conclusion that, during normal homeostasis, the corneal epithelium is maintained by stem cells in the limbus, which produce daughter TACs that migrate centripetally, rather than any stem cells in the corneal epithelium itself. However, if homeostasis is compromised, so the limbal epithelial stem cells are unable to maintain the corneal epithelium, it seems likely that TACs in the basal corneal epithelium can act as long-term progenitors and maintain the tissue for a considerable time in the absence of functional LESCOs.



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## REFERENCES

- 1 **Majo F**, Roachat A, Nicolas M, Jaoudé GA, Barrandon Y. Oligopotent stem cells are distributed throughout the mammalian ocular surface. *Nature* 2008; **456**: 250-254 [PMID: 18830243]
- 2 **Haddad A**, Faria-e-Sousa SJ. Maintenance of the corneal epithelium is carried out by germinative cells of its basal stratum and not by presumed stem cells of the limbus. *Braz J Med Biol Res* 2014; **47**: 470-477 [PMID: 24820068]
- 3 **Chen Z**, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells* 2004; **22**: 355-366 [PMID: 15153612]
- 4 **Schermer A**, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986; **103**: 49-62 [PMID: 2424919]
- 5 **Cotsarelis G**, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell* 1989; **57**: 201-209 [PMID: 2702690]
- 6 **Li W**, Hayashida Y, Chen YT, Tseng SC. Niche regulation of corneal epithelial stem cells at the limbus. *Cell Res* 2007; **17**: 26-36 [PMID: 17211449]
- 7 **Xie HT**, Chen SY, Li GG, Tseng SC. Limbal epithelial stem/progenitor cells attract stromal niche cells by SDF-1/CXCR4 signaling to prevent differentiation. *Stem Cells* 2011; **29**: 1874-1885 [PMID: 21948620 DOI: 10.1002/stem.743]
- 8 **Shortt AJ**, Secker GA, Munro PM, Khaw PT, Tuft SJ, Daniels JT. Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells. *Stem Cells* 2007; **25**: 1402-1409 [PMID: 17332511 DOI: 10.1634/stemcells.2006-0580]
- 9 **Dziasko MA**, Armer HE, Levis HJ, Shortt AJ, Tuft S, Daniels JT. Localisation of epithelial cells capable of holoclone formation in vitro and direct interaction with stromal cells in the native human limbal crypt. *PLoS One* 2014; **9**: e94283 [PMID: 24714106 DOI: 10.1371/journal.pone.0094283]
- 10 **Dua HS**, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol* 2005; **89**: 529-532 [PMID: 15834076]
- 11 **Kulkarni BB**, Tighe PJ, Mohammed I, Yeung AM, Powe DG, Hopkinson A, Shanmuganathan VA, Dua HS. Comparative transcriptional profiling of the limbal epithelial crypt demonstrates its putative stem cell niche characteristics. *BMC Genomics* 2010; **11**: 526 [PMID: 20920242 DOI: 10.1186/1471-2164-11-526]
- 12 **Pajohesh-Ganji A**, Pal-Ghosh S, Tadvalkar G, Stepp MA. Corneal goblet cells and their niche: implications for corneal stem cell deficiency. *Stem Cells* 2012; **30**: 2032-2043 [PMID: 22821715 DOI: 10.1002/stem.1176]
- 13 **Lehrer MS**, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci* 1998; **111**: 2867-2875 [PMID: 9730979]
- 14 **Beebe DC**, Masters BR. Cell lineage and the differentiation of corneal epithelial cells. *Invest Ophthalmol Vis Sci* 1996; **37**: 1815-1825 [PMID: 8759349]
- 15 **Marinari E**, Mehonic A, Curran S, Gale J, Duke T, Baum B. Live-cell delamination counterbalances epithelial growth to limit tissue overcrowding. *Nature* 2012; **484**: 542-545 [PMID: 22504180 DOI: 10.1038/nature10984]
- 16 **Sun TT**, Tseng SC, Lavker RM. Location of corneal epithelial stem cells. *Nature* 2010; **463**: E10; discussion E11 [PMID: 20182462 DOI: 10.1038/nature08805]
- 17 **Douvaras P**, Webb S, Whitaker DA, Dorà N, Hill RE, Dorin JR, West JD. Rare corneal clones in mice suggest an age-related decrease of stem cell activity and support the limbal epithelial stem cell hypothesis. *Stem Cell Res* 2012; **8**: 109-119 [PMID: 22099025 DOI: 10.1016/j.scr.2011.08.007]
- 18 **Mort RL**, Douvaras P, Morley SD, Dorà N, Hill RE, Collinson JM, West JD. Stem cells and corneal epithelial maintenance: insights from the mouse and other animal models. *Results Probl Cell Differ* 2012; **55**: 357-394 [PMID: 22918816 DOI: 10.1007/978-3-642-30406-4\_19]
- 19 **Dor Y**, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 2004; **429**: 41-46 [PMID: 15129273 DOI: 10.1038/nature02520]
- 20 **Clayton E**, Doupé DP, Klein AM, Winton DJ, Simons BD, Jones PH. A single type of progenitor cell maintains normal epidermis. *Nature* 2007; **446**: 185-189 [PMID: 17330052]
- 21 **Giangreco A**, Arwerta EN, Rosewell IR, Snyder J, Watt FM, Stripp BR. Stem cells are dispensable for lung homeostasis but restore airways after injury. *Proc Natl Acad Sci USA* 2009; **106**: 9286-9291 [DOI: 10.1073/pnas.0900668106]
- 22 **Miyajima A**, Tanaka M, Itoh T. Stem/progenitor cells in liver development, homeostasis, regeneration, and reprogramming. *Cell Stem Cell* 2014; **14**: 561-574 [PMID: 24792114 DOI: 10.1016/j.stem.2014.04.010]
- 23 **Tarlow BD**, Finegold MJ, Grompe M. Clonal tracing of Sox9+ liver progenitors in mouse oval cell injury. *Hepatology* 2014; **60**: 278-289 [PMID: 24700457 DOI: 10.1002/hep.27084]
- 24 **Barrandon Y**, Green H. Three clonal types of keratinocyte with different capacities for multiplication. *Proc Natl Acad Sci USA* 1987; **84**: 2302-2306 [PMID: 2436229]
- 25 **Pellegrini G**, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, De Luca M. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J Cell Biol* 1999; **145**: 769-782 [PMID: 10330405]
- 26 **Chung EH**, Bukusoglu G, Zieske JD. Localization of corneal epithelial stem cells in the developing rat. *Invest Ophthalmol Vis Sci* 1992; **33**: 2199-2206 [PMID: 1607230]
- 27 **Collinson JM**, Morris L, Reid AI, Ramaesh T, Keighren MA, Flockhart JH, Hill RE, Tan SS, Ramaesh K, Dhillon B, West JD. Clonal analysis of patterns of growth, stem cell activity, and cell movement during the development and maintenance of the murine corneal epithelium. *Dev Dyn* 2002; **224**: 432-440 [PMID: 12203735]
- 28 **Tanifuji-Terai N**, Terai K, Hayashi Y, Chikama T, Kao WW. Expression of keratin 12 and maturation of corneal epithelium during development and postnatal growth. *Invest Ophthalmol Vis Sci* 2006; **47**: 545-551 [PMID: 16431949]
- 29 **Chang CY**, McGhee JJ, Green CR, Sherwin T. Comparison of stem cell properties in cell populations isolated from human central and limbal corneal epithelium. *Cornea* 2011; **30**: 1155-1162 [PMID: 21849892 DOI: 10.1097/ICO.0b013e318213796b]
- 30 **Lavker RM**, Dong G, Cheng SZ, Kudoh K, Cotsarelis G, Sun TT. Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. *Invest Ophthalmol Vis Sci* 1991; **32**: 1864-1875 [PMID: 2032808]
- 31 **Ebato B**, Friend J, Thoft RA. Comparison of limbal and peripheral human corneal epithelium in tissue culture. *Invest Ophthalmol Vis Sci* 1988; **29**: 1533-1537 [PMID: 3170124]
- 32 **Kenyon KR**, Tseng SC. Limbal autograft transplantation for ocular



- surface disorders. *Ophthalmology* 1989; **96**: 709-722; discussion 722-723 [PMID: 2748125]
- 33 **Tseng SC.** Concept and application of limbal stem cells. *Eye (Lond)* 1989; **3**: 141-157 [PMID: 2695347]
  - 34 **Pellegrini G,** Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 1997; **349**: 990-993 [PMID: 9100626 DOI: 10.1016/s0140-6736(96)11188-0]
  - 35 **Tsai RJ,** Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med* 2000; **343**: 86-93 [PMID: 10891515 DOI: 10.1056/nejm200007133430202]
  - 36 **Sangwan VS,** Vemuganti GK, Iftekhhar G, Bansal AK, Rao GN. Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury: a case report of unique application. *Cornea* 2003; **22**: 478-481 [PMID: 12827056 DOI: 10.1097/00003226-200307000-00016]
  - 37 **Rama P,** Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G. Limbal stem-cell therapy and long-term corneal regeneration. *N Engl J Med* 2010; **363**: 147-155 [PMID: 20573916 DOI: 10.1056/NEJMoa0905955]
  - 38 **Shortt AJ,** Tuft SJ, Daniels JT. Corneal stem cells in the eye clinic. *Br Med Bull* 2011; **100**: 209-225 [PMID: 21926089 DOI: 10.1093/bmb/ldr041]
  - 39 **Huang AJ,** Tseng SC. Corneal epithelial wound healing in the absence of limbal epithelium. *Invest Ophthalmol Vis Sci* 1991; **32**: 96-105 [PMID: 1702774]
  - 40 **Kawakita T,** Higa K, Shimmura S, Tomita M, Tsubota K, Shimazaki J. Fate of corneal epithelial cells separated from limbus in vivo. *Invest Ophthalmol Vis Sci* 2011; **52**: 8132-8137 [PMID: 21896841 DOI: 10.1167/iov.11-7984]
  - 41 **Dua HS,** Miri A, Alomar T, Yeung AM, Said DG. The role of limbal stem cells in corneal epithelial maintenance: testing the dogma. *Ophthalmology* 2009; **116**: 856-863 [PMID: 19410942 DOI: 10.1016/j.ophtha.2008.12.017]
  - 42 **Bi YL,** Bock F, Zhou Q, Cursiefen C. Central corneal epithelium self-healing after ring-shaped glycerin-cryopreserved lamellar keratoplasty in Terrien marginal degeneration. *Int J Ophthalmol* 2013; **6**: 251-252 [PMID: 23638432 DOI: 10.3980/j.issn.2222-3959.2013.02.27]
  - 43 **Castro-Muñozledo F,** Gómez-Flores E. Challenges to the study of asymmetric cell division in corneal and limbal epithelia. *Exp Eye Res* 2011; **92**: 4-9 [PMID: 21056036 DOI: 10.1016/j.exer.2010.11.002]
  - 44 **Bertalanffy FD,** Lau C. Mitotic rate and renewal time of the corneal epithelium in the rat. *Arch Ophthalmol* 1962; **68**: 546-550 [PMID: 13868376]
  - 45 **Douvaras P,** Mort RL, Edwards D, Ramaesh K, Dhillon B, Morley SD, Hill RE, West JD. Increased corneal epithelial turnover contributes to abnormal homeostasis in the Pax6<sup>-/-</sup> mouse model of aniridia. *PLoS One* 2013; **8**: e71117 [PMID: 23967157 DOI: 10.1371/journal.pone.0071117]
  - 46 **Urbanowicz MM,** Zhao J, Nagasaki T. Spatial distribution of cell divisions in the basal epithelium of mouse cornea. *Invest Ophthalmol Vis Sci* 2011; **52** (ARVO Meeting Abstracts April 22, 2011): E-abstract 320
  - 47 **Hanna C,** O'Brien JE. Cell production and migration in the epithelial layer of the cornea. *Arch Ophthalmol* 1960; **64**: 536-539 [PMID: 13711262]
  - 48 **Hanna C,** Bicknell DS, O'Brien JE. Cell turnover in the adult human eye. *Arch Ophthalmol* 1961; **65**: 695-698 [PMID: 13711260]
  - 49 **Håskjold E,** Bjerknes R, Bjerknes E. Migration of cells in the rat corneal epithelium. *Acta Ophthalmol (Copenh)* 1989; **67**: 91-96 [PMID: 2773642]
  - 50 **Cenedella RJ,** Fleschner CR. Kinetics of corneal epithelium turnover in vivo. Studies of lovastatin. *Invest Ophthalmol Vis Sci* 1990; **31**: 1957-1962 [PMID: 2210991]
  - 51 **Haddad A.** Renewal of the rabbit corneal epithelium as investigated by autoradiography after intravitreal injection of 3H-thymidine. *Cornea* 2000; **19**: 378-383 [PMID: 10832703]
  - 52 **Pajooesh-Ganji A,** Pal-Ghosh S, Simmens SJ, Stepp MA. Integrins in slow-cycling corneal epithelial cells at the limbus in the mouse. *Stem Cells* 2006; **24**: 1075-1086 [PMID: 16282441]
  - 53 **Zhao J,** Mo V, Nagasaki T. Distribution of label-retaining cells in the limbal epithelium of a mouse eye. *J Histochem Cytochem* 2009; **57**: 177-185 [PMID: 19001638 DOI: 10.1369/jhc.2008.952390]
  - 54 **Ksander BR,** Kolovou PE, Wilson BJ, Saab KR, Guo Q, Ma J, McGuire SP, Gregory MS, Vincent WJ, Perez VL, Cruz-Guilloty F, Kao WW, Call MK, Tucker BA, Zhan Q, Murphy GF, Lathrop KL, Alt C, Mortensen LJ, Lin CP, Zieske JD, Frank MH, Frank NY. ABCB5 is a limbal stem cell gene required for corneal development and repair. *Nature* 2014; **511**: 353-357 [PMID: 25030174 DOI: 10.1038/nature13426]
  - 55 **Chen W,** Ishikawa M, Yamaki K, Sakuragi S. Wistar rat palpebral conjunctiva contains more slow-cycling stem cells that have larger proliferative capacity: implication for conjunctival epithelial homeostasis. *Jpn J Ophthalmol* 2003; **47**: 119-128 [PMID: 12738543 DOI: 10.1016/s0021-5155(02)00687-1]
  - 56 **Chen W,** Hara K, Tian Q, Zhao K, Yoshitomi T. Existence of small slow-cycling Langerhans cells in the limbal basal epithelium that express ABCG2. *Exp Eye Res* 2007; **84**: 626-634 [PMID: 17254566 DOI: 10.1016/j.exer.2006.11.006]
  - 57 **Budak MT,** Alpdogan OS, Zhou M, Lavker RM, Akinci MA, Wolosin JM. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci* 2005; **118**: 1715-1724 [PMID: 15811951]
  - 58 **Davanger M,** Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature* 1971; **229**: 560-561 [PMID: 4925352]
  - 59 **Kinoshita S,** Friend J, Thoft RA. Sex chromatin of donor corneal epithelium in rabbits. *Invest Ophthalmol Vis Sci* 1981; **21**: 434-441 [PMID: 7024181]
  - 60 **Lemp MA,** Mathers WD. Corneal epithelial cell movement in humans. *Eye (Lond)* 1989; **3**: 438-445 [PMID: 2606218]
  - 61 **Auran JD,** Koester CJ, Kleiman NJ, Rapaport R, Bomann JS, Wirotks BM, Florakis GJ, Koniarek JP. Scanning slit confocal microscopic observation of cell morphology and movement within the normal human anterior cornea. *Ophthalmology* 1995; **102**: 33-41 [PMID: 7831039]
  - 62 **Buck RC.** Measurement of centripetal migration of normal corneal epithelial cells in the mouse. *Invest Ophthalmol Vis Sci* 1985; **26**: 1296-1299 [PMID: 4030257]
  - 63 **Nagasaki T,** Zhao J. Centripetal movement of corneal epithelial cells in the normal adult mouse. *Invest Ophthalmol Vis Sci* 2003; **44**: 558-566 [PMID: 12556383]
  - 64 **Di Girolamo N,** Bobba S, Raviraj V, Delic NC, Slapetova I, Nicovich PR, Halliday GM, Wakefield D, Whan R, Lyons GJ. Tracing the fate of limbal epithelial progenitor cells in the murine cornea. *Stem Cells* 2014 Jun 25; Epub ahead of print [PMID: 24966117 DOI: 10.1002/stem.1769]
  - 65 **Nagasaki T,** Zhao J. Uniform distribution of epithelial stem cells in the bulbar conjunctiva. *Invest Ophthalmol Vis Sci* 2005; **46**: 126-132 [PMID: 15623764]
  - 66 **Bron AJ.** Vortex patterns of the corneal epithelium. *Trans Ophthalmol Soc U K* 1973; **93**: 455-472 [PMID: 4210604]
  - 67 **Sharma A,** Coles WH. Kinetics of corneal epithelial maintenance and graft loss. A population balance model. *Invest Ophthalmol Vis Sci* 1989; **30**: 1962-1971 [PMID: 2674050]
  - 68 **Wolosin JM,** Xiong X, Schütte M, Stegman Z, Tieng A. Stem cells and differentiation stages in the limbo-corneal epithelium. *Prog Retin Eye Res* 2000; **19**: 223-255 [PMID: 10674709 DOI: 10.1016/s1350-9462(99)00005-1]
  - 69 **Foster JW,** Jones RR, Bippes CA, Gouveia RM, Connon CJ. Differential nuclear expression of Yap in basal epithelial cells across the cornea and substrates of differing stiffness. *Exp Eye Res* 2014; **127**: 37-41 [PMID: 24992208 DOI: 10.1016/j.exer.2014.06.020]
  - 70 **Jones MA,** Marfurt CF. Sympathetic stimulation of corneal epithelial proliferation in wounded and nonwounded rat eyes. *Invest Ophthalmol Vis Sci* 1996; **37**: 2535-2547 [PMID: 8977468]



- 71 **McCaig CD**, Rajnicek AM, Song B, Zhao M. Controlling cell behavior electrically: current views and future potential. *Physiol Rev* 2005; **85**: 943-978 [PMID: 15987799 DOI: 10.1152/physrev.00020.2004]
- 72 **Mort RL**, Ramaesh T, Kleinjan DA, Morley SD, West JD. Mosaic analysis of stem cell function and wound healing in the mouse corneal epithelium. *BMC Dev Biol* 2009; **9**: 4 [PMID: 19128502 DOI: 10.1186/1471-213X-9-4]
- 73 **Iannaccone S**, Zhou Y, Walterhouse D, Taborn G, Landini G, Iannaccone P. Three dimensional visualization and fractal analysis of mosaic patches in rat chimeras: cell assortment in liver, adrenal cortex and cornea. *PLoS One* 2012; **7**: e31609 [PMID: 22347498 DOI: 10.1371/journal.pone.0031609]
- 74 **Chong EM**, Campbell RJ, Bourne WM. Vortex keratopathy in a patient with multiple myeloma. *Cornea* 1997; **16**: 592-594 [PMID: 9294696]
- 75 **Dua HS**, Gomes JA. Clinical course of hurricane keratopathy. *Br J Ophthalmol* 2000; **84**: 285-288 [PMID: 10684839]
- 76 **Bradshaw JJ**, Obritsch WF, Cho BJ, Gregerson DS, Holland EJ. Ex vivo transduction of corneal epithelial progenitor cells using a retroviral vector. *Invest Ophthalmol Vis Sci* 1999; **40**: 230-235 [PMID: 9888447]
- 77 **Zhang W**, Zhao J, Chen L, Urbanowicz MM, Nagasaki T. Abnormal epithelial homeostasis in the cornea of mice with a destrin deletion. *Mol Vis* 2008; **14**: 1929-1939 [PMID: 18958303]
- 78 **Hayashi Y**, Watanabe N, Ohashi Y. The "replacement hypothesis": corneal stem cell origin epithelia are replaced by limbal stem cell origin epithelia in mouse cornea during maturation. *Cornea* 2012; **31** Suppl 1: S68-S73 [PMID: 23038039]
- 79 **Endo M**, Zoltick PW, Chung DC, Bennett J, Radu A, Muvarak N, Flake AW. Gene transfer to ocular stem cells by early gestational intraamniotic injection of lentiviral vector. *Mol Ther* 2007; **15**: 579-587 [PMID: 17245352]
- 80 **Waring GO**, Roth AM, Ekins MB. Clinical and pathologic description of 17 cases of corneal intraepithelial neoplasia. *Am J Ophthalmol* 1984; **97**: 547-559 [PMID: 6720832]
- 81 **Reya T**, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105-111 [PMID: 11689955]
- 82 **Burkert J**, Wright NA, Alison MR. Stem cells and cancer: an intimate relationship. *J Pathol* 2006; **209**: 287-297 [PMID: 16770755 DOI: 10.1002/path.2016]
- 83 **Wagers AJ**. The stem cell niche in regenerative medicine. *Cell Stem Cell* 2012; **10**: 362-369 [PMID: 22482502 DOI: 10.1016/j.stem.2012.02.018]
- 84 **Culver JC**, Vadakkan TJ, Dickinson ME. A specialized microvascular domain in the mouse neural stem cell niche. *PLoS One* 2013; **8**: e53546 [PMID: 23308251 DOI: 10.1371/journal.pone.0053546]
- 85 **Barker N**. Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nat Rev Mol Cell Biol* 2014; **15**: 19-33 [PMID: 24326621 DOI: 10.1038/nrm3721]
- 86 **Chaloin-Dufau C**, Pavitt I, Delorme P, Dhoulilly D. Identification of keratins 3 and 12 in corneal epithelium of vertebrates. *Epithelial Cell Biol* 1993; **2**: 120-125 [PMID: 7688259]
- 87 **Liu CY**, Zhu G, Westerhausen-Larson A, Converse R, Kao CW, Sun TT, Kao WW. Cornea-specific expression of K12 keratin during mouse development. *Curr Eye Res* 1993; **12**: 963-974 [PMID: 7508359]
- 88 **Romano AC**, Espana EM, Yoo SH, Budak MT, Wolosin JM, Tseng SC. Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. *Invest Ophthalmol Vis Sci* 2003; **44**: 5125-5129 [PMID: 14638707 DOI: 10.1167/iovs.03-0628]
- 89 **Rees DC**, Johnson E, Lewinson O. ABC transporters: the power to change. *Nat Rev Mol Cell Biol* 2009; **10**: 218-227 [PMID: 19234479 DOI: 10.1038/nrm2646]
- 90 **Bunting KD**. ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* 2002; **20**: 11-20 [PMID: 11796918]
- 91 **Watanabe K**, Nishida K, Yamato M, Umemoto T, Sumide T, Yamamoto K, Maeda N, Watanabe H, Okano T, Tano Y. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. *FEBS Lett* 2004; **565**: 6-10 [PMID: 15135043 DOI: 10.1016/j.febslet.2004.03.064]
- 92 **de Paiva CS**, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells* 2005; **23**: 63-73 [PMID: 15625123 DOI: 10.1634/stemcells.2004-0093]
- 93 **Umemoto T**, Yamato M, Nishida K, Kohno C, Yang J, Tano Y, Okano T. Rat limbal epithelial side population cells exhibit a distinct expression of stem cell markers that are lacking in side population cells from the central cornea. *FEBS Lett* 2005; **579**: 6569-6574 [PMID: 16297384 DOI: 10.1016/j.febslet.2005.10.047]
- 94 **Umemoto T**, Yamato M, Nishida K, Yang J, Tano Y, Okano T. Limbal epithelial side-population cells have stem cell-like properties, including quiescent state. *Stem Cells* 2006; **24**: 86-94 [PMID: 16150918 DOI: 10.1634/stemcells.2005-0064]
- 95 **Krulovala M**, Pokorna K, Lencova A, Fric J, Zajicova A, Filipec M, Forrester JV, Holan V. A rapid separation of two distinct populations of mouse corneal epithelial cells with limbal stem cell characteristics by centrifugation on percoll gradient. *Invest Ophthalmol Vis Sci* 2008; **49**: 3903-3908 [PMID: 18469183 DOI: 10.1167/iovs.08-1987]
- 96 **Di Iorio E**, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M. Isoforms of  $\Delta Np63$  and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci USA* 2005; **102**: 9523-9528 [PMID: 15983386 DOI: 10.1073/pnas.0503437102]
- 97 **Morris RJ**, Liu Y, Marles L, Yang Z, Trempus C, Li S, Lin JS, Sawicki JA, Cotsarelis G. Capturing and profiling adult hair follicle stem cells. *Nat Biotechnol* 2004; **22**: 411-417 [PMID: 15024388 DOI: 10.1038/nbt950]
- 98 **Barker N**, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007; **449**: 1003-1007 [PMID: 17934449]
- 99 **Sangiorgi E**, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* 2008; **40**: 915-920 [PMID: 18536716]
- 100 **Snippert HJ**, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD, Clevers H. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* 2010; **143**: 134-144 [PMID: 20887898 DOI: 10.1016/j.cell.2010.09.016]
- 101 **Ng A**, Tan S, Singh G, Rizk P, Swathi Y, Tan TZ, Huang RY, Leushacke M, Barker N. Lgr5 marks stem/progenitor cells in ovary and tubal epithelia. *Nat Cell Biol* 2014; **16**: 745-757 [PMID: 24997521]
- 102 **Pan YA**, Freundlich T, Weissman TA, Schoppik D, Wang XC, Zimmerman S, Ciruna B, Sanes JR, Lichtman JW, Schier AF. Zebrafish: multispectral cell labeling for cell tracing and lineage analysis in zebrafish. *Development* 2013; **140**: 2835-2846 [PMID: 23757414 DOI: 10.1242/dev.094631]
- 103 **Collinson JM**, Chanas SA, Hill RE, West JD. Corneal development, limbal stem cell function, and corneal epithelial cell migration in the Pax6<sup>+/+</sup> mouse. *Invest Ophthalmol Vis Sci* 2004; **45**: 1101-1108 [PMID: 15037575]
- 104 **Livet J**, Weissman TA, Kang H, Draft RW, Lu J, Bennis RA, Sanes JR, Lichtman JW. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* 2007; **450**: 56-62 [PMID: 17972876]
- 105 **Stepp MA**, Zhu L, Sheppard D, Cranfill RL. Localized distribution of  $\alpha 9$  integrin in the cornea and changes in expression during corneal epithelial cell differentiation. *J Histochem Cytochem* 1995; **43**: 353-362 [PMID: 7534781]
- 106 **Pajoohesh-Ganji A**, Ghosh SP, Stepp MA. Regional distribution of  $\alpha 9 \beta 1$  integrin within the limbus of the mouse ocular surface. *Dev Dyn* 2004; **230**: 518-528 [PMID: 15188436]
- 107 **Pellegrini G**, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F, De Luca M. p63 identifies



- keratinocyte stem cells. *Proc Natl Acad Sci USA* 2001; **98**: 3156-3161 [PMID: 11248048]
- 108 **Schlötzer-Schrehardt U**, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res* 2005; **81**: 247-264 [PMID: 16051216]
- 109 **Yoshida S**, Shimmura S, Kawakita T, Miyashita H, Den S, Shimazaki J, Tsubota K. Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest Ophthalmol Vis Sci* 2006; **47**: 4780-4786 [PMID: 17065488]
- 110 **Hayashi R**, Yamato M, Sugiyama H, Sumide T, Yang J, Okano T, Tano Y, Nishida K. N-Cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. *Stem Cells* 2007; **25**: 289-296 [PMID: 17008425 DOI: 10.1634/stemcells.2006-0167]
- 111 **Barbaro V**, Testa A, Di Iorio E, Mavilio F, Pellegrini G, De Luca M. C/EBPdelta regulates cell cycle and self-renewal of human limbal stem cells. *J Cell Biol* 2007; **177**: 1037-1049 [PMID: 17562792 DOI: 10.1083/jcb.200703003]
- 112 **Moore JE**, McMullen CB, Mahon G, Adamis AP. The corneal epithelial stem cell. *DNA Cell Biol* 2002; **21**: 443-451 [PMID: 12167247]
- 113 **Ramaesh T**, Ramaesh K, Martin Collinson J, Chanas SA, Dhillon B, West JD. Developmental and cellular factors underlying corneal epithelial dysgenesis in the Pax6<sup>-/-</sup> mouse model of aniridia. *Exp Eye Res* 2005; **81**: 224-235 [PMID: 16080917]
- 114 **Goldberg MF**, Bron AJ. Limbal palisades of Vogt. *Trans Am Ophthalmol Soc* 1982; **80**: 155-171 [PMID: 7182957]
- 115 **Townsend WM**. The limbal palisades of Vogt. *Trans Am Ophthalmol Soc* 1991; **89**: 721-756 [PMID: 1808821]
- 116 **Hayashi S**, McMahon AP. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* 2002; **244**: 305-318 [PMID: 11944939]
- 117 **Soriano P**. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999; **21**: 70-71 [PMID: 9916792]

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## Imprinted *Zac1* in neural stem cells

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important role for NSC quiescence, proliferation and differentiation. Overall, transcriptional, epigenomic, and genomic mechanisms seem to coordinate the functional relationships of NSCs and imprinted genes from development to maturation, and possibly aging.

**Key words:** *Zac1*; Cell fate decisions; Neural stem cells; Genomic imprinting; *Igf2-H19*; *Dlk1*; *p57<sup>Kip2</sup>*; *Necdin*; Differentiation; Imprinted gene networks

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**Core tip:** Both neural stem cells (NSCs) and imprinted genes participate in the same developmental processes. Here, we will explore the possibility that these two processes actually interact with each other. We will exemplarily consider the role of single imprinted genes in NSC biology based on their functional relationship to the imprinted gene *Zac1*, which is itself at the focus of this review due to its role in directing neuronal vs astroglial differentiation of NSCs and as a central hub of an imprinted gene network comprising genes important to NSC biology.

### Abstract

Neural stem cells (NSCs) and imprinted genes play an important role in brain development. On historical grounds, these two determinants have been largely studied independently of each other. Recent evidence suggests, however, that NSCs can reset select genomic imprints to prevent precocious depletion of the stem cell reservoir. Moreover, imprinted genes like the transcriptional regulator *Zac1* can fine tune neuronal vs astroglial differentiation of NSCs. *Zac1* binds in a sequence-specific manner to pro-neuronal and imprinted genes to confer transcriptional regulation and furthermore coregulates members of the p53-family in NSCs. At the genome scale, *Zac1* is a central hub of an imprinted gene network comprising genes with an

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### INTRODUCTION

Neural stem cells (NSC), also known as neural precursor cells (NPC), are the common source of all neuronal and glial cells, including astrocytes and oligodendrocytes, in the developing and adult CNS. They arise from the neuroepithelial layers which line the spinal canal and forebrain ventricles at early embryonic stages and reside in circumscribed regions in the postnatal



brain to produce in a spatio-temporal controlled manner a variety of cell-types. Following a series of symmetric proliferative divisions, NSCs progress to asymmetric neurogenic divisions. Hereby, the parent cell maintains the progenitor state while the daughter cell migrates to its final destination, exits from the cell cycle, differentiates and participates in the formation of complex neural networks<sup>[1]</sup>. Although the last decade has witnessed major progress on the pathways and genes coordinating NSC behavior, a potential role of imprinted genes has been largely ignored. In this review, we will consider current evidences for the general impact of imprinted genes in NSC cell fate decisions and differentiation with a particular focus on their relationship with the imprinted gene *Zac1*, as well as the role of *Zac1* itself, which encodes a versatile transcriptional regulator.

## GENOMIC IMPRINTING

Although equivalent complements of paternally and maternally expressed autosomes are transmitted from parent to offspring, select autosomal regions can be lastingly silenced as a result of their parental origin. This process takes place in the respective parental germ cells and is known as genomic imprinting. Germline-derived imprints are preserved during fertilization and somatic development with few remarkable exceptions (see below). Some 120 genes with a verified imprinting status have been identified in mouse, which are largely conserved in human and correspond to less than 1% of the genome<sup>[2]</sup>. A considerably larger number of genes with a strong bias in allele-specific expression has been recently detected in mice brains although an authentic imprinting status has still to be proven<sup>[3,4]</sup>. In this respect we note that the reported number of genes showing allele-specific expression differences in mice actually matches general estimates on tissue-specific and allele-specific differences in human gene expression (approximately 10%) based on common genetic variations (*i.e.*, single nucleotide and copy number polymorphisms)<sup>[5]</sup>. This observation speaks against the hypothesis that most of these allelic differences originate from genomic imprinting.

Multiple molecular mechanisms govern imprinted gene expression, they include CpG methylation of specific DNA sequences, ncRNAs, alterations in chromatin structure, and posttranslational histone modifications (*e.g.*, lysine acetylation, lysine and arginine methylation, serine phosphorylation, and covalent binding of the small peptide ubiquitin)<sup>[6,7]</sup>. Above all, DNA methylation is at the center of the imprinting process and is thought to catalyze the establishment and life-long maintenance of genomic imprints. Differentially methylated regions (DMR), which harbor CpG-rich regulatory sequences, play a critical role in determining parental allele-specific expression. With few exceptions (*i.e.*, *Zac1*) imprinted

genes cluster in huge, conserved chromosomal domains throughout the genome, and their well-balanced expression enables regular development from fetus to early postnatal life<sup>[8,9]</sup>.

## GENOMIC IMPRINTING AND THE BRAIN

Dysregulation of imprinted gene expression can elicit complex neurodevelopmental syndromes in humans, frequently associated with mental retardation [*i.e.*, Angelman syndrome (OMIM 105830) and Prader-Willi syndrome (OMIM 176270)]<sup>[10]</sup>. Moreover, psychotic and autistic spectrum disorders possibly result from more subtle deregulation of imprinted genes. Indeed, mice harboring altered dosage of single or multiple imprinted genes showed various defects in higher brain functions ranging from learning<sup>[11]</sup> and memory formation<sup>[12]</sup> to social and nurturing behaviors<sup>[13-15]</sup>.

## A ROLE OF IMPRINTED GENES IN NSCS?

On a historical ground, both NSCs and imprinted genes have been studied in the context of brain development. While our insight into the role of NSCs in brain development has largely expanded over the last decade<sup>[1]</sup>, the molecular targets and cellular pathways by which imprinted genes participate in brain development remain poorly defined. Importantly, the roles of NSCs and imprinted genes have been commonly investigated independently of each other although they apparently participate in the same developmental processes. Thus, new studies should explore possible reciprocal interactions between imprinted genes and NSCs during neurodevelopment. Here, we highlight recent evidences in the scientific literature on the critical role of imprinted genes in NSCs and major cellular processes they control.

## INSULIN-LIKE GROWTH FACTOR 2

Insulin-like growth factor (*Igf2*) was the first mammalian gene shown to be maternally imprinted as a result of a differentially methylated imprinting control region (ICR) located nearby and upstream of *H19*, which is imprinted in the opposite direction (see below)<sup>[16]</sup>.

The product of *Igf2* is a potent growth factor promoting cell survival, proliferation, and differentiation by binding with high affinity to the insulin-like receptors *Igf1r* or *Igf2r*, but less efficiently to the insulin receptor (*Insr*). Hereby, *Igf2r* has no signaling function and is encoded by the paternally imprinted *Igf2r* gene. This antagonistic functional relationship together with the opposite imprinting of *Igf2* and *Igf2r* originally stimulated the genetic conflict hypothesis for genomic imprinting<sup>[17]</sup>.

During CNS development, *Igf2* is for the most part synthesized by the choroid plexus, and released into the cerebrospinal fluid (CSF), which contacts the primary cilia and apical surfaces of cortical progenitors.



There, CSF-borne Igf2 binding to the Igf1r stimulates neural precursors proliferation<sup>[18]</sup>.

Neurogenesis is maintained at a low level in the adult brain in so-called neurogenic niches, which comprise the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus. Adult neurogenesis resembles in many aspects embryonic neurogenesis and raises the possibility of an additional role of Igf2 in stemness maintenance in the mature brain. In support of this hypothesis, transcriptome analysis of the SGZ evidenced substantially higher expression of Igf2 in stem cells than in immature neurons<sup>[19]</sup>. Igf2 expression localized to radial-glia like NSCs (Nestin<sup>+</sup>, Sox2<sup>+</sup>, and Gfap<sup>+</sup>) and to a significant fraction of dividing cells (Ki67<sup>+</sup>). Interestingly, Igf2 enhances *in vivo* and *in vitro* the proliferation of NSCs isolated from the DG, but not from the SGZ, indicative of a site-specific effect. Finally, the secretion of Igf2 by Nestin<sup>+</sup> progenitors in the external granule cell layer (EGL) potently stimulates neuronal cell proliferation whereas overexpression of Igf2 in granule neurons facilitates tumor formation in rodents<sup>[20]</sup>.

In sum, these reports show that *Igf2* enhances in a tissue- and age-dependent manner NSC proliferation and maintenance.

### ***H19* ncRNA**

The maternally imprinted *H19* locus localizes in tandem with the oppositely imprinted *Igf2* gene<sup>[16]</sup> and encodes high levels of a 2.5 kb long RNA polymerase II-derived transcript. This large intergenic non-coding RNA (lincRNA) is not involved in the imprinting process<sup>[21,22]</sup> but inhibits *in vitro* and *in vivo* tumor growth possibly due to its participation in and regulation of an imprinted gene network (IGN, see below).

Additionally, the first exon of *H19* encodes a micro RNA-containing hairpin that serves as a template for the miRNA 675, which reduces Igf1r expression and Igf2-signalling in the placenta<sup>[23]</sup>. These self-restraining activities of the tandem *Igf2-H19* locus are necessary for normal embryogenesis and protect against parthenogenetic development in mammals<sup>[24]</sup>.

Erasure of imprinting at the *Igf2-H19* DMR is found in primordial germ cells (PGS) and associates with overexpression of *H19* RNAs at the expense of Igf2. This epigenetic switch-off is thought to safeguard PGS quiescence and prevent from teratoma formation<sup>[25]</sup>.

A similar strategy seems to be used by very small embryonic-like stem cells (VSELs), a population of very rare early-development cells with broad differentiation potential<sup>[26]</sup>. VSELs can give rise to neurons, oligodendrocytes, and microglia among other cell types and possibly fulfill a role in physiological tissue rejuvenation and regeneration following cell damage.

In VSELs, the paternally silenced allele of select imprinted genes (*i.e.*, *Igf2-H19* and *Rasgrf1*) is reactivated by demethylation and results in biallelic expression. Conversely, select maternally expressed

alleles (*i.e.*, *Peg1*, *Igfr2*, and *p57<sup>Kip2</sup>*) undergo deactivation and silencing by DNA methylation. Overall, this cell type-specific resetting of a limited number of genomic imprints supports growth-inhibition, cellular quiescence, and preservation of the stem cell population. On the other hand, methylation at the *Igf2-H19* DMR slowly increases with aging and has been suggested to facilitate increased insulin signaling and age-related depletion of the VSELs reservoir<sup>[27]</sup>.

Taken together, integrate imprinting at the *Igf2-H19* tandem locus critically controls growth and cell proliferation in the early embryo as well as in VSELs.

## **DELTA-LIKE HOMOLOG 1**

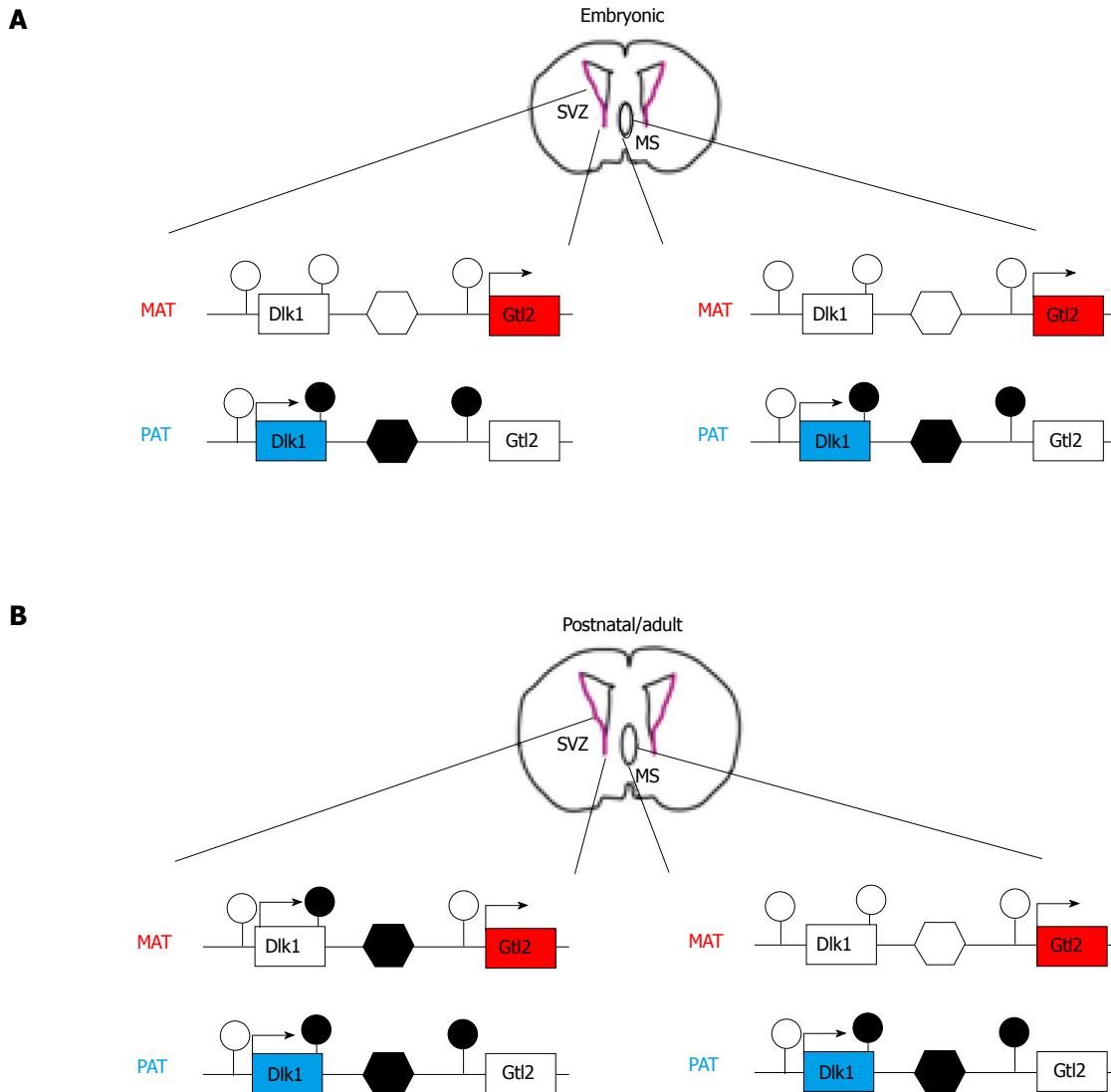
The imprinted *Dlk1-Dio3* domain harbors the delta-like homolog 1 (*Dlk1*) and type III iodothyronine deiodinase (*Dio3*) genes which are expressed from the paternal derived chromosome. Similarly to the *Igf2-H19* locus, *Dlk1* and the close by *Gtl2* (gene trap locus 2, *alias* maternally expressed gene 3, *Meg3*) are imprinted in an opposite manner and locate 80 kb apart from each other. Three DMRs containing specific epigenetic signatures are hypermethylated on the paternal allele in somatic tissues<sup>[28]</sup>. Hereby, those DNA methylation marks which are deposited in the paternal germline are confined to the central DMR. This region contains tandem repeats and localizes in the intergenic region of the tail-to-head orientated *Dlk1* and *Gtl2* genes (Figure 1).

*Dlk1* encodes a transmembrane protein and closely resembles the Notch/Delta/Serrate type family of signaling molecules. Due to minor albeit important structural differences *Dlk1* is thought to compete with canonical Delta-like (DLL) ligands at the Notch receptor and to inhibit downstream signaling<sup>[29]</sup>.

*Dlk1* is broadly expressed in developing mouse tissues and continues to be expressed in some adult neuronal tissues (*i.e.*, ventral striatum, septum, and ventral tegmental area) including the SVZ<sup>[29,30]</sup>. Here, *Dlk1* is detected in NSCs (Nestin<sup>+</sup>, Sox<sup>+</sup>, Gfap<sup>+</sup>) and astrocytes (Sox2<sup>+</sup>, Gfap<sup>+</sup>, S100b<sup>+</sup>) localized to the germinal niche but not in differentiated parenchymal astrocytes (Gfap<sup>+</sup>, S100b<sup>+</sup>) and neuroblasts ( $\beta$  III-tubulin<sup>+</sup>). NSCs mainly express the membrane-bound form of *Dlk1* that is poorly active on its own but necessary for the response to secreted *Dlk1* produced by nearby astrocytes.

Postnatal deletion of *Dlk1* enhances NSC proliferation in the SVZ, causing the depletion of the quiescent NSC pool and reduced neurogenesis at later ages. Interestingly, this process takes place irrespectively of the parental origin of the deleted allele<sup>[30]</sup>, suggesting that *Dlk1* can be expressed from either parental allele. Indeed, both alleles of *Dlk1* are expressed from postnatal day 7 onward in NSCs and niche astrocytes as a result of an increase in methylation at the germ line targeted ICR of the maternal allele (Figure 1). This postnatal epigenetic switch at the *Dlk1* locus is





**Figure 1 Genomic imprinting at the *delta-like homolog 1* locus is reset in the subventricular zone.** A: *Delta-like homolog 1* (*Dlk1*) is monoallelically expressed from the paternal allele during development. Silencing of the maternal allele takes place in non-neuronal and various neuronal tissues such as the subventricular zone (SVZ) of the lateral ventricle (SVZ) and the medial septum (MS). At the molecular level, maternal silencing results from the absence of DNA methylation at the intergenic, germ line-controlled differentially methylated regions (DMR) (unfilled hexagon), which resides between the *Dlk1* and *Gtl2* genes and their associated DMRs (unfilled lollipops). Conversely, methylation at the intergenic DMR (filled hexagon), the 3' end *Dlk1* DMR and the *Gtl2* DMR (filled lollipops) associates with expression from the paternal allele. The same methylation patterns are present in the SVZ (left scheme) and MS (right scheme) at embryonic ages; B: *Dlk1* shows biallelic expression from postnatal day 7 onward towards adulthood in the SVZ, but not in the MS. Hereby, the maternal methylation pattern closely resembles the one on the paternal allele (*i.e.*, methylation at the 3' end *Dlk1* DMR, the intergenic DMR, but not the *Gtl2* DMR). At opposite, the methylation pattern in the MS is preserved and determines monoallelic *Dlk1* expression.

confined to the neurogenic areas, whereas all other tissues continue to express *Dlk1* exclusively from the paternal allele from embryogenesis to adulthood.

These findings suggest that genomic imprints at select loci (*i.e.*, *Igf2-H19* and *Dlk1*) are dynamically regulated in NSCs during specific developmental time windows, possibly to match the need for cell stemness vs cell differentiation.

## NECDIN

Human and mouse *necdin* genes are maternally imprinted and localize to chromosome 15 q11.2 and a syntenic segment on chromosome 7, respectively<sup>[10]</sup>, within a cluster of paternally expressed genes.

Paternal deletion of this chromosomal segment in human underlies the neurodevelopmental Prader-Willi syndrome, which manifests with feeding anomalies, gross obesity, and hypogonadism<sup>[10]</sup>.

*Necdin* (*Ndn*) is broadly expressed in postmitotic neurons from early embryonic to adult ages with particular high expression in the developing hypothalamus, medulla oblongata, pons, and midbrain. Knock out mice show variable neonatal lethality, reductions in oxytocin and luteinizing hormone-releasing hormone producing hypothalamic neurons, impairments in serotonergic and catecholaminergic projections, and decreased tangential migration of neocortical interneurons from the basal forebrain<sup>[31]</sup>.

*Ndn* was originally discovered in a screen for



genes induced in neurally differentiated embryonic carcinoma cells<sup>[32]</sup>. Functionally, Ndn potently inhibits proliferation in favor of differentiation by virtue of its interaction with various proteins critically involved in cell cycle progression and survival<sup>[33,34]</sup>. Similar to the retinoblastoma tumor suppressor gene, Ndn binds to the carboxyl-terminal transactivation domain of E2F1 to repress its activity and consequently cell cycle progression<sup>[33]</sup>. On the other hand, Ndn interacts with the amino-terminal transactivation domain of p53 to abolish its proapoptotic function without interfering with p53-dependent cell cycle arrest<sup>[34]</sup>. Ndn also recruits the deacetylase sirtuin 1 to promote deacetylation of p53 leading to its inactivation and protection against DNA damage induced neuronal apoptosis<sup>[35]</sup>.

Furthermore, Ndn interacts and promotes the degradation of the hypoxia inducible factor-1 alpha (HIF) under normoxia<sup>[36]</sup>, whereas hypoxia enhances degradation of Ndn in primary NSCs through the HIF-associated ubiquitin-proteasome system<sup>[37]</sup>.

Interestingly, a growing number of reports (e.g.,<sup>[38-40]</sup>) suggest that NSC proliferation is increased under hypoxia. Accordingly, Ndn-deficient NSCs show increased proliferation and apoptosis under normoxia, but not under hypoxia, which triggers degradation of endogenous Ndn in wild-type NSCs. Moreover, Ndn null mice show higher rates of NSC proliferation and apoptosis [e.g., in the embryonic (E14.5) ganglion eminence], strengthening Ndn's dual role in the suppression of proliferation and apoptosis<sup>[33-35]</sup>.

Ndn controls additionally the proliferation of NSCs in the ventricular zone of the embryonic cortex as evidenced by an upregulation of the stem cell marker Sox2, a downregulation of the cyclin-dependent kinase inhibitor p16<sup>Ink4</sup>, and significantly increased proliferation rates in Ndn null mice<sup>[41]</sup>. Interestingly, Ndn binds *in vitro* and *in vivo* to the polycomb protein Bmi1 and counteracts Bmi1-dependent inhibition of the p16<sup>Ink4</sup> promoter and consequently promotes NPC proliferation. Conversely, overexpression of Bmi1 prevents Ndn-mediated inhibition of E2F1-driven Cdk1 promoter activity<sup>[41]</sup>.

Together, these findings suggest that Ndn controls NSC proliferation and apoptosis in an oxygen-dependent manner through interaction with various proteins driving cell proliferation (E2F1, Bmi1) and apoptosis (p53, Sirt1).

## CYCLIN-DEPENDENT KINASE INHIBITOR P57<sup>KIP2</sup>

The catalytic activity of cyclin-dependent kinases (CDK) is regulated by the binding of cyclins which oscillate periodically during the cell cycle and drive the orderly progression through consecutive phases. Conversely, inhibition of these complexes by CKIs induces transient or permanent cell cycle arrest,

differentiation, quiescence, senescence, or apoptosis.

The formation of early progenitors from neuro-epithelial cells and the transition from proliferative symmetric to neurogenic asymmetric division is accompanied by a lengthening of the cell cycle, preferentially in G1-phase, implicating an involvement of CKIs<sup>[42]</sup>.

Paternally imprinted *p57<sup>KIP2</sup>* gene encodes a cyclin-dependent kinase inhibitor (CKI)<sup>[43]</sup> expressed in the VZ and SVZ, midbrain, thalamus, hypothalamus, cortical plate, septum, basal ganglia, cortex, and mantle zone of the hippocampus during development<sup>[44,45]</sup>. By means of controlling cortical progenitor cell cycle exit, *p57<sup>KIP2</sup>* influences the migration and differentiation of neuronal precursors. Absence of *p57<sup>KIP2</sup>*<sup>(+/m-)</sup> during late embryogenesis and postnatal life gives rise to cortical hyperplasia. *P57<sup>KIP2</sup>*<sup>(+/m-)</sup> deficiency leads to an increased proliferation of radial glia cells (RGC) and intermediate precursors (IPC) and promotes re-entry into the cell cycle during corticogenesis<sup>[46]</sup>. Cell cycle analysis of RGCs and IPCs evidenced an abnormal short cell cycle length favoring precursor proliferation and aberrant migration into cortical layers.

In mice adult hippocampus, *p57<sup>KIP2</sup>* deficiency causes the severe depletion of the NSC reservoir by enhancing neuronal differentiation of NSCs at early stages of life<sup>[47]</sup>. Moreover, consistent with a role of *p57<sup>KIP2</sup>* in restraining NSC proliferation, neurogenic stimuli such as extensive running elicit a stronger activation of NSCs in mid-aged *p57<sup>KIP2</sup>*<sup>(+/m-)</sup> animals indicating that *p57<sup>KIP2</sup>* might also play a critical role in the life long plasticity of brain functions<sup>[47]</sup>.

In addition to their canonical role in cell cycle control, CKIs have further functions including transcriptional regulation<sup>[48]</sup>. As an example, nuclear *p57<sup>KIP2</sup>* expression rises transiently during early telencephalic progenitor proliferation [embryonic day (E) 12.5] without inducing cell cycle exit. Instead, *p57<sup>KIP2</sup>* interacts with the pro-neuronal basic helix-loop-helix (bHLH) factor Mash1 and blocks its transcriptional activity. As a result, *p57<sup>KIP2</sup>* delays neuronal differentiation of telencephalic progenitors by antagonizing Mash1.

Taken together, *p57<sup>KIP2</sup>* through inhibition of cell cycle progression and unrelated transcriptional mechanisms regulates many key processes in NSCs, including proliferation, cell cycle exit, differentiation, cell fate decisions, and stem cell quiescence in a cell type- and age-specific manner.

## ZINC FINGER PROTEIN REGULATING APOPTOSIS AND CELL CYCLE ARREST

The *Zac1* gene is maternally imprinted and maps on chromosome 10 in mice and chromosome 6q24 in human<sup>[49,50]</sup>. The presence of a canonical C2H2 zinc finger domain and the potent induction of apoptosis and cell-cycle arrest in transformed tumor cells inspired originally the naming "*Zac1*"<sup>[51]</sup>.



An increased dosage of ZAC1 due to chromosomal anomalies or imprinting defects at the DMR is the most frequent genetic defect underlying transient neonatal diabetes mellitus (TNDM)<sup>[52]</sup>. This disease manifests with intrauterine growth retardation (IUGR), dehydration, hypoinsulinemia, and early-onset hyperglycemia in term, newborn infants. Transgenic mice, which harbor an extra copy of the human ZAC1 locus, display key symptoms of the human condition and show at the cellular level an impaired proliferation and maturation of  $\beta$ -cell progenitors<sup>[53]</sup>. In naïve pancreata, Zac1 is preferentially expressed in insulin-positive progenitor cells and increases strongly perinatally with the onset of terminal differentiation. At the same time, Zac1 confers repression following binding to specific DNA elements (see below) at the proximal promoter of the paternally imprinted *Rasgrf1* gene, an important modulator of various growth factor pathways. As a result, stimulus-induced activation of mitogen-activated protein kinase and phosphoinositide 3-kinase pathways and, ultimately, insulin secretion is impaired under conditions of increased Zac1 dosage<sup>[54]</sup>.

Zac1 confers transcriptional regulation either by DNA-binding<sup>[50,55-57]</sup> or as coregulator of the nuclear receptor family, in particular of those members belonging to the subgroup of steroid receptors<sup>[58]</sup>, and furthermore, by coactivation of members of the p53 family<sup>[59,60]</sup>. Transcription factors typically comprise separable, modular DNA-binding and transcriptional domains; the latter confer gene activation and repression in a context-dependent manner<sup>[61]</sup>. Zac1 matches well to these criteria, whereby its diverse transcriptional functions are tightly controlled by the interaction of single zinc fingers at the level of protein-protein and protein-DNA interactions<sup>[55,62]</sup>.

The N-terminal zinc finger domain, containing seven canonical C2H2 zinc fingers, is highly conserved in mouse and human Zac1 proteins and across the *Zac1* gene family<sup>[63]</sup>. Mouse and human proteins diverge, however, by a central region rich in proline residues and a C-terminal cluster of glutamic acid residues both of which exist exclusively in mice. The proline-rich region together with the adjacent upstream region, termed linker region, confers potent transactivation<sup>[64]</sup>. This function is further enhanced by the simultaneous recruitment of the general coactivators p300/CBP through the C-terminus. In contrast to the separable function of multiple domains in mice, transactivation and p300 recruitment map indistinguishably to the C-terminus of human Zac1<sup>[62]</sup>.

Zac1 can bind as a monomer to GC-rich palindromic DNA-elements or as a dimer to direct and reverse repeat elements to confer transactivation. Conversely, Zac1 binding to a half-site of a repeat element causes repression<sup>[55]</sup>. Mechanistically, the zinc fingers assist in the recruitment of p300 and regulate p300's catalytic activities in a manner dependent on the nature of the bound DNA element<sup>[62]</sup>. Hereby, single zinc fingers can

participate selectively in DNA binding and/or regulation of coactivator activities.

Moreover, Zac1 can also act as a coregulator for unrelated transcription factors comprising nuclear steroid or thyroid hormone receptors or various members of the p53 family (including p53 itself, p63, and p73). All of these transcription factors bind to specific DNA elements at their target genes to critically control cell proliferation and differentiation in a cell type-specific manner<sup>[58,59]</sup>. For example, Zac1 is recruited jointly with the coactivators p300 and PCAF by the tumor suppressor p73 at the *p21<sup>Cip1</sup>* promoter during early neuronal differentiation (see below). In addition to serving as a scaffold for coactivator assembly, Zac1 furthermore regulates PCAF's catalytic functions similar to the ones of p300<sup>[65]</sup>. Overall, this close relationship between Zac1 DNA-binding and enzymatic regulation of transcription might contribute to the precise and efficient regulation of target genes.

In brief, Zac1's transcriptional activities are coordinated by sequence-specific DNA binding or the coregulation of unrelated transcription factors.

### ***Zac1 expression during neurodevelopment***

Zac1 is highly expressed in progenitor/stem cells of neuroectodermal and mesodermal tissues during early embryogenesis<sup>[66]</sup>. Zones of active cellular proliferation, comprising the telencephalic vesicles and the infundibular recess of the third ventricle (the origin of the neurohypophysis) show robust Zac1 expression at E9.5 and E12.5. Moreover, Zac1 is also detected in mitotically active regions of the developing nervous system including the neural tube at E9.5 and the neural retina at E10.5. At later stage of neurodevelopment, high levels of Zac1 expression appeared in the mitotically active cell layers lining the VZ of the 3<sup>rd</sup> and 4<sup>th</sup> ventricle, the EGL of the cerebellum, and different neuroepithelia (*e.g.*, infundibulum, ventral hypothalamic sulcus)<sup>[67,68]</sup>.

As noted before, Zac1 expression induced apoptosis and cell cycle arrest in transformed tumor cells<sup>[50,51,56]</sup> raising the question of its function in neural progenitor cells. In this respect, the analysis of Zac1 deficient mice (*Zac1<sup>+/-</sup>*) provided interesting insight into the role of Zac1 in the brain<sup>[69]</sup>. Specifically, these animals revealed a high incidence of hydrocephalus, a significant decrease in brain size, and a substantial increase in the proliferation rate of progenitor cells in the germinative zones of the dentate gyrus, the RMS of the olfactory system, and the dentate gyrus<sup>[70]</sup>. At the same time, the number of Nestin<sup>+</sup> cells was largely unaffected, compatible with the view that Zac1 rather controls the proliferation of progenitor cells than of stem cells. Yet, a limitation to this work is the fact that Zac1 deficient mice develop IUGR, various skeletal anomalies, and postnatal lung failure pointing to additional defects at the level of single organs and whole systems that are likely to impact on brain development. As an alternative to the multilayered



phenotype of *Zac1* knock out mice, we conducted genome wide expression profiling (see below) in order to identify *Zac1* target genes and analyzed their roles in well-defined *in vitro* and *in vivo* systems to elucidate *Zac1*'s function at the cellular level.

### ***Zac1* target genes in NSCs**

**Pituitary adenylate cyclase activating polypeptide receptor 1:** The first *Zac1* target gene to be identified was the G-protein coupled receptor for the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP)<sup>[51,57,71]</sup>, which controls various neuroendocrine functions in addition to its role as potent neurotrophic factor<sup>[72-74]</sup>. Mechanistically, *Zac1* binds to an imperfect palindromic DNA element localized in the proximal polypeptide receptor 1 (*Pac1*) promoter to confer transactivation in a cell-type specific manner and to compete at the same time with the estrogen receptor dependent activation of *PAC1*<sup>[75]</sup>.

PACAP and *PAC1* are broadly expressed in the fetal brain and reduce the proliferation rate of certain neural precursor populations. At E13 in rat, PACAP can be detected hippocampus, hypothalamus, cortex, amygdala, dorsal root ganglia, and spinal cord, whereas *PAC1* is expressed in the neural plate, the neuroepithelia of the mesencephalon and rhombencephalon at E9, and in the neuroepithelia of the cortex, hippocampal formation, and cerebellum at E11, and in the basal telencephalon and olfactory bulb from E13 and E16 forward, respectively<sup>[76]</sup>. PACAP induces a sharp increase in *p57<sup>Kip2</sup>* expression in embryonic cortical precursors resulting in decreased CDK2 kinase activity, S-phase entry, and DNA synthesis. Furthermore, PACAP promotes the association of *p57<sup>Kip2</sup>* with the kinase complex supporting its anti-mitogenic activity in neural progenitors. In accord with the specific role of *p57<sup>Kip2</sup>* in cortical neurogenesis (see above), the expression levels of CDK2, cyclin E, or of the CKIs *p21<sup>Cip2</sup>* and *p27<sup>Kip1</sup>* remain unaffected by PACAP.

**Coregulation of *p53* and *p73* target genes:** As noted before, *Zac1* can serve as a coregulator for *p53* and *p73* due to its scaffolding function and regulation of coactivator activities<sup>[59,65]</sup>. The *p53* family consisting of *p53* itself, *p63*, and *p73* encodes transcription factors with a key role in proliferation, differentiation, apoptosis, stem cell renewal and cell fate commitment<sup>[77]</sup>.

*Zac1* coregulation of *p53* has been originally discovered in a cervical carcinoma cell line<sup>[59]</sup> and led us to investigate its potential role in NSCs. Consistent with previous studies<sup>[78,79]</sup>, we observed high levels of *p53* in the undifferentiated state in ESCs which reside primarily in the cytoplasm<sup>[80]</sup> and decline upon differentiation<sup>[65]</sup>. At the same time, *p53* binds to the promoter of the pluripotency genes *Nanog* and *Oct-4*<sup>[81,82]</sup>, represses their transcription, and triggers the transition from self-renewal to differentiation.

Although *Zac1* has been reported to co-repress nuclear receptors in a cell-type specific manner, no evidence exists for a comparable role towards members of the *p53* family<sup>[58,59]</sup>.

In the developing brain, *p53* is expressed in progenitor cells of the SVZ<sup>[83]</sup> where it induces cell-cycle arrest, DNA repair and cell death following genotoxic stress<sup>[84]</sup>. *p53*-deficient mice show higher cell proliferation in the SVZ and enhanced neurosphere formation *in vitro*<sup>[83,85]</sup> consistent with a role of *p53* as negative regulator of NSC self-renewal. Moreover, *p53* seems to be involved in different aspects of NSC differentiation including repression of self-renewal and promotion of gliogenesis<sup>[86,87]</sup>.

Mounting experimental evidence indicates that endogenous reactive oxygen species (ROS) play a critical role in cell signaling and NSC physiology including appropriate timing of neurogenesis. In this respect, the pattern of ROS generation matches the one of *p53* immunoreactivity in the developing telencephalon. Similarly to *Zac1*, nuclear *p53* is detected in Nestin<sup>+</sup> NSCs in E11 and E13 proliferative germinal zones, and its expression decreases towards the cortical plate<sup>[88]</sup>. Loss of *p53* function causes enhanced ROS production and premature neurogenesis, which is partly reversed by reinstatement of *p53* or antioxidant treatment<sup>[88]</sup>.

Despite these interesting findings, a role of *Zac1* in any of these *p53*-dependent processes is presently unknown and future studies are needed to address this topic.

*p73* has been recognized for its critical role in brain development as evidenced by the highly penetrant phenotype in *p73* null mice which display cortical hypoplasia, hydrocephalus, and hippocampal dysgenesis<sup>[89,90]</sup>. The hippocampal anomaly corresponds with either a complete absence or truncation of the lower blade of the neurogenic DG and an abnormal gyration of the Ammon's horn. Isoform-specific *p73* knock-out mice showed that this phenotype results in major part from the absence of the activation-proficient *p73* protein (TAp73) as opposed to the activation-deficient *p73* protein (ΔNp73). The latter isoform lacks *Zac1* coactivation<sup>[65]</sup> and is thought to act as a potent prosurvival protein in neurons by counteracting the proapoptotic function of *p53*<sup>[91,92]</sup>. Therefore, it would be of interest to investigate the possibility of *Zac1*-dependent coregulation of TAp73 in NSC in greater depth.

Withdrawal of leukemia inhibitory factor (Lif1) potentially induces neuronal differentiation of ESCs and strongly upregulates *Zac1* and *p73* expression concomitantly to a rapid decline in *p53* mRNA and protein<sup>[65]</sup>. At the same time, *p73* isoforms switch from activation-deficient ΔNp73, prevailing under the undifferentiated state, to activation-proficient TAp73 and caused a strong up-regulation of the *p21<sup>Cip1</sup>* and *p57<sup>Kip2</sup>* genes, two well-known direct *p73* target genes<sup>[93]</sup>. As referred to above, DNA bound *p73* recruits *Zac1* to select target genes, where it



serves as a scaffold for coactivator assembly and enhancement of catalytic functions. As a result, *Zac1* enhances p73 transcriptional activities in a site-specific manner<sup>[65]</sup>. These findings suggest a joint role for *Zac1* and p73 in inducing cell cycle exit of ESCs and in differentiation towards neural cells.

*In vivo* studies evidenced a reduced proliferation of neurogenic cells isolated from the E16 and E18 VZ/SVZ and a smaller size of the perinatal SVZ in p73 deficient mice when compared to controls. As a result, p73<sup>(-/-)</sup> mice suffer from a depletion of the stem cell compartment at birth pointing to a role of p73 in NSC self-renewal and maintenance<sup>[94]</sup>. This function of p73 extends also to the adult neurogenesis in the DG. At the molecular level, p73 deficiency elicits perturbations in the canonical Sox2 and Notch signaling pathways driving NSC proliferation<sup>[95]</sup>. Additionally, a reduced transcription of *Hey2*, a negative regulator of activator bHLH proteins, impairs the long-term maintenance of neural precursors in the absence of p73<sup>[95]</sup>.

Because *Zac1* is expressed in embryonic and adult neural stem cells it could potentially interact with and coregulate TAp73 functions. Presently, the actual evidence remains limited to *Zac1* coactivation of TAp73 during neuronal differentiation of ESCs. However, further studies should address the role of *Zac1* coregulation for NSC self-renewal, maintenance and differentiation at different developmental stages though.

**Suppressor of cytokine signaling 3:** Genome-wide expression profiling in a cerebellar neural stem cell line (C17.2) using a Tet-off system led to the identification of suppressor of cytokine signaling 3 (*Socs3*), a negative regulator of Jak/Stat3 signaling, as potential *Zac1* target gene<sup>[96]</sup>.

The transition from neuronal cell types to glial subtype-specific precursors is critically controlled by preset developmental programs and extracellular signals like the cytokine driven Jak/Stat3 pathway, which is largely inactive at early, neurogenic stages and takes on at later gliogenic stages, when the expression of neurogenic factors progressively declines<sup>[97]</sup>.

*Zac1* recognizes a cluster of GC-rich DNA elements in the proximal promoter and intronic region of the mice and human *Socs3* genes to confer transcriptional activation<sup>[96]</sup>.

Two radial glial-like NSC lines, derived from E15 or the adult SVZ of mice, showed a transient upregulation of *Zac1* mRNA and protein upon neuronal or astroglial differentiation, whereby transactivation of *Socs3* occurred solely under the latter condition indicative of a role of *Socs3* as a lineage-specific target gene. Consistent with this finding, DNA methylation at the *Socs3* gene decreased during astroglial differentiation but remained unaltered during neuronal differentiation. *Zac1* and *Socs3* are expressed simultaneously exclusively during the early

stage of astroglial differentiation and associated with a strong decline in receptor activation-dependent tyrosine phosphorylation of Stat3. In agreement with these results, *Zac1* and *Socs3* are highly expressed in the neocortical ventricular zone at E18, which corresponds to the onset of astroglial differentiation<sup>[96]</sup>.

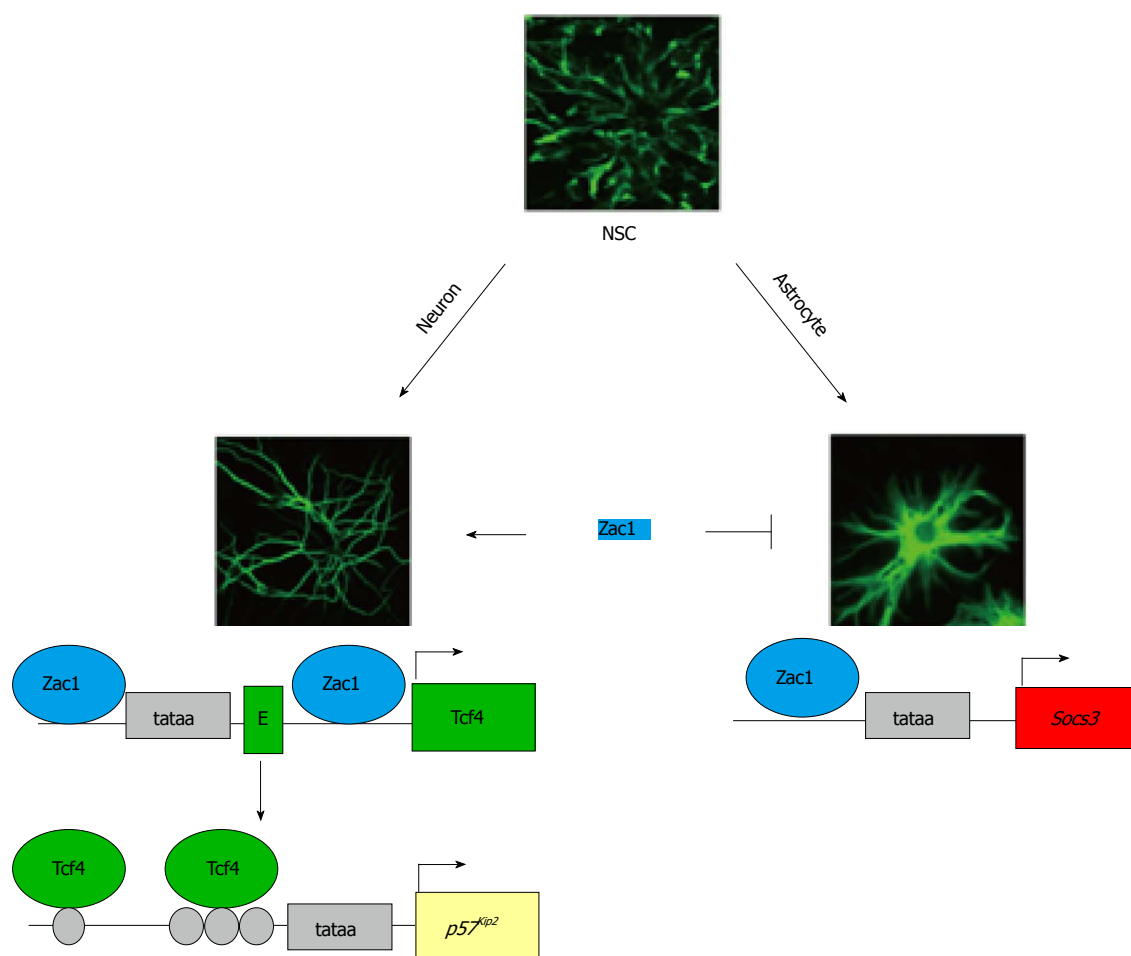
Astroglial differentiation is triggered by various cytokines namely cardiotrophin-1, ciliary neurotrophic factor, or leukemia inhibitory factor, which activate Jak/Stat3 signaling<sup>[97-100]</sup>. Conversely, genetic deletions in this pathway (*i.e.*, gp130, LIF and Stat3) reduce astroglial differentiation<sup>[97,101,102]</sup>. Importantly, overexpression of *Socs3* inhibits Stat3 signaling and impairs astroglial differentiation<sup>[103]</sup>, whereas conditional deletion of *Socs3* leads to enhanced astroglial differentiation in neonatal mouse brain and primary neuroepithelial cells<sup>[100]</sup>. These findings led us to investigate whether *Zac1*-dependent induction of *Socs3* could provide a negative feedback loop to inhibit Jak/Stat3 signaling during early astroglial differentiation of NSCs.

In accord with an inhibitory role in Jak/Stat3 signaling, *Zac1* overexpression delayed astroglial differentiation, independent of a simultaneous increase in the number of cells in transition into G<sub>1</sub> arrest<sup>[96]</sup>. Conversely, knock-down of *Zac1* in NSCs facilitated astroglial differentiation and postponed cell cycle arrest. Although lengthening of the G<sub>1</sub> phase has been suggested to initiate differentiation, this mechanism seems not to apply to *Zac1*'s cell cycle arrest function in NSCs and supports the self-sufficient role of *Socs3* in the control of astroglial differentiation. Compatible with this view, knock-down of *Socs3* in *Zac1* overexpressing cells reinstated timely astroglial differentiation. Similar results were obtained in primary E18 NSCs strengthening the role of *Zac1*-dependent regulation of *Socs3* in early astroglial differentiation of NSCs<sup>[96]</sup>.

Overall, this study provides detailed insight into the molecular mechanisms by which an imprinted gene can fine tune cell-fate decisions and differentiation in NSCs and assigns to *Zac1* a critical role in the prevention of precocious astroglial differentiation (Figure 2).

**Transcription factor 4:** *Zac1* expression robustly increased under astroglial as well as neuronal differentiation, which led us to question *Zac1*'s function during the latter condition<sup>[96]</sup>. The family of bHLH proteins plays a prominent role in cell fate decisions and differentiation. These proteins share the eponymic bHLH domain which is necessary for homo- or heterodimerization and for binding to a specific DNA sequence named E-box motif<sup>[104]</sup>. bHLH proteins expressed in the brain can be classified into two groups; the so-called specification factors (*i.e.*, Math, Mash, neurogenin, and NeuroD), which are expressed in a spatiotemporally controlled manner, and their ubiquitously expressed dimerization partners, the E proteins<sup>[105,106]</sup>. Under the undifferentiated, proliferative state proneural factors are weakly





**Figure 2 Imprinted *Zac1* favors neuronal differentiation of neural stem cells.** The *Zac1* gene is maternally imprinted and encodes a versatile transcriptional regulator. *Zac1* expression is strongly induced during neuronal and astroglial differentiation of embryonic and adult neural stem cells (NSCs). During neuronal differentiation (left scheme), *Zac1* binds to GC-rich DNA binding sites at the *Tcf4* promoter and first intron to confer synergistic transactivation. As a result, enhanced *Tcf4* expression promotes binding to and transactivation of the cyclin kinase inhibitor *p57<sup>Kip2</sup>*, which induces G1 arrest. Moreover, *Zac1* binds to GC-rich DNA elements at the *Socs3* promoter during astroglial differentiation (right scheme). *Socs3* encodes a potent inhibitor of prodifferentiative Jak/Stat3 signaling and prevents precocious astroglial differentiation. The *tataa*-elements are boxed in light grey and the transcriptional start sites are symbolized by arrows. The first exon in the *Tcf4* locus is depicted as a green box (labeled E) and coding exons of *Tcf4* and *Socs3* as green and red boxes, respectively. *Tcf4* binds to various E-box motifs (light grey circles) localizing to the *p57<sup>Kip2</sup>* regulatory region.

expressed, prior to a rapid increase in concert with the E-proteins at the initiation of neurogenesis. E-protein family members comprise two splice variants of *E2A* (*E12* and *E47*), *HEB*, and transcription factor 4 (*Tcf4*) (*alias E2-2*, *SEF2*, or *ITF2*), which upon heterodimerization with a specification factor, bind to the promoter of their target genes, in order to promote neurogenesis, and inhibit astroglial differentiation and NSC proliferation<sup>[107,108]</sup>.

Expression profiling in a NSC line (C17.2) based on inducible *Zac1* expression showed a robust upregulation of *Tcf4* mRNA and protein<sup>[109]</sup>. Thereby, *Zac1* bound simultaneously to the proximal promoter and first intron of *Tcf4* and binding at either site was necessary to confer synergistic transactivation.

Neuronal differentiation of ESCs caused a strong *Zac1* and *Tcf4* upregulation and associated with *Zac1* binding at the *Tcf4* gene. As noted before, *Zac1* upregulation following differentiation of embryonic and adult NSC lines occurred under either astroglial

or neuronal differentiation, whereby induction of *Socs3* was confined to the astroglial lineage. Oppositely, *Zac1*-dependent upregulation of *Tcf4* was specific to neuronal differentiation, associated with an overall increase in active chromatin marks but no change in DNA methylation, at the *Tcf4* locus<sup>[109]</sup>. *Zac1*-dependent *Tcf4* regulation was also confined to neuronal differentiation of primary NSCs; moreover, *Zac1* binding to and transactivation of the *Tcf4* locus occurred exclusively during periods of neuronal differentiation in the neocortical ventricular zone<sup>[109]</sup>.

Among known *Tcf4* target genes, *p57<sup>Kip2</sup>* is of particular interest as it is co-regulated with *Zac1* in the framework of an imprinted gene network (see below), it critically controls differentiation and migration of radial glia cells, and shares with *Zac1* and *Tcf4* a cell cycle arrest function (see above).

*Zac1* and *Tcf4* are broadly expressed in neuronal progenitor cell populations during early (E11) and midneurogenesis (E15) such as in the caudal brain



regions, the pallium, and the prethalamus eminence<sup>[109]</sup>.

$P57^{Kip2}$  expression is more localized and mapped strongly to the subpallium and peduncular hypothalamus where all three factors were detected.

Functionally, *Zac1* enhances G1-cell cycle arrest in NSC lines and primary NSCs (E15) during neuronal differentiation, by inducing  $p57^{Kip2}$  expression through *Tcf4*<sup>[109]</sup>.

Taken together, these results suggest a role to *Zac1* in the prevention of precocious astroglial differentiation through *Socs3* induction and in advancing at the same time neuronal differentiation through *Tcf4* induction and *Tcf4*-mediated upregulation of  $p57^{Kip2}$  (Figure 2).

## ZAC1 IMPRINTED GENE NETWORK

Recent evidence indicates that many imprinted genes might work in an integrated network of imprinted genes. In this respect, Arima *et al.*<sup>[110]</sup> noted that *Zac1* and  $p57^{Kip2}$  show a conspicuously similar expression pattern in mesenchymal and neuroepithelial tissues, suggesting a functional interaction between these genes. Interestingly, the Beckwith-Wiedemann syndrome (loss of  $p57^{Kip2}$  imprinting) and TNDM (loss of *ZAC1* imprinting) represent with partly opposite phenotypes including gigantism vs IUGR or hypoglycemia vs hyperglycemia.

At the molecular level, *ZAC1* binds in a methylation-sensitive manner within the promoter CpG island of *LIT1* (KCNQ1OT1), which encodes a paternally expressed, anti-sense RNA thought to repress  $p57^{Kip2}$  in *cis*<sup>[16]</sup>. *ZAC1* confers transactivation to *LIT1* promoter constructs suggesting that *ZAC1* might down-regulate  $p57^{Kip2}$  via *LIT1* anti-sense<sup>[110]</sup>. Oppositely, we demonstrated that *Zac1* upregulates  $p57^{Kip2}$  via *Tcf4* in NSCs<sup>[109]</sup>.

Some aspects of the *Zac1* knock-out phenotype, such as growth retardation, perinatal death, and incomplete bone formation appear difficult to reconcile with *Zac1*'s antiproliferative activities<sup>[69]</sup>. A possible explanation to this puzzle is provided by a meta-analysis of microarray data, which indicates that *Zac1* coordinates a network of genes that consists of a remarkable huge number of imprinted genes. These include *Igf2*, *H19*, *Dlk1*, *Ndn*, and  $p57^{Kip2}$ , which share an important role in NSC maintenance and differentiation, among others (*Grb10*, *Gnas*, *Meg3*, *Mest*, and *Sgce*). Moreover, *Zac1*-deficient liver tissue and *Zac1* overexpression experiments in neuroblastoma cells showed an opposite regulation of *Igf2*, *H19*, *Dlk1*, and  $p57^{Kip2}$ . Interestingly, *Zac1* bound to the downstream *H19* enhancer and conferred transactivation to both the *H19* and *Igf2* promoters (Figure 3).

More recently, Lui *et al.*<sup>[111]</sup> showed that IGNs are possibly involved in mammalian somatic growth control. Early postnatal life is a period of fast somatic growth, which slows down with maturation and finally arrests in adulthood. A group of 11 imprinted genes involved in cell proliferation (including *Zac1*, *Igf2*, *H19*, *Dlk1*, *Ndn*, and  $p57^{Kip2}$ ) and part of the *Zac1*-IGN is expressed in multiple tissues at levels that

correlate with trajectories of overall somatic growth and decrease coordinately with age. Although this study does not explicitly address the role of IGNs in NSCs, it importantly suggests that the function of IGNs is not reserved to embryonic life but potentially extends across lifespan.

Another recent study focused on gene expression profiling in murine long-term hematopoietic stem cells (LT-HSC) vs their differentiated progeny. These experiments showed that imprinted genes (including *Zac1*, *Igf2*, *H19*, *Dlk1*, *Ndn*, and  $p57^{Kip2}$ ) were more uniformly expressed in progenitors when compared to the differentiated counterparts<sup>[112]</sup>. Moreover, stem/progenitor cells from adult skeletal muscle and epidermis show a higher expression of these genes when compared to their differentiated derivatives.

Taken together, the *Zac1*-associated IGN network comprises, among others, imprinted genes that play a critical role in NSC maintenance and differentiation. Some of them, like *Igf2* and *H19* seem to be under the direct transcriptional control of *Zac1* while others like *Dlk1* might be regulated more indirectly as exemplified by *Zac1*-dependent upregulation of  $p57^{Kip2}$  via *Tcf4* (Figure 3).

## CONCLUSION

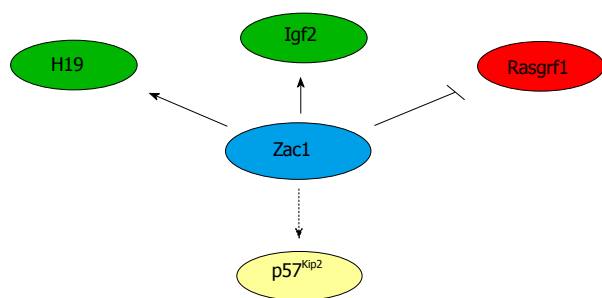
A wide range of genes acts in a concerted manner during brain development to regulate NSC proliferation, migration, and differentiation. Among these, imprinted genes have gained increasing apprehension in NSC biology due to their critical roles in quiescence, stemness, and cellular differentiation.

The molecular processes controlling both NSC fate and imprinted gene expression are manifold and include transcriptional regulation, epigenetic, and genomic interactions.

For instance, imprinted genes can regulate the expression or the transcriptional activities of proneural bHLH proteins in NSCs. *Zac1* activates *Tcf4* during neuronal differentiation of NSCs, while  $p57^{Kip2}$  inhibits the transcriptional activity of *Mash1*. Conversely, the proneuronal bHLH-protein neurogenin regulates the imprinted *Dlk1-Gtl2* locus in the dorsal telencephalon<sup>[113]</sup> indicating bidirectional interactions between imprinted genes and proneuronal proteins.

Genomic imprinting defects have been originally recognized for their role in early development of the embryo and placenta<sup>[9]</sup> and more recently for postnatal life<sup>[114]</sup>. New evidences suggest an additional role of variation in genomic imprinting in the mediation of environmental exposures, which is thought to associate with less severe consequences than those resulting from loss of genomic imprinting and might represent an important component of complex traits<sup>[115]</sup>. For example, newborn of obese parents show altered DNA methylation profiles of multiple imprinted genes, which may be carried onto the next generation and confer an increased risk for metabolic diseases





**Figure 3** Zac1-dependent transcriptional regulation of imprinted genes. Zac1 binds to the downstream enhancer of *H19* and transactivates *H19* and *Igf2* expression in neuronal cells. In pancreatic progenitor cell, Zac1 binds to the promoter of the paternally imprinted *Rasgrf1* gene and confers repression. Following neuronal differentiation of NSCs, Zac1 induces *Tcf4*, which binds at the promoter of the imprinted *p57<sup>Kip2</sup>* gene and enhances G1 cell cycle arrest.

in adulthood<sup>[116]</sup>. Moreover, the degree of methylation of *ZAC1* associates with pre- and postnatal growth in healthy infants as well as with maternal nutrition and lasts at least until the first year of life<sup>[117]</sup>. Similarly, individuals prenatally exposed to famine showed 6 decades later less DNA methylation of *IGF2* compared with their unexposed, same-sex siblings<sup>[118]</sup>, although variation in DNA methylation at this locus is thought to increase as a result of the aging process itself<sup>[119]</sup>. Collectively, these findings indicate the need for further studies on genetic and epigenetic variation at imprinted loci in response to environmental exposures and across lifetime.

Remarkably, in NSCs and other discrete stem cell populations, recent findings indicate that genomic imprinting can be epigenetically switched off at defined developmental time windows as shown for *Igf2-H19* and *Dlk1*. Such temporary changes in allele-specific transcription of imprinted genes alter gene dosage in a cell-type and tissue-specific manner and are required to prevent precocious depletion of the stem cell pool. The influence of various environmental exposures on epigenetic switches in NSCs is presently unknown and might contribute to brain function and aging in individuals at risk.

Interestingly, recent literature suggests that imprinted genes do not operate in isolation, but as complex network of genes, whose expression is dynamically controlled by epigenetic mechanisms that extend from prenatal to postnatal development and possibly during aging. For instance, *Zac1* is a central hub in an IGN comprising *Igf2*, *H19*, *Dlk1*, *p57<sup>Kip2</sup>*, and *Ndn*, which share a role in NSC maintenance and differentiation.

Though, additionally studies are necessary to explore in more detail the role of IGNs in NSCs across lifespan as well as in response to environmental exposures and to elicit their molecular basis. Collectively, a better understanding of the complex interactions governing imprinted genes expression promises new insight into the biology of NSC and associated conditions ranging from imprinting disorders to age-related diseases.

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## REFERENCES

- Ming GL, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* 2011; **70**: 687-702 [PMID: 21609825 DOI: 10.1016/J.NEURON.2011.05.001]
- Jirtle RL. Geneimprint: Genes by species. Available from: <http://www.geneimprint.com/site/genes-by-species>
- Gregg C, Zhang J, Weissbourd B, Luo S, Schroth GP, Haig D, Dulac C. High-resolution analysis of parent-of-origin allelic expression in the mouse brain. *Science* 2010; **329**: 643-648 [PMID: 20616232 DOI: 10.1126/SCIENCE.1190830]
- Gregg C, Zhang J, Butler JE, Haig D, Dulac C. Sex-specific parent-of-origin allelic expression in the mouse brain. *Science* 2010; **329**: 682-685 [PMID: 20616234 DOI: 10.1126/SCIENCE.1190831]
- Zhang K, Li JB, Gao Y, Egli D, Xie B, Deng J, Li Z, Lee JH, Aach J, Leproust EM, Eggan K, Church GM. Digital RNA allelotyping reveals tissue-specific and allele-specific gene expression in human. *Nat Methods* 2009; **6**: 613-618 [PMID: 19620972 DOI: 10.1038/NMETH.1357]
- Bhaumik SR, Smith E, Shilatifard A. Covalent modifications of histones during development and disease pathogenesis. *Nat Struct Mol Biol* 2007; **14**: 1008-1016 [PMID: 17984963 DOI: 10.1038/nmsb1337]
- Kouzarides T. Chromatin modifications and their function. *Cell* 2007; **128**: 693-705 [PMID: 17320507 DOI: 10.1016/j.cell.2007.02.005]
- Eggermann T, Leisten I, Binder G, Begemann M, Spengler S. Disturbed methylation at multiple imprinted loci: an increasing observation in imprinting disorders. *Epigenomics* 2011; **3**: 625-637 [PMID: 22126250 DOI: 10.2217/EPI.11.84]
- Constância M, Kelsey G, Reik W. Resourceful imprinting. *Nature* 2004; **432**: 53-57 [PMID: 15525980 DOI: 10.1038/432053A]
- Kernohan KD, Jiang Y, Tremblay DC, Bonvissuto AC, Eubanks JH, Mann MR, Bérubé NG. ATRX partners with cohesin and MeCP2 and contributes to developmental silencing of imprinted genes in the brain. *Dev Cell* 2010; **18**: 191-202 [PMID: 20159591 DOI: 10.2217/EPI.10.61]
- Drake NM, DeVito LM, Cleland TA, Soloway PD. Imprinted *Rasgrf1* expression in neonatal mice affects olfactory learning and memory. *Genes Brain Behav* 2011; **10**: 392-403 [PMID: 21251221 DOI: 10.1111/J.1601-183X.2011.00678.X]
- Chen DY, Stern SA, Garcia-Osta A, Saunier-Rebori B, Pollonini G, Bambach-Mukku D, Blitzer RD, Alberini CM. A critical role for IGF-II in memory consolidation and enhancement. *Nature* 2011; **469**: 491-497 [PMID: 21270887 DOI: 10.1038/NATURE09667]
- Lefebvre L, Viville S, Barton SC, Ishino F, Keverne EB, Surani MA. Abnormal maternal behaviour and growth retardation associated with loss of the imprinted gene *Mest*. *Nat Genet* 1998; **20**: 163-169 [PMID: 9771709 DOI: 10.1038/2464]
- Li L, Keverne EB, Aparicio SA, Ishino F, Barton SC, Surani MA. Regulation of maternal behavior and offspring growth by paternally expressed *Peg3*. *Science* 1999; **284**: 330-333 [PMID: 10195900 DOI: 10.1126/science.284.5412.330]
- Champagne FA, Curley JP, Swaney WT, Hasen NS, Keverne EB. Paternal influence on female behavior: the role of *Peg3* in exploration, olfaction, and neuroendocrine regulation of maternal behavior of female mice. *Behav Neurosci* 2009; **123**: 469-480 [PMID: 19485553 DOI: 10.1037/A0015060]
- Edwards CA, Ferguson-Smith AC. Mechanisms regulating imprinted genes in clusters. *Curr Opin Cell Biol* 2007; **19**: 281-289 [PMID: 17467259 DOI: 10.1016/J.CEB.2007.04.013]
- Moore T, Haig D. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet* 1991; **7**: 45-49 [PMID: 2035190 DOI: 10.1016/0168-9525(91)90230-N]



- 18 **Lehtinen MK**, Zappaterra MW, Chen X, Yang YJ, Hill AD, Lun M, Maynard T, Gonzalez D, Kim S, Ye P, D'Ercole AJ, Wong ET, LaMantia AS, Walsh CA. The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. *Neuron* 2011; **69**: 893-905 [PMID: 21382550 DOI: 10.1016/J.NEURON.2011.01.023]
- 19 **Bracko O**, Singer T, Aigner S, Knobloch M, Winner B, Ray J, Clemenson GD, Suh H, Couillard-Despres S, Aigner L, Gage FH, Jessberger S. Gene expression profiling of neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult hippocampal neurogenesis. *J Neurosci* 2012; **32**: 3376-3387 [PMID: 22399759 DOI: 10.1523/JNEUROSCI.4248-11.2012]
- 20 **Rao G**, Pedone CA, Del Valle L, Reiss K, Holland EC, Fufts DW. Sonic hedgehog and insulin-like growth factor signaling synergize to induce medulloblastoma formation from nestin-expressing neural progenitors in mice. *Oncogene* 2004; **23**: 6156-6162 [PMID: 15195141 DOI: 10.1038/SJ.ONC.1207818]
- 21 **Hao Y**, Crenshaw T, Moulton T, Newcomb E, Tycko B. Tumour-suppressor activity of H19 RNA. *Nature* 1993; **365**: 764-767 [PMID: 7692308 DOI: 10.1038/365764A0]
- 22 **Yoshimizu T**, Miroglio A, Ripoche MA, Gabory A, Vernucci M, Riccio A, Colnot S, Godard C, Terris B, Jammes H, Dandolo L. The H19 locus acts in vivo as a tumor suppressor. *Proc Natl Acad Sci USA* 2008; **105**: 12417-12422 [PMID: 18719115 DOI: 10.1073/PNAS.0801540105]
- 23 **Keniry A**, Oxley D, Monnier P, Kyba M, Dandolo L, Smits G, Reik W. The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. *Nat Cell Biol* 2012; **14**: 659-665 [PMID: 22684254 DOI: 10.1038/NCB2521]
- 24 **Kono T**, Obata Y, Wu Q, Niwa K, Ono Y, Yamamoto Y, Park ES, Seo JS, Ogawa H. Birth of parthenogenetic mice that can develop to adulthood. *Nature* 2004; **428**: 860-864 [PMID: 15103378 DOI: 10.1038/NATURE02402]
- 25 **Surani MA**, Hayashi K, Hajkova P. Genetic and epigenetic regulators of pluripotency. *Cell* 2007; **128**: 747-762 [PMID: 17320511 DOI: 10.1016/J.CELL.2007.02.010]
- 26 **Ratajczak MZ**, Zuba-Surma E, Wojakowski W, Suszynska M, Mierzejewska K, Liu R, Ratajczak J, Shin DM, Kucia M. Very small embryonic-like stem cells (VSELs) represent a real challenge in stem cell biology: recent pros and cons in the midst of a lively debate. *Leukemia* 2014; **28**: 473-484 [PMID: 24018851 DOI: 10.1038/LEU.2013.255]
- 27 **Ratajczak MZ**, Shin DM, Schneider G, Ratajczak J, Kucia M. Parental imprinting regulates insulin-like growth factor signaling: a Rosetta Stone for understanding the biology of pluripotent stem cells, aging and cancerogenesis. *Leukemia* 2013; **27**: 773-779 [PMID: 23135355 DOI: 10.1038/LEU.2012.322]
- 28 **Takada S**, Paulsen M, Tevendale M, Tsai CE, Kelsey G, Cattanach BM, Ferguson-Smith AC. Epigenetic analysis of the Dlk1-Gtl2 imprinted domain on mouse chromosome 12: implications for imprinting control from comparison with Igf2-H19. *Hum Mol Genet* 2002; **11**: 77-86 [PMID: 11773001 DOI: 10.1093/hmg/11.1.77]
- 29 **Falix FA**, Aronson DC, Lamers WH, Gaemers IC. Possible roles of DLK1 in the Notch pathway during development and disease. *Biochim Biophys Acta* 2012; **1822**: 988-995 [PMID: 22353464 DOI: 10.1016/J.BBADIS.2012.02.003]
- 30 **Ferrón SR**, Charalambous M, Radford E, McEwen K, Wildner H, Hind E, Morante-Redolat JM, Laborda J, Guillemot F, Bauer SR, Fariñas I, Ferguson-Smith AC. Postnatal loss of Dlk1 imprinting in stem cells and niche astrocytes regulates neurogenesis. *Nature* 2011; **475**: 381-385 [PMID: 21776083 DOI: 10.1038/NATURE10229]
- 31 **Takumi T**. The neurobiology of mouse models syntenic to human chromosome 15q. *J Neurodev Disord* 2011; **3**: 270-281 [PMID: 21789598 DOI: 10.1007/s11689-011-9088-1]
- 32 **Maruyama K**, Usami M, Aizawa T, Yoshikawa K. A novel brain-specific mRNA encoding nuclear protein (necdin) expressed in neurally differentiated embryonal carcinoma cells. *Biochem Biophys Res Commun* 1991; **178**: 291-296 [PMID: 2069569 DOI: 10.1016/0006-291X(91)91812-Q]
- 33 **Taniura H**, Taniguchi N, Hara M, Yoshikawa K. Necdin, a postmitotic neuron-specific growth suppressor, interacts with viral transforming proteins and cellular transcription factor E2F1. *J Biol Chem* 1998; **273**: 720-728 [PMID: 9422723 DOI: 10.1074/jbc.273.2.720]
- 34 **Taniura H**, Matsumoto K, Yoshikawa K. Physical and functional interactions of neuronal growth suppressor necdin with p53. *J Biol Chem* 1999; **274**: 16242-16248 [PMID: 10347180 DOI: 10.1074/jbc.274.23.16242]
- 35 **Hasegawa K**, Yoshikawa K. Necdin regulates p53 acetylation via Sirtuin1 to modulate DNA damage response in cortical neurons. *J Neurosci* 2008; **28**: 8772-8784 [PMID: 18753379 DOI: 10.1523/JNEUROSCI.3052-08.2008]
- 36 **Moon HE**, Ahn MY, Park JA, Min KJ, Kwon YW, Kim KW. Negative regulation of hypoxia inducible factor-1alpha by necdin. *FEBS Lett* 2005; **579**: 3797-3801 [PMID: 15978586 DOI: 10.1016/j.febslet.2005.05.072]
- 37 **Huang Z**, Fujiwara K, Minamide R, Hasegawa K, Yoshikawa K. Necdin controls proliferation and apoptosis of embryonic neural stem cells in an oxygen tension-dependent manner. *J Neurosci* 2013; **33**: 10362-10373 [PMID: 23785149 DOI: 10.1523/JNEUROSCI.5682-12.2013]
- 38 **Chen HL**, Pistollato F, Hoepfner DJ, Ni HT, McKay RD, Panchision DM. Oxygen tension regulates survival and fate of mouse central nervous system precursors at multiple levels. *Stem Cells* 2007; **25**: 2291-2301 [PMID: 17556599 DOI: 10.1634/stemcells.2006-0609]
- 39 **Clarke L**, van der Kooy D. Low oxygen enhances primitive and definitive neural stem cell colony formation by inhibiting distinct cell death pathways. *Stem Cells* 2009; **27**: 1879-1886 [PMID: 19544448 DOI: 10.1002/stem.96]
- 40 **Zhao T**, Zhang CP, Liu ZH, Wu LY, Huang X, Wu HT, Xiong L, Wang X, Wang XM, Zhu LL, Fan M. Hypoxia-driven proliferation of embryonic neural stem/progenitor cells--role of hypoxia-inducible transcription factor-1alpha. *FEBS J* 2008; **275**: 1824-1834 [PMID: 18341590 DOI: 10.1111/j.1742-4658.2008.06340.x]
- 41 **Minamide R**, Fujiwara K, Hasegawa K, Yoshikawa K. Antagonistic interplay between necdin and Bmi1 controls proliferation of neural precursor cells in the embryonic mouse neocortex. *PLoS One* 2014; **9**: e84460 [PMID: 24392139 DOI: 10.1371/journal.pone.0084460]
- 42 **Ohnuma S**, Harris WA. Neurogenesis and the cell cycle. *Neuron* 2003; **40**: 199-208 [PMID: 14556704 DOI: 10.1016/S0896-6273(03)00632-9]
- 43 **Zhang P**, Liégeois NJ, Wong C, Finegold M, Hou H, Thompson JC, Silverman A, Harper JW, DePinho RA, Elledge SJ. Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. *Nature* 1997; **387**: 151-158 [PMID: 9144284 DOI: 10.1038/387151A0]
- 44 **Carey RG**, Li B, DiCicco-Bloom E. Pituitary adenylate cyclase activating polypeptide anti-mitogenic signaling in cerebral cortical progenitors is regulated by p57Kip2-dependent CDK2 activity. *J Neurosci* 2002; **22**: 1583-1591 [PMID: 11880488]
- 45 **Ye W**, Mairet-Coello G, Pasoreck E, DiCicco-Bloom E. Patterns of p57Kip2 expression in embryonic rat brain suggest roles in progenitor cell cycle exit and neuronal differentiation. *Dev Neurobiol* 2009; **69**: 1-21 [PMID: 18814313 DOI: 10.1002/DNEU.20680]
- 46 **Mairet-Coello G**, Tury A, Van Buskirk E, Robinson K, Genestine M, DiCicco-Bloom E. p57(KIP2) regulates radial glia and intermediate precursor cell cycle dynamics and lower layer neurogenesis in developing cerebral cortex. *Development* 2012; **139**: 475-487 [PMID: 22223678 DOI: 10.1242/DEV.067314]
- 47 **Furutachi S**, Matsumoto A, Nakayama KI, Gotoh Y. p57 controls adult neural stem cell quiescence and modulates the pace of lifelong neurogenesis. *EMBO J* 2013; **32**: 970-981 [PMID: 23481253 DOI: 10.1038/EMBOJ.2013.50]
- 48 **Besson A**, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. *Dev Cell* 2008; **14**: 159-169 [PMID: 18267085 DOI: 10.1016/J.DEVCEL.2008.01.013]
- 49 **Piras G**, El Kharroubi A, Kozlov S, Escalante-Alcalde D, Hernandez L, Copeland NG, Gilbert DJ, Jenkins NA, Stewart CL. *Zac1* (*Lot1*), a potential tumor suppressor gene, and the gene for



- epsilon-sarcoglycan are maternally imprinted genes: identification by a subtractive screen of novel uniparental fibroblast lines. *Mol Cell Biol* 2000; **20**: 3308-3315 [PMID: 10757814 DOI: 10.1128/MCB.20.9.3308-3315.2000]
- 50 **Varrault A**, Ciani E, Apiou F, Bilanges B, Hoffmann A, Pantaloni C, Bockaert J, Spengler D, Journot L. hZAC encodes a zinc finger protein with antiproliferative properties and maps to a chromosomal region frequently lost in cancer. *Proc Natl Acad Sci USA* 1998; **95**: 8835-8840 [PMID: 9671765]
- 51 **Spengler D**, Villalba M, Hoffmann A, Pantaloni C, Houssami S, Bockaert J, Journot L. Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain. *EMBO J* 1997; **16**: 2814-2825 [PMID: 9184226 DOI: 10.1093/EMBOJ/16.10.2814]
- 52 **Aguilar-Bryan L**, Bryan J. Neonatal diabetes mellitus. *Endocr Rev* 2008; **29**: 265-291 [PMID: 18436707 DOI: 10.1210/ER.2007-0029]
- 53 **Ma D**, Shield JP, Dean W, Leclerc I, Knauf C, Burcelin R Ré, Rutter GA, Kelsey G. Impaired glucose homeostasis in transgenic mice expressing the human transient neonatal diabetes mellitus locus, TNDM. *J Clin Invest* 2004; **114**: 339-348 [PMID: 15286800 DOI: 10.1172/JCI19876]
- 54 **Hoffmann A**, Spengler D. Transient neonatal diabetes mellitus gene Zac1 impairs insulin secretion in mice through Rasgrf1. *Mol Cell Biol* 2012; **32**: 2549-2560 [PMID: 22547676 DOI: 10.1128/MCB.06637-11]
- 55 **Hoffmann A**, Ciani E, Boeckardt J, Holsboer F, Journot L, Spengler D. Transcriptional activities of the zinc finger protein Zac are differentially controlled by DNA binding. *Mol Cell Biol* 2003; **23**: 988-1003 [PMID: 12529403 DOI: 10.1128/MCB.23.3.988-1003.2003]
- 56 **Bilanges B**, Varrault A, Mazumdar A, Pantaloni C, Hoffmann A, Bockaert J, Spengler D, Journot L. Alternative splicing of the imprinted candidate tumor suppressor gene ZAC regulates its antiproliferative and DNA binding activities. *Oncogene* 2001; **20**: 1246-1253 [PMID: 11313869 DOI: 10.1038/SJ.ONC.1204237]
- 57 **Ciani E**, Hoffmann A, Schmidt P, Journot L, Spengler D. Induction of the PAC1-R (PACAP-type I receptor) gene by p53 and Zac. *Brain Res Mol Brain Res* 1999; **69**: 290-294 [PMID: 10366751 DOI: 10.1016/S0169-328X(99)00116-3]
- 58 **Huang SM**, Stallcup MR. Mouse Zac1, a transcriptional coactivator and repressor for nuclear receptors. *Mol Cell Biol* 2000; **20**: 1855-1867 [PMID: 10669760 DOI: 10.1128/MCB.20.5.1855-1867.2000]
- 59 **Huang SM**, Schönthal AH, Stallcup MR. Enhancement of p53-dependent gene activation by the transcriptional coactivator Zac1. *Oncogene* 2001; **20**: 2134-2143 [PMID: 11360197 DOI: 10.1038/SJ.ONC.1204298]
- 60 **Rozenfeld-Granot G**, Krishnamurthy J, Kannan K, Toren A, Amariglio N, Givol D, Rechavi G. A positive feedback mechanism in the transcriptional activation of Apaf-1 by p53 and the coactivator Zac-1. *Oncogene* 2002; **21**: 1469-1476 [PMID: 11896574 DOI: 10.1038/sj.onc.1205218]
- 61 **Ptashne M**, Gann A. Transcriptional activation by recruitment. *Nature* 1997; **386**: 569-577 [PMID: 9121580 DOI: 10.1038/386569A0]
- 62 **Hoffmann A**, Barz T, Spengler D. Multitasking C2H2 zinc fingers link Zac DNA binding to coordinated regulation of p300-histone acetyltransferase activity. *Mol Cell Biol* 2006; **26**: 5544-5557 [PMID: 16809786 DOI: 10.1128/MCB.02270-05]
- 63 **Abdollahi A**. LOT1 (ZAC1/PLAGL1) and its family members: mechanisms and functions. *J Cell Physiol* 2007; **210**: 16-25 [PMID: 17063461 DOI: 10.1002/JCP.20835]
- 64 **Theodoropoulou M**, Stalla GK, Spengler D. ZAC1 target genes and pituitary tumorigenesis. *Mol Cell Endocrinol* 2010; **326**: 60-65 [PMID: 20117169 DOI: 10.1016/J.MCE.2010.01.033]
- 65 **Hoffmann A**, Spengler D. A new coactivator function for Zac1's C2H2 zinc finger DNA-binding domain in selectively controlling PCAF activity. *Mol Cell Biol* 2008; **28**: 6078-6093 [PMID: 18663001 DOI: 10.1128/MCB.00842-08]
- 66 **Valente T**, Junyent F, Auladell C. Zac1 is expressed in progenitor/stem cells of the neuroectoderm and mesoderm during embryogenesis: differential phenotype of the Zac1-expressing cells during development. *Dev Dyn* 2005; **233**: 667-679 [PMID: 15844099 DOI: 10.1002/DVDY.20373]
- 67 **Valente T**, Auladell C. Expression pattern of Zac1 mouse gene, a new zinc-finger protein that regulates apoptosis and cellular cycle arrest, in both adult brain and along development. *Mech Dev* 2001; **108**: 207-211 [PMID: 11578877 DOI: 10.1016/S0925-4773(01)00492-0]
- 68 **Alam S**, Zinyk D, Ma L, Schuurmans C. Members of the Plag gene family are expressed in complementary and overlapping regions in the developing murine nervous system. *Dev Dyn* 2005; **234**: 772-782 [PMID: 16193498 DOI: 10.1002/DVDY.20577]
- 69 **Varrault A**, Gueydan C, Delalbre A, Bellmann A, Houssami S, Aknin C, Severac D, Chotard L, Kahl M, Le Digarcher A, Pavlidis P, Journot L. Zac1 regulates an imprinted gene network critically involved in the control of embryonic growth. *Dev Cell* 2006; **11**: 711-722 [PMID: 17084362 DOI: 10.1016/J.DEVCEL.2006.09.003]
- 70 **Valente T**. Expressió de la Zac1 durant el desenvolupament de ratolí. Paper de la Zac1 en el sistema nerviós central: ISBN: 8468924865 [PhD thesis]. Barcelona: Universitat de Barcelona, 2005. Available from: URL: <http://www.tdx.cat/handle/10803/840>
- 71 **Hoffmann A**, Ciani E, Houssami S, Brabet P, Journot L, Spengler D. Induction of type I PACAP receptor expression by the new zinc finger protein Zac1 and p53. *Ann N Y Acad Sci* 1998; **865**: 49-58 [PMID: 9927996 DOI: 10.1111/j.1749-6632.1998.tb11162.x]
- 72 **Arimura A**. Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. *Jpn J Physiol* 1998; **48**: 301-331 [PMID: 9852340 DOI: 10.2170/jphysiol.48.301]
- 73 **Li M**, Arimura A. Neuropeptides of the pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide/growth hormone-releasing hormone/secretin family in testis. *Endocrine* 2003; **20**: 201-214 [PMID: 12721498 DOI: 10.1385/ENDO.20.3.201]
- 74 **Mustafa T**. Pituitary adenylate cyclase-activating polypeptide (PACAP): a master regulator in central and peripheral stress responses. *Adv Pharmacol* 2013; **68**: 445-457 [PMID: 24054157 DOI: 10.1016/B978-0-12-411512-5.00021-X]
- 75 **Rodríguez-Henche N**, Jamen F, Leroy C, Bockaert J, Brabet P. Transcription of the mouse PAC1 receptor gene: cell-specific expression and regulation by Zac1. *Biochim Biophys Acta* 2002; **1576**: 157-162 [PMID: 12031496 DOI: 10.1016/S0167-4781(02)00303-2]
- 76 **Shen S**, Gehlert DR, Collier DA. PACAP and PAC1 receptor in brain development and behavior. *Neuropeptides* 2013; **47**: 421-430 [PMID: 24220567 DOI: 10.1016/J.NPEP.2013.10.005]
- 77 **Vousden KH**, Lane DP. p53 in health and disease. *Nat Rev Mol Cell Biol* 2007; **8**: 275-283 [PMID: 17380161 DOI: 10.1038/NRM2147]
- 78 **Sabapathy K**, Klemm M, Jaenisch R, Wagner EF. Regulation of ES cell differentiation by functional and conformational modulation of p53. *EMBO J* 1997; **16**: 6217-6229 [PMID: 9321401 DOI: 10.1093/EMBOJ/16.20.6217]
- 79 **Solozobova V**, Blattner C. Regulation of p53 in embryonic stem cells. *Exp Cell Res* 2010; **316**: 2434-2446 [PMID: 20542030 DOI: 10.1016/J.YEXCR.2010.06.006]
- 80 **Han MK**, Song EK, Guo Y, Ou X, Mantel C, Broxmeyer HE. SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. *Cell Stem Cell* 2008; **2**: 241-251 [PMID: 18371449 DOI: 10.1016/J.STEM.2008.01.002]
- 81 **Lin T**, Chao C, Saito S, Mazur SJ, Murphy ME, Appella E, Xu Y. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* 2005; **7**: 165-171 [PMID: 15619621 DOI: 10.1038/NCB1211]
- 82 **Qin H**, Yu T, Qing T, Liu Y, Zhao Y, Cai J, Li J, Song Z, Qu X, Zhou P, Wu J, Ding M, Deng H. Regulation of apoptosis and differentiation by p53 in human embryonic stem cells. *J Biol Chem* 2007; **282**: 5842-5852 [PMID: 17179143 DOI: 10.1074/JBC.M610464200]
- 83 **Meletis K**, Wirta V, Hede SM, Nistér M, Lundberg J, Frisén J. p53 suppresses the self-renewal of adult neural stem cells. *Development* 2006; **133**: 363-369 [PMID: 16368933 DOI: 10.1242/DEV.02208]



- 84 **Tedeschi A**, Di Giovanni S. The non-apoptotic role of p53 in neuronal biology: enlightening the dark side of the moon. *EMBO Rep* 2009; **10**: 576-583 [PMID: 19424293 DOI: 10.1038/EMBOR.2009.89]
- 85 **Gil-Perotin S**, Marin-Husstege M, Li J, Soriano-Navarro M, Zindy F, Roussel MF, Garcia-Verdugo JM, Casaccia-Bonnett P. Loss of p53 induces changes in the behavior of subventricular zone cells: implication for the genesis of glial tumors. *J Neurosci* 2006; **26**: 1107-1116 [PMID: 16436596 DOI: 10.1523/JNEUROSCI.3970-05.2006]
- 86 **Nagao M**, Campbell K, Burns K, Kuan CY, Trumpp A, Nakafuku M. Coordinated control of self-renewal and differentiation of neural stem cells by Myc and the p19ARF-p53 pathway. *J Cell Biol* 2008; **183**: 1243-1257 [PMID: 19114593 DOI: 10.1083/JCB.200807130]
- 87 **Solozobova V**, Blattner C. p53 in stem cells. *World J Biol Chem* 2011; **2**: 202-214 [PMID: 21949570 DOI: 10.4331/WJBC.V2.I9.202]
- 88 **Forsberg K**, Wuttke A, Quadrato G, Chumakov PM, Wizenmann A, Di Giovanni S. The tumor suppressor p53 fine-tunes reactive oxygen species levels and neurogenesis via PI3 kinase signaling. *J Neurosci* 2013; **33**: 14318-14330 [PMID: 24005285 DOI: 10.1523/JNEUROSCI.1056-13.2013]
- 89 **Meyer G**, Cabrera Socorro A, Perez Garcia CG, Martinez Millan L, Walker N, Caput D. Developmental roles of p73 in Cajal-Retzius cells and cortical patterning. *J Neurosci* 2004; **24**: 9878-9887 [PMID: 15525772 DOI: 10.1523/JNEUROSCI.3060-04.2004]
- 90 **Yang A**, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, Vagner C, Bonnet H, Dikkes P, Sharpe A, McKeon F, Caput D. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* 2000; **404**: 99-103 [PMID: 10716451 DOI: 10.1038/35003607]
- 91 **Pozniak CD**, Radinovic S, Yang A, McKeon F, Kaplan DR, Miller FD. An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. *Science* 2000; **289**: 304-306 [PMID: 10894779 DOI: 10.1126/science.289.5477.304]
- 92 **Tissir F**, Ravni A, Achouri Y, Riethmacher D, Meyer G, Goffinet AM. DeltaNp73 regulates neuronal survival in vivo. *Proc Natl Acad Sci USA* 2009; **106**: 16871-16876 [PMID: 19805388 DOI: 10.1073/PNAS.0903191106]
- 93 **Balint E**, Phillips AC, Kozlov S, Stewart CL, Vousden KH. Induction of p57(KIP2) expression by p73beta. *Proc Natl Acad Sci USA* 2002; **99**: 3529-3534 [PMID: 11891335 DOI: 10.1073/PNAS.062491899]
- 94 **Talos F**, Abraham A, Vaseva AV, Holembowski L, Tsirka SE, Scheel A, Bode D, Dobbelstein M, Brück W, Moll UM. p73 is an essential regulator of neural stem cell maintenance in embryonal and adult CNS neurogenesis. *Cell Death Differ* 2010; **17**: 1816-1829 [PMID: 21076477 DOI: 10.1038/CDD.2010.131]
- 95 **Fujitani M**, Cancino GI, Dugani CB, Weaver IC, Gauthier-Fisher A, Paquin A, Mak TW, Wojtowicz MJ, Miller FD, Kaplan DR. TAp73 acts via the bHLH Hey2 to promote long-term maintenance of neural precursors. *Curr Biol* 2010; **20**: 2058-2065 [PMID: 21074438 DOI: 10.1016/J.CUB.2010.10.029]
- 96 **Schmidt-Edelkraut U**, Hoffmann A, Daniel G, Spengler D. Zac1 regulates astroglial differentiation of neural stem cells through Socs3. *Stem Cells* 2013; **31**: 1621-1632 [PMID: 23630160 DOI: 10.1002/stem.1405]
- 97 **Bonni A**, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, Stahl N, Yancopoulos GD, Greenberg ME. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science* 1997; **278**: 477-483 [PMID: 9334309 DOI: 10.1002/STEM.1405]
- 98 **He F**, Ge W, Martinowich K, Becker-Catania S, Coskun V, Zhu W, Wu H, Castro D, Guillemot F, Fan G, de Vellis J, Sun YE. A positive autoregulatory loop of Jak-STAT signaling controls the onset of astroglial differentiation. *Nat Neurosci* 2005; **8**: 616-625 [PMID: 15852015 DOI: 10.1038/NN1440]
- 99 **Freeman MR**. Specification and morphogenesis of astrocytes. *Science* 2010; **330**: 774-778 [PMID: 21051628 DOI: 10.1126/SCIENCE.1190928]
- 100 **Fukuda S**, Abematsu M, Mori H, Yanagisawa M, Kagawa T, Nakashima K, Yoshimura A, Taga T. Potentiation of astroglial differentiation by STAT3-mediated activation of bone morphogenetic protein-Smad signaling in neural stem cells. *Mol Cell Biol* 2007; **27**: 4931-4937 [PMID: 17452461 DOI: 10.1128/MCB.02435-06]
- 101 **Bugga L**, Gadiant RA, Kwan K, Stewart CL, Patterson PH. Analysis of neuronal and glial phenotypes in brains of mice deficient in leukemia inhibitory factor. *J Neurobiol* 1998; **36**: 509-524 [PMID: 9740023]
- 102 **Nakashima K**, Wiese S, Yanagisawa M, Arakawa H, Kimura N, Hisatsune T, Yoshida K, Kishimoto T, Sendtner M, Taga T. Developmental requirement of gp130 signaling in neuronal survival and astrocyte differentiation. *J Neurosci* 1999; **19**: 5429-5434 [PMID: 10377352 DOI: 10.1002/(SICI)1097-4695(19980915)3]
- 103 **Cao F**, Hata R, Zhu P, Ma YJ, Tanaka J, Hanakawa Y, Hashimoto K, Niinobe M, Yoshikawa K, Sakanaka M. Overexpression of SOCS3 inhibits astroglial differentiation and promotes maintenance of neural stem cells. *J Neurochem* 2006; **98**: 459-470 [PMID: 16805839 DOI: 10.1111/J.1471-4159.2006.03890.x]
- 104 **Murre C**, McCaw PS, Baltimore D. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell* 1989; **56**: 777-783 [PMID: 2493990 DOI: 10.1016/0092-8674(89)90682-X]
- 105 **Bertrand N**, Castro DS, Guillemot F. Proneural genes and the specification of neural cell types. *Nat Rev Neurosci* 2002; **3**: 517-530 [PMID: 12094208 DOI: 10.1038/nrn874]
- 106 **Bhattacharya A**, Baker NE. A network of broadly expressed HLH genes regulates tissue-specific cell fates. *Cell* 2011; **147**: 881-892 [PMID: 22078884 DOI: 10.1016/j.cell.2011.08.055]
- 107 **Ross SE**, Greenberg ME, Stiles CD. Basic helix-loop-helix factors in cortical development. *Neuron* 2003; **39**: 13-25 [PMID: 12848929 DOI: 10.1016/S0896-6273(03)00365-9]
- 108 **Sun Y**, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hua X, Fan G, Greenberg ME. Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* 2001; **104**: 365-376 [PMID: 11239394 DOI: 10.1016/S0092-8674(01)00224-0]
- 109 **Schmidt-Edelkraut U**, Daniel G, Hoffmann A, Spengler D. Zac1 regulates cell cycle arrest in neuronal progenitors via Tcf4. *Mol Cell Biol* 2014; **34**: 1020-1030 [PMID: 24396065 DOI: 10.1128/MCB.01195-13]
- 110 **Arima T**, Kamikihara T, Hayashida T, Kato K, Inoue T, Shirayoshi Y, Oshimura M, Soejima H, Mukai T, Wake N. ZAC, LIT1 (KCNQ1OT1) and p57KIP2 (CDKN1C) are in an imprinted gene network that may play a role in Beckwith-Wiedemann syndrome. *Nucleic Acids Res* 2005; **33**: 2650-2660 [PMID: 15888726 DOI: 10.1093/NAR/GKI555]
- 111 **Lui JC**, Finkelstein GP, Barnes KM, Baron J. An imprinted gene network that controls mammalian somatic growth is down-regulated during postnatal growth deceleration in multiple organs. *Am J Physiol Regul Integr Comp Physiol* 2008; **295**: R189-R196 [PMID: 18448610 DOI: 10.1152/AJPREGU.00182.2008]
- 112 **Berg JS**, Lin KK, Sonnet C, Boles NC, Weksberg DC, Nguyen H, Holt LJ, Rickwood D, Daly RJ, Goodell MA. Imprinted genes that regulate early mammalian growth are coexpressed in somatic stem cells. *PLoS One* 2011; **6**: e26410 [PMID: 22039481 DOI: 10.1371/JOURNAL.PONE.0026410]
- 113 **Seibt J**, Armant O, Le Digarcher A, Castro D, Ramesh V, Journot L, Guillemot F, Vanderhaeghen P, Bouchet T. Expression at the imprinted dlk1-gtl2 locus is regulated by proneural genes in the developing telencephalon. *PLoS One* 2012; **7**: e48675 [PMID: 23139813 DOI: 10.1371/JOURNAL.PONE.0048675]
- 114 **Cowley M**, Garfield AS, Madon-Simon M, Charalambous M, Clarkson RW, Smalley MJ, Kendrick H, Isles AR, Parry AJ, Carney S, Oakley RJ, Heisler LK, Moorwood K, Wolf JB, Ward A. Developmental programming mediated by complementary roles of imprinted Grb10 in mother and pup. *PLoS Biol* 2014; **12**: e1001799 [PMID: 24586114 DOI: 10.1371/journal.pbio.1001799]
- 115 **Lawson HA**, Cheverud JM, Wolf JB. Genomic imprinting and parent-of-origin effects on complex traits. *Nat Rev Genet* 2013; **14**: 609-617 [PMID: 23917626 DOI: 10.1038/nrg3543]



- 116 **Soubry A**, Murphy SK, Wang F, Huang Z, Vidal AC, Fuemmeler BF, Kurtzberg J, Murtha A, Jirtle RL, Schildkraut JM, Hoyo C. Newborns of obese parents have altered DNA methylation patterns at imprinted genes. *Int J Obes (Lond)* 2013 Oct 25; Epub ahead of print [PMID: 24158121 DOI: 10.1038/ijo.2013.193]
- 117 **Azzi S**, Sas TC, Koudou Y, Le Bouc Y, Souberbielle JC, Dargent-Molina P, Netchine I, Charles MA. Degree of methylation of ZAC1 (PLAGL1) is associated with prenatal and post-natal growth in healthy infants of the EDEN mother child cohort. *Epigenetics* 2014; 9: 338-345 [PMID: 24316753 DOI: 10.4161/epi.27387]
- 118 **Heijmans BT**, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE, Lumey LH. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci USA* 2008; **105**: 17046-17049 [PMID: 18955703 DOI: 10.1073/pnas.0806560105]
- 119 **Pirazzini C**, Giuliani C, Bacalini MG, Boattini A, Capri M, Fontanesi E, Marasco E, Mantovani V, Pierini M, Pini E, Luiselli D, Franceschi C, Garagnani P. Space/population and time/age in DNA methylation variability in humans: a study on IGF2/H19 locus in different Italian populations and in mono- and di-zygotic twins of different age. *Aging (Albany NY)* 2012; **4**: 509-520 [PMID: 22879348]

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## Induced pluripotent stem cells: Mechanisms, achievements and perspectives in farm animals

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unlimited self-renewal, and they can be triggered to differentiate into desired specialized cell types. These features provide the basis for an unlimited cell source for innovative cell therapies. Pluripotent cells also allow to study developmental pathways, and to employ them or their differentiated cell derivatives in pharmaceutical testing and biotechnological applications. *Via* blastocyst complementation, pluripotent cells are a favoured tool for the generation of genetically modified mice. The recently established technology to generate an induced pluripotency status by ectopic co-expression of the transcription factors Oct4, Sox2, Klf4 and c-Myc allows to extending these applications to farm animal species, for which the derivation of genuine embryonic stem cells was not successful so far. Most induced pluripotent stem (iPS) cells are generated by retroviral or lentiviral transduction of reprogramming factors. Multiple viral integrations into the genome may cause insertional mutagenesis and may increase the risk of tumour formation. Non-integration methods have been reported to overcome the safety concerns associated with retro and lentiviral-derived iPS cells, such as transient expression of the reprogramming factors using episomal plasmids, and direct delivery of reprogramming mRNAs or proteins. In this review, we focus on the mechanisms of cellular reprogramming and current methods used to induce pluripotency. We also highlight problems associated with the generation of iPS cells. An increased understanding of the fundamental mechanisms underlying pluripotency and refining the methodology of iPS cell generation will have a profound impact on future development and application in regenerative medicine and reproductive biotechnology of farm animals.

**Key words:** Reprogramming; Large animal models; Stemness; Chimera; Germline transmission; Induced pluripotent stem cells; Gene delivery

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### Abstract

Pluripotent stem cells are unspecialized cells with

**Core tip:** The generation of an induced status of pluripotency



in somatic cells by ectopic expression of core transcription factors allows to extending advanced genetic modifications and reproductive techniques to species, for which the derivation of genuine embryonic stem cells was not successful till now. The commonly employed viral gene transfer may be genotoxic and therefore non-viral methods for iPS cell derivation are intensively studied. In this review, we focus on the mechanisms of cellular reprogramming and current methods used to induce pluripotency.

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## INTRODUCTION

Induced pluripotent stem (iPS) cells are defined as differentiated cells that have been experimentally reprogrammed to an embryonic stem (ES) cell-like state. The first generation of murine iPS cells was achieved<sup>[1]</sup> by retroviral transduction of four core reprogramming factors: Oct4, Sox2, Klf4, and c-Myc. Subsequently, human iPS cells were produced by viral transduction of adult fibroblasts<sup>[2,3]</sup>. Also a combination of Oct4, Sox2, Nanog and Lin28, was effective for the generation of human iPS cells<sup>[4]</sup>. An overview of reprogramming cells into iPS cells is shown in Figure 1.

Subsequently, the core reprogramming factors have been successfully used to derive pluripotent cells in various other species, including rhesus monkey<sup>[5]</sup>, rat<sup>[6]</sup>, pig<sup>[7]</sup>, dog<sup>[8]</sup>, cattle<sup>[9]</sup>, horse<sup>[10]</sup>, sheep<sup>[11]</sup>, goat<sup>[12]</sup> and buffalo<sup>[13]</sup>. A summary of the generation of iPS cells from different species of livestock is enumerated in Table 1. Importantly, iPS cells could be isolated from several species, in which the isolation of authentic ES cells was not successful despite several attempts since many years<sup>[14,15]</sup>. In particular, for economically important species, such as farm animals, the availability of authentic iPS cells would have important consequences for reproductive biology and approaches for genetic modification. For agricultural purposes, iPS cells from farm animal species can serve as a valuable genetic engineering tool to boost the generation of livestock with advantageous genes that are important for economic, reproductive and disease resistant traits, or for the study of functional genomics in mammals.

So far, iPS cells have been successfully produced from fibroblasts<sup>[16]</sup>, pancreas cells<sup>[17]</sup>, leukocytes<sup>[18]</sup>, hepatocytes<sup>[19]</sup>, keratinocytes<sup>[20]</sup>, neural stem cells<sup>[21]</sup>, cord blood cells<sup>[22]</sup>, and other cell types. Together these data suggest that most cell types can be reprogrammed to a pluripotent state, and that the unidirectional lineage commitment can be experimentally overwritten.

Certain cell types, such as neuronal progenitors, which exhibit basal expression of one or more of the core reprogramming factors, seem to be ideal for reprogramming<sup>[21]</sup>.

Rodent iPS cells are almost identical to their ES cell counterparts, sharing typical hallmarks of pluripotency such as colony morphology, unlimited self-renewal, *in vitro* and *in vivo* differentiation potentials, and contribution to the germline<sup>[23,24]</sup>. Most iPS lines from farm animal species have not been tested in chimera complementation assays; however some preliminary reports suggest that chimeras and germline transmission can be achieved in sheep and pig<sup>[25,26]</sup>. iPS cells derived from rodents, humans, monkeys and farm animals share the features of high telomerase activity, expression of alkaline phosphatase, and expression of stemness genes, such as *OCT4*, *SOX2*, *UTF1* and *REX1*. The epigenetic status of murine iPS cells has been analysed by bisulfite sequencing and chromatin immuno-precipitation DNA-Sequencing (ChIP-Seq)<sup>[27]</sup>. Thus the hallmarks for iPS cell characterisation can be enumerated as (1) unlimited self-renewal; (2) *in vitro* differentiation capacity; (3) *in vivo* differentiation capacity; (4) chimera contribution; and (5) subsequently germline transmission.

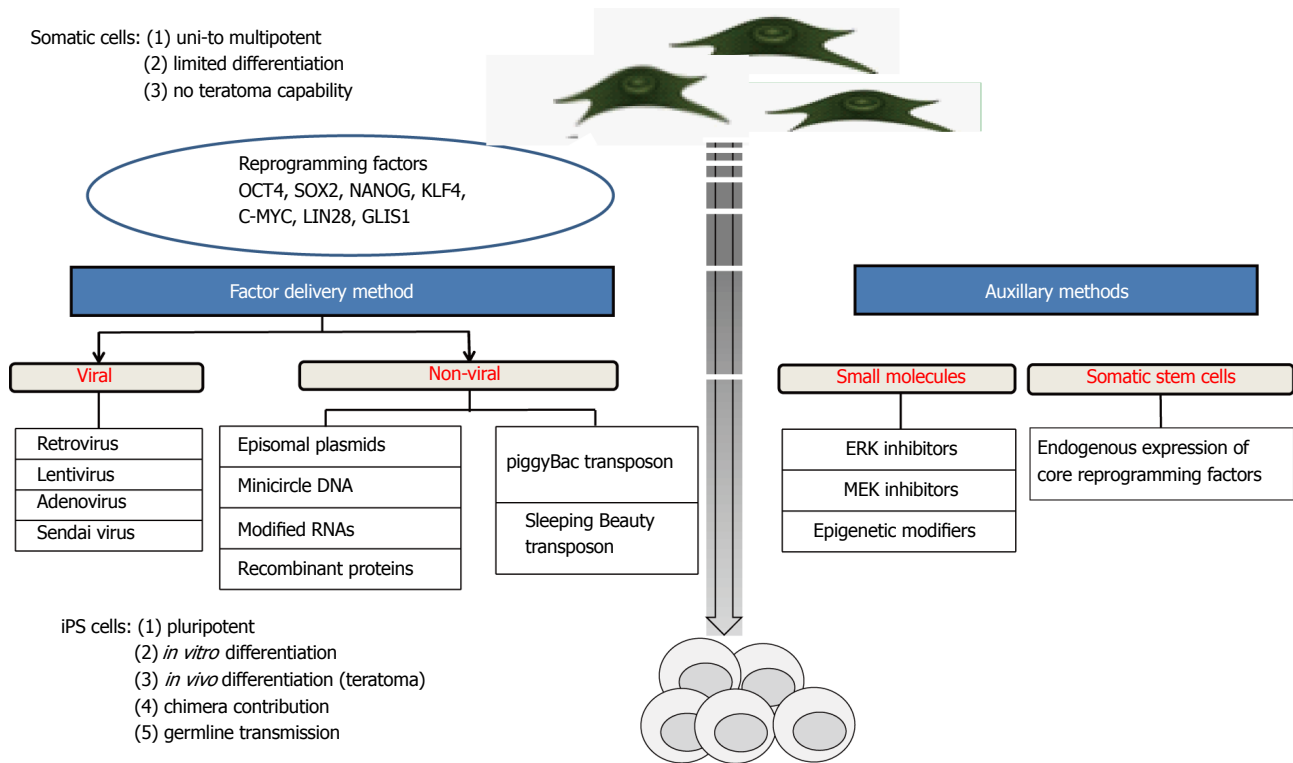
Apart from scientific and ethical hindrances, religious concerns restricted the derivation of human ES cells. To circumvent these concerns, alternative approaches to generate pluripotent cells have been assessed. The alternative approaches include culture of somatic cells with cell extracts isolated from ES cells<sup>[28]</sup> or oocytes<sup>[29]</sup>, and fusion of somatic cell with pluripotent cell<sup>[30]</sup>. However, extremely low efficiencies, high technical difficulties and aberrant ploidies of the resulting cells<sup>[31,32]</sup> did reduce the enthusiasm for these attempts. At the moment, the derivation of iPS cells from human tissues seems to be the most promising alternative. Prior to clinical application of iPS-derivatives, cell survival, functional integration of the cellular transplant and safety of the cell products have to be assessed in informative animal models.

The progress in iPS cell development in farm animals lags behind those in rodents, but large mammalian models may be instrumental for pre-clinical tests of novel cell therapies (Table 2), enhanced pharmaceutical studies and regenerative studies, including the restoration of fertility.

## HISTORICAL PERSPECTIVE

Ontogenesis of an organism and cellular differentiation were thought to be a unidirectional process, where stem and progenitor cells progressively develop to terminally differentiated cells, for example neurons, muscle, and epithelial cells. During ontogenesis the nuclear DNA of most cell types is unchanged, but different epigenetic marks, such as DNA methylation and histon modifications, are set, and lock the





**Figure 1** Methodological toolbox for generating induced pluripotent stem cells. iPS: Induced pluripotent stem.

cellular potency and cell lineage commitment. This is depicted by the “epigenetic landscape” proposed by Waddington<sup>[33]</sup>.

Already in 1962, Gurdon<sup>[34]</sup> questioned this view by amphibian cloning; he transplanted nuclei from intestinal cells into irradiated oocytes and obtained vital tadpoles. More than three decades later, the successful cloning of a sheep (Dolly) by SCNT of a mammary epithelial cell to an enucleated oocyte, showed that even mammalian cells can be reprogrammed<sup>[35]</sup>. This success demonstrated that differentiated cells contain the genetic information to direct ontogenesis of an entire mammalian organisms, and that enucleated oocytes contain pivotal factors for reprogramming of differentiated cell nuclei. However, the identity of the oocyte reprogramming factors remained elusive.

The discoveries that ectopic expression of Antennapedia-a transcription factor was able and sufficient to induce leg structures in *Drosophila*<sup>[36]</sup>, and that ectopic expression of the mammalian transcription factor MyoD1 converted fibroblasts into myocytes<sup>[37]</sup> led to the concept of “master genes”. A master gene was defined as a key transcription factor that in a hierarchical manner regulates a cascade of critical genes, which in a concerted action induce the cell commitment.

## DISCOVERY OF INDUCED PLURIPOTENCY

In 2006, Takahashi *et al.*<sup>[1]</sup> proved that not a single

master factor, but a combination of four reprogramming factors, Oct4, Sox2, Klf4 and c-Myc, was sufficient to induce the pluripotent status in somatic mammalian cells. The resulting cells were called iPS cells<sup>[1]</sup>. This discovery offers new opportunities to study developmental biology, regenerative medicine, as well as reproductive biology and biotechnology of farm animals.

iPS cells from farm animals will likely serve as a bridging link between well developed rodent iPS and poorly characterised human iPS (Table 2), supporting the translation of innovative cell therapies from experimental studies to curative treatments. At the moment, human iPS cell application seems to be too risky because of basic lack of knowledge and ethical consideration which forbid certain tests such as chimera assays.

In contrast, research on iPS cells derived from farm animal species is not tainted with ethical concerns. Furthermore, the methodology for generation of iPS cells is relative simple and is thought to be easily transferable to other mammalian species. Thus farm animal models may turn out to be ideally suited to determine required cell doses, to assess long-term performance, tumorigenicity, applications methods and fate of transplanted cells<sup>[38-41]</sup>.

Recent advances in genetic engineering of farm animals allow the generation of precise genetic modifications<sup>[42-47]</sup>, such as the production of immunodeficient pigs<sup>[48]</sup> which will be instrumental for further advances in preclinical testings of new cell therapies. A boost of recent



**Table 1** Most advanced achievements in induced pluripotent stem cells from domestic animals

Domestic species	Cell type	Transduction	Reprogramming factors	Culture medium	Differentiation		Chimera	Germline contribution	Ref.
					<i>In vitro</i>	<i>In vivo</i>			
Buffalo	Fetal fibroblasts	Retrovirus	OSKM	A	EBs	Teratoma	NA	NA	[13]
Cattle	Fetal fibroblasts	Retrovirus	OSKM, OSKMLN, OSKM	B	EBs	Teratoma	NA	NA	[9]
Dog	Fetal fibroblasts	Plasmid	OSKM	C	EBs	Teratoma	NA	NA	[53]
	Skin fibroblasts	Lentivirus	Human OKSM	J	EBs	Teratoma	NA	NA	[56]
	Skin fibroblasts	Retrovirus	Mouse OKSM	K	EBs	Teratoma	NA	NA	[54]
Goat	Fibroblasts	Inducible lentivirus	OSKM, SV40 large T and hTERT	A	EBs	Teratoma	NA	NA	[12]
Horse	Fetal fibroblasts	PiggyBac transposon	OSKM	E	EBs	Teratoma	NA	NA	[10]
	Adult fibroblasts	Retrovirus	OSK	F	EBs	Teratoma	NA	NA	[61]
Pig	Mesenchymal stem cells from bone marrow	Lentivirus	OSNKLM	G	EBs	NA	Low grade	Two offspring	[26]
	Fetal fibroblasts	Sleeping Beauty transposon	Mouse OSKM	I	Neuronal lineage	Teratoma	NA	NA	[91]
Rabbit	Skin fibroblasts	Retrovirus	Human OKSM	I	EBs	Teratoma	NA	NA	[72]
Sheep	Fetal fibroblasts	Retrovirus	MKOS	D	EBs	Teratoma	Low grade	NA	[25]

A: DMEM, ESC FBS, L-glutamine, NEAA,  $\beta$ -Me, bFGF, LIF and MEFs; B: DMEM, KSR, L-glutamine, NEAA,  $\beta$ -Me, bFGF and MEFs; C: DMEM/F12 + N2 and Neurobasal with B27, L-glutamine, hLIF, PD0325901, CHIR99021 and MEFs; D: KO-DMEM, SR, L-glutamine, NEAA, 2-Me, human bFGF and MEFs; E: DMEM, FBS, L-Glutamine, NEAA,  $\beta$ -Me, Sodium Pyruvate, LIF, bFGF, Doxycycline, CHIR99021, PD0325901, A83-01, Thiazovivin, B431542 and 1:1 MEFs and EEFs; F:  $\alpha$ -MEM, FBS, deoxyribonucleosides, ribonucleoside, glutamax, NEAA,  $\beta$ -Me, ITS, human LIF,  $\beta$ FGF, EGF and MEFs; G: DMEM/F12, KSR, L-glutamine, NEAA,  $\beta$ -Me, FGF and MEFs; H: KO DMEM, KSR, glutamax-L, NEAA, 2-Me, pLIF, forskolin and collagen I; I: DMEM/F12, KSR, L-glutamine, NEAA,  $\beta$ -Me, bFGF and MEFs or gelatinized plates; J: KO DMEM, ESC FBS, bFGF, hLIF and MEFs; K: DMEM/F12, KSR, bFGF, hLIF, PD0325901, CHIR99021 and MEFs. DMEM: Dulbecco's modified Eagle's medium; LIF: Leukemia inhibitory factor; IGF1: Insulin-like growth factor 1; NEAA: Nonessential amino acids; FBS: Fetal bovine serum; KO: Knockout; MEM: Minimum essential medium; ITS: Insulin-transferring selenium; bFGF: Basic fibroblastic growth factor; DOX: Doxycycline; EB: Embryonic body; FCS: Fetal calf serum; hSCF: Human stem cell factor; KSR: Knockout serum replacement; MEFs: Mouse embryonic fibroblasts; OKSM: Oct-4, Klf4, Sox2, and c-Myc; OKSMLN: Oct-4, Klf4, Sox2, c-Myc, Lin28 and Nanog; VPA: Valproic acid; Me: Mercaptoethanol.

publications describe iPS cells from buffalo<sup>[13]</sup>, cattle<sup>[9,49-53]</sup>, dog<sup>[8,54-56]</sup>, goat<sup>[11,57]</sup>, horse<sup>[10,58-62]</sup>, pig<sup>[7,63-71]</sup>, rabbit<sup>[72-74]</sup> and sheep<sup>[11,75,76]</sup>. The majority of these iPS cells from farm animals showed typical hallmarks of pluripotency, such as differentiation *in vivo* and teratoma formation. However, most farm animal iPS cultures were not assessed for chimera contribution so far. Preliminary results that porcine iPS cells can contribute to chimera formation in blastocyst complementation were provided recently<sup>[71]</sup>. Similarly, ovine iPS cells contributed moderately to chimeric lambs after injection into eight-cell stage embryos or blastocysts<sup>[25]</sup>. These experiments represent an important step in the understanding of mechanistic nature of pluripotency in farm animals. The iPS technology may become instrumental for advanced transgenesis in large mammals (Figure 2).

## METHODS TO DERIVE IPS CELLS

In recent years, several methods have been established for iPS cell generation (Figure 1), employing the core reprogramming factors as genes, mRNAs or proteins, and auxillary chemical agents, which infer with the involved signalling pathways. Here, the main approaches for the generation of iPS cells are summarized.

### Virally-induced iPS cells

There has been extensive amount of work carried out to obtain virally-derived iPS cells employing either retroviruses, lentiviruses, and non-integrating viruses. The first iPS cells have been generated through retroviral transduction of Oct4, Sox2, Klf4 and c-Myc<sup>[1]</sup>. Disarmed, optimized retro- or lentiviruses can infect mammalian cells with high efficiencies. The use of the pantropic vesicular stomatitis virus G protein (VSVG) was instrumental for viral transduction of a broad spectrum of receptive cells. Interestingly, unstimulated T cells, B cells and hematopoietic stem cells could not be efficiently transduced with the VSVG lentiviruses<sup>[77]</sup>.

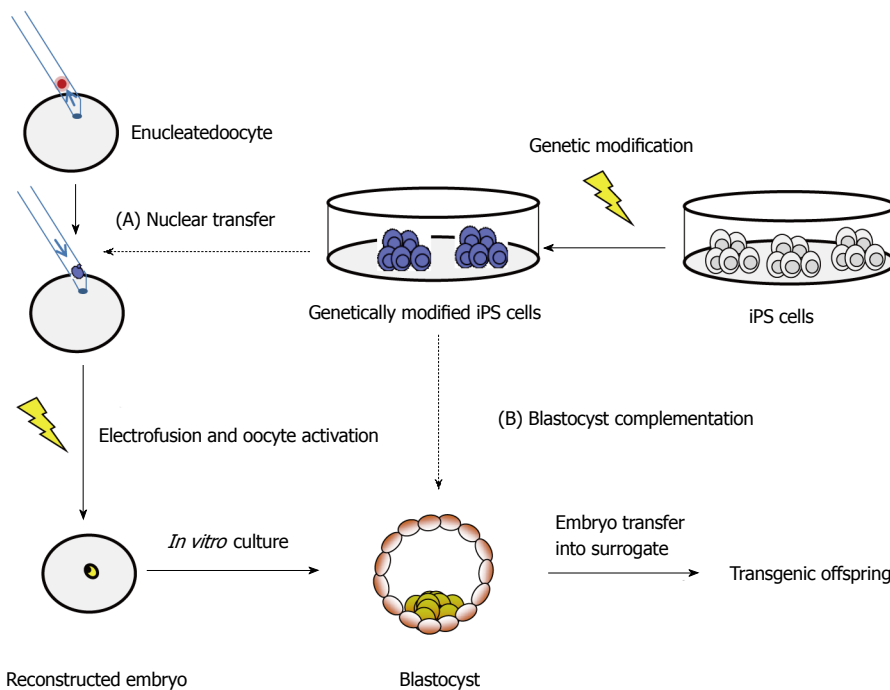
Retro- and lentiviruses integrate into the host genome allowing for high expression of the encoded cargo genes. The expression can be temporally confined by employing viral promoters, such as the 5' long terminal repeat, which are usually silenced by epigenetic mechanisms. Disadvantages of the viral approach include the limited cargo capacity of maximally 7 kb, the induction of immune responses and potential genotoxic effects. Retro- and lentiviral integrations do not happen randomly in the genome, but show a strong bias for promoter and exonic regions, which may result in dysregulation of endogenous genes. In a retrovirus-based clinical gene therapy of the X-linked



**Table 2 Achievements with induced pluripotent stem cells from rodents, farm animals and humans**

	Rodents	Farm animals	Human
iPS cells	√√	√√	√√
<i>In vivo</i> differentiation	√√	√√	√√
<i>In vitro</i> differentiation	√√	√√	√√
Chimera	√√	√/-	Ethically not allowed
Germline transmission	√√	√/-	Ethically not allowed
Follow up generations	√√	--	Ethically not allowed
Transplantation of iPS cell-derived cells	√√	√/-	No clinical studies to date <sup>1</sup>

√√: Fully proven; √/-: Partially proven; --: Not achieved yet; <sup>1</sup>The first clinical study was recently initiated ([http://www.riken.jp/en/pr/press/2013/20130730\\_1](http://www.riken.jp/en/pr/press/2013/20130730_1)). iPS: Induced pluripotent stem.



**Figure 2 Application of induced pluripotent stem cells for advanced generation of transgenic animals.** iPS: Induced pluripotent stem.

severe combined immunodeficiency (X-SCID), two of the treated children independently developed T-cell lymphomas due to viral integration in the neighborhood of the LIM domain only 2 gene<sup>[78]</sup>. These data highlight the risks of viral-based therapies<sup>[78]</sup>. Somatic cells derived from retrovirally reprogrammed iPS cells are apparently inconspicuous, provided that the c-Myc transgene is silenced<sup>[19,79]</sup>. Retroviral reprogramming may evoke an immunogenicity of iPS cells<sup>[80]</sup>. Human iPS cell-like cells can be formed through transduction with lentiviruses, which do not carry reprogramming factors. The “pseudo” iPS cells were induced by viral encoded microRNA expression<sup>[81]</sup>.

Alternative to integrating retroviruses, non-integrating adenoviruses can be used for reprogramming<sup>[17,82]</sup>. Another non-integrating virus is represented by the Sendai virus system. Sendai viruses enable efficient production of iPS cells and later on elimination of the viral vector<sup>[83]</sup>. Though viral mediated gene transfer offers high efficiency in generation of iPS cells, they require specific safety conditions for their handling.

### Non-virally-derived iPS cells

The generation of iPS cells without viral transduction is preferable for regenerative medicine. Non-viral methods of reprogramming include episomal vectors<sup>[84]</sup>, minicircle DNAs<sup>[85]</sup>, plasmid vectors<sup>[86]</sup>, small molecules<sup>[87]</sup>, mRNAs<sup>[88]</sup>, recombinant proteins<sup>[89]</sup> and transposons like piggyBac<sup>[90]</sup> and Sleeping Beauty<sup>[91]</sup>. In comparison to viral systems, non-viral approaches such as transposons are able to carry large DNA cargo into the host cell, they are non-infectious and do not evoke immune responses.

### Episomal vectors

Episomal vectors for reprogramming of somatic cells were recently described<sup>[84]</sup>. In this method, reprogramming of fibroblasts was carried out by transfecting with the episomal vector oriP/Epstein-Barr nuclear antigen-1. This vector was chosen because it can be removed after reprogramming by a drug selection method. The iPS cells generated through this method show similar morphology and expression patterns to ES cells. Further, they were able to form



teratomas in immunocompromised mice. As there was no integration into the host genome, transgene free iPS cells may be selected through further sub-cloning. Despite these advantages, this method yields low reprogramming efficiency in human fibroblasts at about three to six iPS colonies per  $10^6$  input cells<sup>[84]</sup>.

### **Minicircle vectors**

Minicircle vectors are produced by the recombinatorial elimination of the bacterial backbone of the original plasmids. Minicircles containing the four reprogramming factors Oct4, Nanog, Lin28, and Sox2 in addition to an enhanced green fluorescent protein were used to obtain human iPS cells<sup>[85]</sup>. The group excised the bacterial backbone from the plasmid by taking advantage of the PhiC31-based intramolecular recombination system, which cleaves away the undesired bacterial plasmid backbones, leaving minicircle DNA to be purified containing the desired reprogramming factors<sup>[85]</sup>. It was claimed that minicircle DNA benefited from higher transfection efficiency compared to the parental plasmids. They also have longer ectopic expression, which is due to the lower activation of exogenous silencing mechanisms. Later, other groups reproduced the minicircle approach for reprogramming<sup>[92,93]</sup>.

### **Small molecules**

Nowadays, small molecules and chemicals are assessed to enhance reprogramming efficiency and iPS cell generation. The idea behind their use is to substitute core reprogramming factors with small molecules, which will serve to enhance the reprogramming. Shi *et al.*<sup>[94]</sup> showed that neural progenitor cells, which endogenously express Sox2, were reprogrammed only by ectopic expression of Oct4 and Klf4. They also showed that this process was supported by the G9a histone methyltransferase inhibitor, BIX-01294 (BIX). Ichida *et al.*<sup>[95]</sup> used small molecules (RepSox2) for replacing transcription factors (Sox2) by inhibiting transforming growth factor- $\beta$  signalling. In this direction, Lee *et al.*<sup>[96]</sup> used magnetic nanoparticle-based transfection method that employs biodegradable cationic polymer PEI-coated super paramagnetic nanoparticles for iPS cells generation. Recently, the L-channel calcium agonist, BayK8644, was assessed to improve generation of iPS cells<sup>[87]</sup> and it was claimed that BayK8644 does not directly cause epigenetic modifications as it works upstream in cell signalling pathways and can therefore avoid unwanted modifications. A more comprehensive list of small molecules involved in the iPS cells generation and their mechanism has been reviewed recently<sup>[97]</sup>.

### **Transposon systems**

The recent development of hyperactive transposase enzymes makes transposon systems an interesting alternative to viral based methods. The commonly employed Sleeping Beauty, piggyBac and Tol2

transposon systems are relatively simple organized, and the essential components can be separated on two plasmids. One plasmid carries the inverted terminal repeats (ITR) flanking the transgene, the other plasmid carries an expression cassette for the respective transposase enzyme. Upon co-transfer of both plasmids into a cell, the transposase becomes expressed and subsequently transposes the ITR-flanked transgene into the genome. Importantly, only the desired transgenes becomes integrated by a cut-and-paste mechanism, whereas the plasmid backbones are degraded. On a genomic scale transposon integrations appear to happen at random, without a bias for promoter and gene-containing regions. The integrated transposon can be removed seamlessly by supplying the transposase in trans<sup>[98]</sup>, which makes the system more attractive and relevant in producing the safe and clean iPS cells. Up to six reprogramming factors have been connected by self-cleaving peptide sequences allowing for coexpression from a single cassette<sup>[91,99-103]</sup>. Individual proteins are then produced by the self-cleaving peptide<sup>[104-106]</sup>.

### **Reprogramming with protein factors**

The discussed transposon and episomal systems still require the introduction of cargo DNA into the cells<sup>[106]</sup>. Delivery of reprogramming factors as proteins is an obvious alternative. In 2009, transgene-free iPS cells were produced with proteins of reprogramming factors<sup>[107]</sup>. Therefor recombinant reprogramming proteins were produced as fusion proteins containing cell penetrating peptides. Repeated supplementation of the culture media of fibroblasts converted them to iPS cells. However, the protein-based reprogramming approach has not found widespread use, mainly due to relative low reprogramming efficiencies, and high costs for repeated treatments with protein factors.

### **mRNAs and microRNAs**

The most recent trend in the field of non-viral iPS generation is reprogramming by using RNA molecules. Recently, modified mRNAs encoding reprogramming factors were employed to generate iPS cells with high efficiency<sup>[108]</sup>. Messenger RNAs are an ideal vehicle for reprogramming, because they do not bear the risk of integrational mutagenesis, they can be transduced to cells with high efficiency, and they can be combined in desired ratios of the individual factor encoding transcripts<sup>[108]</sup>. Disadvantages of mRNAs are the short half-life of -10 h, and that innate immune responses must be inhibited to allow for the full effects<sup>[109]</sup>.

Recently, it was shown that micro RNAs (miR) expression is sufficient to induce pluripotency<sup>[110-112]</sup>. Two independent groups reported iPS cell generation by delivery of miR302, or miR200c, miR302, and miR369<sup>[113,114]</sup>. These miR-derived iPS cells were indistinguishable from conventionally generated iPS cells. MicroR reprogramming seems to have advantages



for cellular reprogramming<sup>[114-116]</sup>, for example it avoids the need of transducing proto-oncogenic transcription factors<sup>[117,118]</sup>. However, it needs to be assessed whether this approach will be successful in other species, since the underlying mechanisms are not well understood<sup>[119]</sup>.

## MOLECULAR FACTORS REGULATING REPROGRAMMING

The core factors for reprogramming are Oct4, Nanog, Sox2, Klf4, c-Myc and Lin28. These genetic factors reprogram cells by regulating critical signalling pathways, epigenetic modifications and micro RNAs<sup>[114]</sup>.

### *Reprogramming by core transcription factors*

Oct4 is the best studied regulator of pluripotency. Oct4 expression is confined to early embryonic cells, germ line cells and cultured pluripotent stem cells, where it activates the gene transcription of stemness gene<sup>[120]</sup>. Oct4 protein cooperates with stemness factors such as Nanog and Sox2, but it also interacts with Polycomb group proteins<sup>[120]</sup>, which are important repressors of transcription. Sox2 is a transcription factor that acts as coactivator of Oct4<sup>[121]</sup>. Binding of Oct4/Sox2 dimers to the promoter sequences of *Oct4* and *Nanog* genes upregulate their transcription<sup>[122]</sup>. Nanog is a homeobox-containing transcription factor stabilizing the stemness network<sup>[122]</sup>. Klf4 is a zinc finger-containing transcription factor which regulates the expression of Oct4, Sox2 and Nanog<sup>[123-125]</sup>. Over-expression of Klf4 in ES cells increased the expression of Oct4 which further improve the self-renewal ability<sup>[126]</sup>. c-Myc enhances the efficiency and speed of reprogramming<sup>[127]</sup>. LIN28 promotes the expression of Oct4 at the posttranscriptional level by direct binding to its mRNA<sup>[128]</sup>. Recently, Glis1 has been identified as a substitute for c-Myc<sup>[129]</sup>. Glis1 transactivate the genes of Wnt ligands, Lin28a, Nanog, Myn, Mycl1, and Foxa2<sup>[129]</sup>.

The aspect of whether the species-specificity of reprogramming factors is relevant for proper reprogramming, is not well understood. In principle, the essential domains of the reprogramming factors are highly conserved between mammalian species, and several publications showed successful reprogramming with human and murine sequences in other species<sup>[5-13,130]</sup>.

## APPLICATIONS OF IPS CELLS

### *Modeling of human diseases and preclinical trials*

The potential applications of iPS cells will impact regenerative medicine, pharmaceutical industry, and animal biotechnology<sup>[131]</sup>. Human iPS cells could be utilized for curative treatments, to studying onset and disease progression *in vitro*, and to test potential therapeutic in high throughput screens<sup>[114,131,132]</sup>. The production of disease-specific iPS cells has found

widespread use in recent years<sup>[133-136]</sup>. Disease-specific iPS cells provide a unique resource to obtain a molecular understanding of disease onset and progression<sup>[131,132]</sup>. Induced PS-derived differentiated cells will allow to carry out *in vitro* drug screening (Figure 3), and to test therapeutic interventions<sup>[131]</sup>. In mice, Fanconi anemia and sickle cell anemia have been successfully corrected by using iPS cells<sup>[131,133-136]</sup>.

However with regard to potential curative treatments, the functionality, safety, and lack of tumorigenicity of iPS-derived cells have to be assessed in appropriate animal models bearing significant physiological and anatomical similarities to humans (Table 2). Hence, animal models could be contributed tremendously to a better understanding of disease mechanisms and therapeutic interventions. In addition, iPS cells from monkey<sup>[5]</sup>, porcine<sup>[41,26]</sup>, canine<sup>[8]</sup> and cattle<sup>[9]</sup> would be useful in animal biotechnology such as making precise genetic engineering for improved production traits and products<sup>[137,138]</sup>.

### *Advanced transgenesis in large mammals*

Transgenic farm animals can serve as excellent models of human diseases and during the past few years transgenic farm animals have gained renewed popularity. This is due to the availability of annotated genome depositories of the major domestic species and other organisms (for example: [www.ensembl.org](http://www.ensembl.org); or [www.ncbi.nlm.nih.gov/genome](http://www.ncbi.nlm.nih.gov/genome)), and due the introduction of active methods of transgenesis, which dramatically increased the success rates<sup>[42,43]</sup>. The repertoire of molecular tools now allows the precise modification of large mammalian genomes at rapid pace and has led to a recent boost in this area. The development of genuine iPS cells from domestic species will contribute to these advances and allow to perform desired genetic modifications *via* high throughput screens *in vitro*, and then use either SCNT<sup>[47]</sup> or blastocyst complementation for the generation of transgenic offspring (Figure 3). However at the moment most of the iPS cells cultures from different domestic species have not been tested for their capability to contribute to chimera formation, and only preliminary data are available<sup>[25,26]</sup>. Thus reinforced efforts to assess the potential of current livestock iPS cell lines for chimera contribution and germ cell differentiation are required. The majority of current livestock iPS cell lines are generated with retro- or lentiviral reprogramming approaches (Table 1), and the opportunities to assess alternative non-viral approaches are not widely assessed<sup>[10,56,106]</sup>. Also the potential of auxiliary small molecular inhibitors of stemness signaling pathway is not exploited for livestock iPS cells. Potentially, high throughput screens to identify small molecules with species-specific activity are required. It is anticipated that these approaches will lead to livestock iPS cells, which will make a significant impact for future genetic modifications of these species.



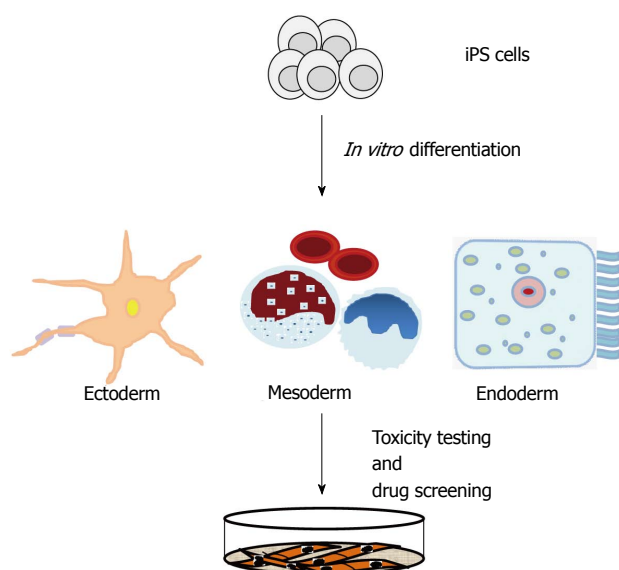


Figure 3 IPS cell technology contributes to disease modelling and drug discovery. iPS: Induced pluripotent stem.

### Preservation of genetic resources and endangered breeds

The iPS technology has the potential to preserve endangered animals and highly valuable genotypes in the near future<sup>[139]</sup>. Cryopreservation of cells and tissues is an important and useful approach for genetic preservation of valuable breeds and for conservation of endangered wild and domestic species. For highly endangered species, the derivation of iPS cells may become a method to prevent extinction. For example, iPS cells have been produced from endangered snow leopard<sup>[140]</sup>, drill and white rhinoceros<sup>[139]</sup>. The iPS cells generated can be easily expanded for banking of genetic material, or used as donor cells for SCNT. Potentially, iPS cells from endangered species may be differentiated into mature oocytes and spermatozoa (Figure 4), which might be employed for *in vitro* embryo production<sup>[139,140]</sup>. The differentiation of livestock iPS cells to functional gametes *in vitro* have not been achieved yet, however the current pace in developing fine-tuned protocols for *in vitro* differentiation of desired cell types, and the progress in inducing meiosis support the notion that the generation of fully functional spermatozoa and oocytes may be feasible. The possibility to obtain fully functional spermatozoa and oocytes from iPS cells of domestic and wild species would have far reaching consequences for maintenance of endangered species, as well as for breeding and genomic selection programs of domestic species. Even potential applications for infertility treatments in humans may become feasible<sup>[141,142]</sup>.

### PROSPECTS OF FARM ANIMAL IPS CELLS IN PRECLINICAL STUDIES

The generation of iPS cells has opened new vista to

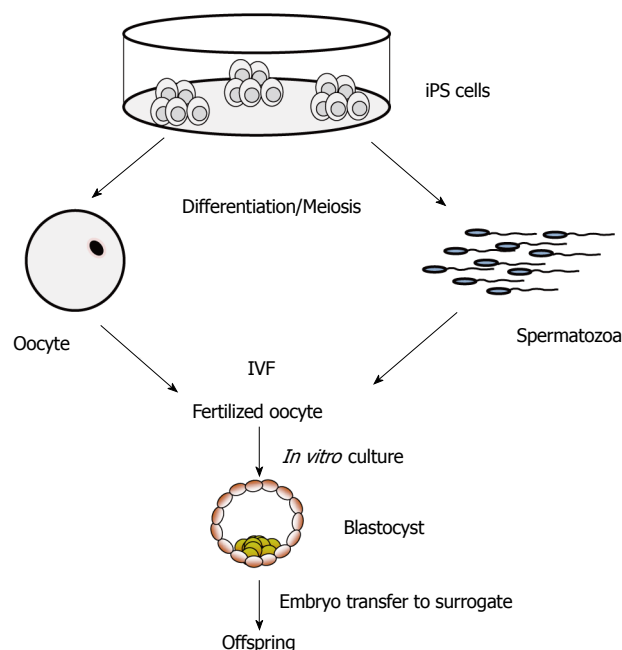


Figure 4 Application of induced pluripotent stem cells for *in vitro* generation of gametes. iPS: Induced pluripotent stem; IVF: *In vitro* fertilization.

understand pluripotency, disease onset and progression, and to develop regenerative medicine<sup>[132]</sup>. However, before the clinical application of iPS cell-derived therapies can be envisioned, the low efficiency and kinetics of iPS cell formation, the risks of insertional mutagenesis, reactivation of silenced ectopic transgenes and potential tumor formation have to be assessed and solved<sup>[131]</sup>. An important aspect is the biosafety of transplanted derivatives of iPS cells<sup>[132]</sup>. A number of reports showed that iPS cell lines could contain genetic mutations, copy number variations, and epigenetic mutations<sup>[132,143-145]</sup>. These aberrant changes may increase the tumorigenicity of iPS and iPS-derived cells. Retro- and lentiviruses are commonly used to introduce the reprogramming factors into differentiated cells, which can increase the immunogenicity<sup>[146]</sup>.

Farm animals represent informative model organisms, which seem to be suitable to assess obstacles and risks in longitudinal pre-clinical studies<sup>[147]</sup>. In contrast to rodent models, they are more similar to humans with respect to life-span, physiology, metabolism and pathophysiology<sup>[148,149]</sup>. Large mammalian models will allow to determine required cell doses to obtain therapeutic effects, to follow the fate of transplanted cells and their functional integration in the host tissue<sup>[150]</sup>. Thus the research on pluripotent stem cells from farm animals will contribute to the development of innovative cell therapies for human patients.

### REFERENCES

- 1 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined



- factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]
- 2 **Okita K**, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007; **448**: 313-317 [PMID: 17554338 DOI: 10.1038/nature05934]
  - 3 **Takahashi K**, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: 18035408 DOI: 10.1016/j.cell.2007.11.019]
  - 4 **Yu J**, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007; **318**: 1917-1920 [PMID: 18029452 DOI: 10.1126/science.1151526]
  - 5 **Liu H**, Zhu F, Yong J, Zhang P, Hou P, Li H, Jiang W, Cai J, Liu M, Cui K, Qu X, Xiang T, Lu D, Chi X, Gao G, Ji W, Ding M, Deng H. Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. *Cell Stem Cell* 2008; **3**: 587-590 [PMID: 19041774 DOI: 10.1016/j.stem.2008.10.014]
  - 6 **Liao J**, Cui C, Chen S, Ren J, Chen J, Gao Y, Li H, Jia N, Cheng L, Xiao H, Xiao L. Generation of induced pluripotent stem cell lines from adult rat cells. *Cell Stem Cell* 2009; **4**: 11-15 [PMID: 19097959 DOI: 10.1016/j.stem.2008.11.013]
  - 7 **Esteban MA**, Xu J, Yang J, Peng M, Qin D, Li W, Jiang Z, Chen J, Deng K, Zhong M, Cai J, Lai L, Pei D. Generation of induced pluripotent stem cell lines from Tibetan miniature pig. *J Biol Chem* 2009; **284**: 17634-17640 [PMID: 19376775 DOI: 10.1074/jbc.M109.008938]
  - 8 **Shimada H**, Nakada A, Hashimoto Y, Shigeno K, Shionoya Y, Nakamura T. Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. *Mol Reprod Dev* 2010; **77**: 2 [PMID: 19890968 DOI: 10.1002/mrd.21117]
  - 9 **Han X**, Han J, Ding F, Cao S, Lim SS, Dai Y, Zhang R, Zhang Y, Lim B, Li N. Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells. *Cell Res* 2011; **21**: 1509-1512 [PMID: 21826109 DOI: 10.1038/cr.2011.125]
  - 10 **Nagy K**, Sung HK, Zhang P, Laflamme S, Vincent P, Agha-Mohammadi S, Woltjen K, Monetti C, Michael IP, Smith LC, Nagy A. Induced pluripotent stem cell lines derived from equine fibroblasts. *Stem Cell Rev* 2011; **7**: 693-702 [PMID: 21347602 DOI: 10.1007/s12015-011-9239-5]
  - 11 **Bao L**, He L, Chen J, Wu Z, Liao J, Rao L, Ren J, Li H, Zhu H, Qian L, Gu Y, Dai H, Xu X, Zhou J, Wang W, Cui C, Xiao L. Reprogramming of ovine adult fibroblasts to pluripotency via drug-inducible expression of defined factors. *Cell Res* 2011; **21**: 600-608 [PMID: 21221129 DOI: 10.1038/cr.2011.6]
  - 12 **Ren J**, Pak Y, He L, Qian L, Gu Y, Li H, Rao L, Liao J, Cui C, Xu X, Zhou J, Ri H, Xiao L. Generation of hircine-induced pluripotent stem cells by somatic cell reprogramming. *Cell Res* 2011; **21**: 849-853 [PMID: 21403680 DOI: 10.1038/cr.2011.37]
  - 13 **Deng Y**, Liu Q, Luo C, Chen S, Li X, Wang C, Liu Z, Lei X, Zhang H, Sun H, Lu F, Jiang J, Shi D. Generation of induced pluripotent stem cells from buffalo (*Bubalus bubalis*) fetal fibroblasts with buffalo defined factors. *Stem Cells Dev* 2012; **21**: 2485-2494 [PMID: 22420535 DOI: 10.1089/scd.2012.0018]
  - 14 **Nowak-Imialek M**, Kues W, Carnwath JW, Niemann H. Pluripotent stem cells and reprogrammed cells in farm animals. *Microsc Microanal* 2011; **17**: 474-497 [PMID: 21682936 DOI: 10.1017/S1431927611000080]
  - 15 **Brevini TA**, Pennarossa G, Gandolfi F. No shortcuts to pig embryonic stem cells. *Theriogenology* 2010; **74**: 544-550 [PMID: 20570327 DOI: 10.1016/j.theriogenology.2010.04.020]
  - 16 **Lowry WE**, Richter L, Yachechko R, Pyle AD, Tchieu J, Sridharan R, Clark AT, Plath K. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci USA* 2008; **105**: 2883-2888 [PMID: 18287077 DOI: 10.1073/pnas.0711983105]
  - 17 **Stadtfeld M**, Brennand K, Hochedlinger K. Reprogramming of pancreatic beta cells into induced pluripotent stem cells. *Curr Biol* 2008; **18**: 890-894 [PMID: 18501604 DOI: 10.1016/j.cub.2008.05.010]
  - 18 **Hanna J**, Markoulaki S, Schorderet P, Carey BW, Beard C, Wernig M, Creyghton MP, Steine EJ, Cassady JP, Foreman R, Lengner CJ, Dausman JA, Jaenisch R. Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. *Cell* 2008; **133**: 250-264 [PMID: 18423197 DOI: 10.1016/j.cell.2008.03.028]
  - 19 **Aoi T**, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, Yamanaka S. Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science* 2008; **321**: 699-702 [PMID: 18276851 DOI: 10.1126/science.1154884]
  - 20 **Aasen T**, Raya A, Barrero MJ, Garreta E, Consiglio A, Gonzalez F, Vassena R, Bilić J, Pekarik V, Tiscornia G, Edel M, Boué S, Izpisua Belmonte JC. Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol* 2008; **26**: 1276-1284 [PMID: 18931654 DOI: 10.1038/nbt.1503]
  - 21 **Kim JB**, Greber B, Araúzo-Bravo MJ, Meyer J, Park KI, Zaehres H, Schöler HR. Direct reprogramming of human neural stem cells by OCT4. *Nature* 2009; **461**: 649-643 [PMID: 19718018 DOI: 10.1038/nature08436]
  - 22 **Takenaka C**, Nishishita N, Takada N, Jakt LM, Kawamata S. Effective generation of iPS cells from CD34+ cord blood cells by inhibition of p53. *Exp Hematol* 2010; **38**: 154-162 [PMID: 19922768 DOI: 10.1016/j.exphem.2009.11.003]
  - 23 **Daley GQ**, Lensch MW, Jaenisch R, Meissner A, Plath K, Yamanaka S. Broader implications of defining standards for the pluripotency of iPSCs. *Cell Stem Cell* 2009; **4**: 200-201; author reply 202 [PMID: 19265657 DOI: 10.1016/j.stem.2009.02.009]
  - 24 **Ellis J**, Bruneau BG, Keller G, Lemischka IR, Nagy A, Rossant J, Srivastava D, Zandstra PW, Stanford WL. Alternative induced pluripotent stem cell characterization criteria for in vitro applications. *Cell Stem Cell* 2009; **4**: 198-199; author reply 202 [PMID: 19265656 DOI: 10.1016/j.stem.2009.02.010]
  - 25 **Sartori C**, DiDomenico AI, Thomson AJ, Milne E, Lillico SG, Burdon TG, Whitelaw CB. Ovine-induced pluripotent stem cells can contribute to chimeric lambs. *Cell Reprogram* 2012; **14**: 8-19 [PMID: 22217199 DOI: 10.1089/cell.2011.0050]
  - 26 **West FD**, Uhl EW, Liu Y, Stowe H, Lu Y, Yu P, Gallegos-Cardenas A, Pratt SL, Stice SL. Brief report: chimeric pigs produced from induced pluripotent stem cells demonstrate germline transmission and no evidence of tumor formation in young pigs. *Stem Cells* 2011; **29**: 1640-1643 [PMID: 22039609 DOI: 10.1002/stem.713]
  - 27 **Pawlak M**, Jaenisch R. De novo DNA methylation by Dnmt3a and Dnmt3b is dispensable for nuclear reprogramming of somatic cells to a pluripotent state. *Genes Dev* 2011; **25**: 1035-1040 [PMID: 21576263 DOI: 10.1101/gad.2039011]
  - 28 **Xu YN**, Guan N, Wang ZD, Shan ZY, Shen JL, Zhang QH, Jin LH, Lei L. ES cell extract-induced expression of pluripotent factors in somatic cells. *Anat Rec (Hoboken)* 2009; **292**: 1229-1234 [PMID: 19645026 DOI: 10.1002/ar.20919]
  - 29 **Miyamoto K**, Tsukiyama T, Yang Y, Li N, Minami N, Yamada M, Imai H. Cell-free extracts from mammalian oocytes partially induce nuclear reprogramming in somatic cells. *Biol Reprod* 2009; **80**: 935-943 [PMID: 19164171 DOI: 10.1095/biolreprod.108.073676]
  - 30 **Silva J**, Chambers I, Pollard S, Smith A. Nanog promotes transfer of pluripotency after cell fusion. *Nature* 2006; **441**: 997-1001 [PMID: 16791199 DOI: 10.1038/nature04914]
  - 31 **Tada M**, Takahama Y, Abe K, Nakatsuji N, Tada T. Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Curr Biol* 2001; **11**: 1553-1558 [PMID: 11591326 DOI: 10.1016/S0960-9822(01)00459-6]
  - 32 **Ying QL**, Nichols J, Evans EP, Smith AG. Changing potency by spontaneous fusion. *Nature* 2002; **416**: 545-548 [PMID: 11932748 DOI: 10.1038/nature729]
  - 33 **Waddington CH**. The Strategy of the genes. A discussion of some aspects of theoretical biology. London: Allen and Unwin, 1957
  - 34 **Gurdon JB**. The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *J Embryol Exp*



- Morphol* 1962; **10**: 622-640 [PMID: 13951335]
- 35 **Wilmot I**, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; **385**: 810-813 [PMID: 9039911 DOI: 10.1038/385810a0]
  - 36 **Schneuwly S**, Klemenz R, Gehring WJ. Redesigning the body plan of *Drosophila* by ectopic expression of the homoeotic gene Antennapedia. *Nature* 1987; **325**: 816-818 [PMID: 3821869 DOI: 10.1038/325816a0]
  - 37 **Davis RL**, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 1987; **51**: 987-1000 [PMID: 3690668 DOI: 10.1016/0092-8674(87)90585-X]
  - 38 **Kawamura M**, Miyagawa S, Miki K, Saito A, Fukushima S, Higuchi T, Kawamura T, Kuratani T, Daimon T, Shimizu T, Okano T, Sawa Y. Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. *Circulation* 2012; **126**: S29-S37 [PMID: 22965990]
  - 39 **Kawamura M**, Miyagawa S, Fukushima S, Saito A, Miki K, Ito E, Sougawa N, Kawamura T, Daimon T, Shimizu T, Okano T, Toda K, Sawa Y. Enhanced survival of transplanted human induced pluripotent stem cell-derived cardiomyocytes by the combination of cell sheets with the pedicled omental flap technique in a porcine heart. *Circulation* 2013; **128**: S87-S94 [PMID: 24030425 DOI: 10.1161/CIRCULATIONAHA.112.000366]
  - 40 **Zhang F**, Song G, Li X, Gu W, Shen Y, Chen M, Yang B, Qian L, Cao K. Transplantation of iPSc ameliorates neural remodeling and reduces ventricular arrhythmias in a post-infarcted swine model. *J Cell Biochem* 2014; **115**: 531-539 [PMID: 24122925 DOI: 10.1002/jcb.24687]
  - 41 **Mizukami Y**, Abe T, Shibata H, Makimura Y, Fujishiro SH, Yanase K, Hishikawa S, Kobayashi E, Hanazono Y. MHC-matched induced pluripotent stem cells can attenuate cellular and humoral immune responses but are still susceptible to innate immunity in pigs. *PLoS One* 2014; **9**: e98319 [PMID: 24927426 DOI: 10.1371/journal.pone.0098319]
  - 42 **Garrels W**, Ivics Z, Kues WA. Precision genetic engineering in large mammals. *Trends Biotechnol* 2012; **30**: 386-393 [PMID: 22521716 DOI: 10.1016/j.tibtech.2012.03.008]
  - 43 **Le Provost F**, Lillico S, Passet B, Young R, Whitelaw B, Vilotte JL. Zinc finger nuclease technology heralds a new era in mammalian transgenesis. *Trends Biotechnol* 2010; **28**: 134-141 [PMID: 20015561 DOI: 10.1016/j.tibtech.2009.11.007]
  - 44 **Matsushita H**, Sano A, Wu H, Jiao JA, Kasinathan P, Sullivan EJ, Wang Z, Kuroiwa Y. Triple immunoglobulin gene knockout transchromosomal cattle: bovine lambda cluster deletion and its effect on fully human polyclonal antibody production. *PLoS One* 2014; **9**: e90383 [PMID: 24603704 DOI: 10.1371/journal.pone.0090383]
  - 45 **Yu Y**, Wang Y, Tong Q, Liu X, Su F, Quan F, Guo Z, Zhang Y. A site-specific recombinase-based method to produce antibiotic selectable marker free transgenic cattle. *PLoS One* 2013; **8**: e62457 [PMID: 23658729 DOI: 10.1371/journal.pone.0062457]
  - 46 **Ivics Z**, Garrels W, Mátés L, Yau TY, Bashir S, Zidek V, Landa V, Geurts A, Pravenec M, Rülicke T, Kues WA, Izsvák Z. Germline transgenesis in pigs by cytoplasmic microinjection of Sleeping Beauty transposons. *Nat Protoc* 2014; **9**: 810-827 [PMID: 24625780 DOI: 10.1038/nprot.2014.010]
  - 47 **Fan N**, Chen J, Shang Z, Dou H, Ji G, Zou Q, Wu L, He L, Wang F, Liu K, Liu N, Han J, Zhou Q, Pan D, Yang D, Zhao B, Ouyang Z, Liu Z, Zhao Y, Lin L, Zhong C, Wang Q, Wang S, Xu Y, Luan J, Liang Y, Yang Z, Li J, Lu C, Vajta G, Li Z, Ouyang H, Wang H, Wang Y, Yang Y, Liu Z, Wei H, Luan Z, Esteban MA, Deng H, Yang H, Pei D, Li N, Pei G, Liu L, Du Y, Xiao L, Lai L. Piglets cloned from induced pluripotent stem cells. *Cell Res* 2013; **23**: 162-166 [PMID: 23247628 DOI: 10.1038/cr.2012.176]
  - 48 **Lee K**, Kwon DN, Ezashi T, Choi YJ, Park C, Ericsson AC, Brown AN, Samuel MS, Park KW, Walters EM, Kim DY, Kim JH, Franklin CL, Murphy CN, Roberts RM, Prather RS, Kim JH. Engraftment of human iPS cells and allogeneic porcine cells into pigs with inactivated RAG2 and accompanying severe combined immunodeficiency. *Proc Natl Acad Sci USA* 2014; **111**: 7260-7265 [PMID: 24799706 DOI: 10.1073/pnas.1406376111]
  - 49 **Cao H**, Yang P, Pu Y, Sun X, Yin H, Zhang Y, Zhang Y, Li Y, Liu Y, Fang F, Zhang Z, Tao Y, Zhang X. Characterization of bovine induced pluripotent stem cells by lentiviral transduction of reprogramming factor fusion proteins. *Int J Biol Sci* 2012; **8**: 498-511 [PMID: 22457605 DOI: 10.7150/ijbs.3723]
  - 50 **Hu PF**, Guan WJ, Li XC, Ma YH. Construction of recombinant proteins for reprogramming of endangered Luxi cattle fibroblast cells. *Mol Biol Rep* 2012; **39**: 7175-7182 [PMID: 22311040 DOI: 10.1007/s11033-012-1549-4]
  - 51 **Malaver-Ortega LF**, Sumer H, Liu J, Verma PJ. The state of the art for pluripotent stem cells derivation in domestic ungulates. *Theriogenology* 2012; **78**: 1749-1762 [PMID: 22578625 DOI: 10.1016/j.theriogenology.2012.03.031]
  - 52 **Sumer H**, Liu J, Malaver-Ortega LF, Lim ML, Khodadadi K, Verma PJ. NANOG is a key factor for induction of pluripotency in bovine adult fibroblasts. *J Anim Sci* 2011; **89**: 2708-2716 [PMID: 21478453 DOI: 10.2527/jas.2010-3666]
  - 53 **Huang B**, Li T, Alonso-Gonzalez L, Gorre R, Keatley S, Green A, Turner P, Kallingappa PK, Verma V, Obach B. A virus-free poly-promoter vector induces pluripotency in quiescent bovine cells under chemically defined conditions of dual kinase inhibition. *PLoS One* 2011; **6**: e24501 [PMID: 21912700 DOI: 10.1371/journal.pone.0024501]
  - 54 **Koh S**, Thomas R, Tsai S, Bischoff S, Lim JH, Breen M, Olby NJ, Piedrahita JA. Growth requirements and chromosomal instability of induced pluripotent stem cells generated from adult canine fibroblasts. *Stem Cells Dev* 2013; **22**: 951-963 [PMID: 23016947 DOI: 10.1089/scd.2012.0393]
  - 55 **Whitworth DJ**, Ovchinnikov DA, Wolvetang EJ. Generation and characterization of LIF-dependent canine induced pluripotent stem cells from adult dermal fibroblasts. *Stem Cells Dev* 2012; **21**: 2288-2297 [PMID: 22221227 DOI: 10.1089/scd.2011.0608]
  - 56 **Luo J**, Suhr ST, Chang EA, Wang K, Ross PJ, Nelson LL, Venta PJ, Knott JG, Cibelli JB. Generation of leukemia inhibitory factor and basic fibroblast growth factor-dependent induced pluripotent stem cells from canine adult somatic cells. *Stem Cells Dev* 2011; **20**: 1669-1678 [PMID: 21495906 DOI: 10.1089/scd.2011.0127]
  - 57 **Song H**, Li H, Huang M, Xu D, Gu C, Wang Z, Dong F, Wang F. Induced pluripotent stem cells from goat fibroblasts. *Mol Reprod Dev* 2013; **80**: 1009-1017 [PMID: 24123501 DOI: 10.1002/mrd.22266]
  - 58 **Donadeu FX**. Equine induced pluripotent stem cells or how to turn skin cells into neurons: horse tissues a la carte? *Equine Vet J* 2014; **46**: 534-537 [PMID: 25099189 DOI: 10.1111/evj.12300]
  - 59 **Hackett CH**, Greve L, Novakofski KD, Fortier LA. Comparison of gene-specific DNA methylation patterns in equine induced pluripotent stem cell lines with cells derived from equine adult and fetal tissues. *Stem Cells Dev* 2012; **21**: 1803-1811 [PMID: 21988203 DOI: 10.1089/scd.2011.0055]
  - 60 **Breton A**, Sharma R, Diaz AC, Parham AG, Graham A, Neil C, Whitelaw CB, Milne E, Donadeu FX. Derivation and characterization of induced pluripotent stem cells from equine fibroblasts. *Stem Cells Dev* 2013; **22**: 611-621 [PMID: 22897112 DOI: 10.1089/scd.2012.0052]
  - 61 **Whitworth DJ**, Ovchinnikov DA, Sun J, Fortuna PR, Wolvetang EJ. Generation and characterization of leukemia inhibitory factor-dependent equine induced pluripotent stem cells from adult dermal fibroblasts. *Stem Cells Dev* 2014; **23**: 1515-1523 [PMID: 24555755]
  - 62 **Sharma R**, Livesey MR, Wyllie DJ, Proudfoot C, Whitelaw CB, Hay DC, Donadeu FX. Generation of functional neurons from feeder-free, keratinocyte-derived equine induced pluripotent stem cells. *Stem Cells Dev* 2014; **23**: 1524-1534 [PMID: 24548115]
  - 63 **Wu Z**, Chen J, Ren J, Bao L, Liao J, Cui C, Rao L, Li H, Gu Y, Dai H, Zhu H, Teng X, Cheng L, Xiao L. Generation of pig induced



- pluripotent stem cells with a drug-inducible system. *J Mol Cell Biol* 2009; **1**: 46-54 [PMID: 19502222 DOI: 10.1093/jmcb/mjp003]
- 64 **Ruan W**, Han J, Li P, Cao S, An Y, Lim B, Li N. A novel strategy to derive iPS cells from porcine fibroblasts. *Sci China Life Sci* 2011; **54**: 553-559 [PMID: 21706416 DOI: 10.1007/s11427-011-4179-5]
  - 65 **Ezashi T**, Matsuyama H, Telugu BP, Roberts RM. Generation of colonies of induced trophoblast cells during standard reprogramming of porcine fibroblasts to induced pluripotent stem cells. *Biol Reprod* 2011; **85**: 779-787 [PMID: 21734265]
  - 66 **Cheng D**, Guo Y, Li Z, Liu Y, Gao X, Gao Y, Cheng X, Hu J, Wang H. Porcine induced pluripotent stem cells require LIF and maintain their developmental potential in early stage of embryos. *PLoS One* 2012; **7**: e51778 [PMID: 23251622 DOI: 10.1371/journal.pone.0051778]
  - 67 **Lahm H**, Doppler S, Dreßen M, Werner A, Adamczyk K, Schrambke D, Brade T, Laugwitz KL, Deutsch MA, Schiemann M, Lange R, Moretti A, Krane M. Live fluorescent RNA-based detection of pluripotency gene expression in embryonic and induced pluripotent cells of different species. *Stem Cells* 2015; **33**: 392-402 [PMID: 25335772 DOI: 10.1002/stem.1872.]
  - 68 **Liu K**, Ji G, Mao J, Liu M, Wang L, Chen C, Liu L. Generation of porcine-induced pluripotent stem cells by using OCT4 and KLF4 porcine factors. *Cell Reprogram* 2012; **14**: 505-513 [PMID: 23035653 DOI: 10.1089/cell.2012.0047]
  - 69 **Hall VJ**, Kristensen M, Rasmussen MA, Ujhelly O, Dinnyés A, Hyttel P. Temporal repression of endogenous pluripotency genes during reprogramming of porcine induced pluripotent stem cells. *Cell Reprogram* 2012; **14**: 204-216 [PMID: 22578162 DOI: 10.1089/cell.2011.0089]
  - 70 **Ezashi T**, Telugu BP, Alexenko AP, Sachdev S, Sinha S, Roberts RM. Derivation of induced pluripotent stem cells from pig somatic cells. *Proc Natl Acad Sci USA* 2009; **106**: 10993-10998 [PMID: 19541600 DOI: 10.1073/pnas.0905284106]
  - 71 **West FD**, Terlouw SL, Kwon DJ, Mumaw JL, Dhara SK, Hasneen K, Dobrinsky JR, Stice SL. Porcine induced pluripotent stem cells produce chimeric offspring. *Stem Cells Dev* 2010; **19**: 1211-1220 [PMID: 20380514 DOI: 10.1089/scd.2009.0458]
  - 72 **Osteil P**, Taponnier Y, Markossian S, Godet M, Schmaltz-Panneau B, Jounneau L, Cabau C, Joly T, Blachère T, Gócza E, Bernat A, Yerle M, Acloque H, Hidot S, Bosze Z, Duranthon V, Savatier P, Afanassieff M. Induced pluripotent stem cells derived from rabbits exhibit some characteristics of naïve pluripotency. *Biol Open* 2013; **2**: 613-628 [PMID: 23789112 DOI: 10.1242/bio.20134242.]
  - 73 **Tancos Z**, Nemes C, Polgar Z, Gocza E, Daniel N, Stout TA, Maraghechi P, Pirity MK, Osteil P, Taponnier Y, Markossian S, Godet M, Afanassieff M, Bosze Z, Duranthon V, Savatier P, Dinnyes A. Generation of rabbit pluripotent stem cell lines. *Theriogenology* 2012; **78**: 1774-1786 [PMID: 22925641 DOI: 10.1016/j.theriogenol.2012.06.017]
  - 74 **Honda A**, Hirose M, Hatori M, Matoba S, Miyoshi H, Inoue K, Ogura A. Generation of induced pluripotent stem cells in rabbits: potential experimental models for human regenerative medicine. *J Biol Chem* 2010; **285**: 31362-31369 [PMID: 20670936 DOI: 10.1074/jbc.M110.150540]
  - 75 **Li Y**, Cang M, Lee AS, Zhang K, Liu D. Reprogramming of sheep fibroblasts into pluripotency under a drug-inducible expression of mouse-derived defined factors. *PLoS One* 2011; **6**: e15947 [PMID: 21253598 DOI: 10.1371/journal.pone.0015947]
  - 76 **Liu J**, Balehosur D, Murray B, Kelly JM, Sumer H, Verma PJ. Generation and characterization of reprogrammed sheep induced pluripotent stem cells. *Theriogenology* 2012; **77**: 338-46.e1 [PMID: 21958637 DOI: 10.1016/j.theriogenology.2011.08.006]
  - 77 **Amirache F**, Lévy C, Costa C, Mangeot PE, Torbett BE, Wang CX, Nègre D, Cosset FL, Verhoeven E. Mystery solved: VSV-G-LVs do not allow efficient gene transfer into unstimulated T cells, B cells, and HSCs because they lack the LDL receptor. *Blood* 2014; **123**: 1422-1424 [PMID: 24578496 DOI: 10.1182/blood-2013-11-540641]
  - 78 **Hacein-Bey-Abina S**, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, Sorensen R, Forster A, Fraser P, Cohen JL, de Saint Basile G, Alexander I, Wintergerst U, Frebourg T, Aurias A, Stoppa-Lyonnet D, Romana S, Radford-Weiss I, Gross F, Valensi F, Delabesse E, Macintyre E, Sigaux F, Soulier J, Leiva LE, Wissler M, Prinz C, Rabbitts TH, Le Deist F, Fischer A, Cavazzana-Calvo M. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 2003; **302**: 415-419 [PMID: 14564000 DOI: 10.1126/science.1088547]
  - 79 **Nakagawa M**, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* 2008; **26**: 101-106 [PMID: 18059259 DOI: 10.1038/nbt1374]
  - 80 **Zhao T**, Zhang ZN, Rong Z, Xu Y. Immunogenicity of induced pluripotent stem cells. *Nature* 2011; **474**: 212-215 [PMID: 21572395 DOI: 10.1038/nature10135]
  - 81 **Kane NM**, Nowrouzi A, Mukherjee S, Blundell MP, Greig JA, Lee WK, Houslay MD, Milligan G, Mountford JC, von Kalle C, Schmidt M, Thrasher AJ, Baker AH. Lentivirus-mediated reprogramming of somatic cells in the absence of transgenic transcription factors. *Mol Ther* 2010; **18**: 2139-2145 [PMID: 20978477 DOI: 10.1038/mt.2010.231]
  - 82 **Tashiro K**, Inamura M, Kawabata K, Sakurai F, Yamanishi K, Hayakawa T, Mizuguchi H. Efficient adipocyte and osteoblast differentiation from mouse induced pluripotent stem cells by adenoviral transduction. *Stem Cells* 2009; **27**: 1802-1811 [PMID: 19544436 DOI: 10.1002/stem.108]
  - 83 **Fusaki N**, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci* 2009; **85**: 348-362 [PMID: 19838014 DOI: 10.2183/pjab.85.348]
  - 84 **Yu J**, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, Thomson JA. Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 2009; **324**: 797-801 [PMID: 19325077 DOI: 10.1126/science.1172482]
  - 85 **Jia F**, Wilson KD, Sun N, Gupta DM, Huang M, Li Z, Panetta NJ, Chen ZY, Robbins RC, Kay MA, Longaker MT, Wu JC. A nonviral minicircle vector for deriving human iPS cells. *Nat Methods* 2010; **7**: 197-199 [PMID: 20139967 DOI: 10.1038/nmeth.1426]
  - 86 **Okita K**, Hong H, Takahashi K, Yamanaka S. Generation of mouse-induced pluripotent stem cells with plasmid vectors. *Nat Protoc* 2010; **5**: 418-428 [PMID: 20203661 DOI: 10.1038/nprot.2009.231]
  - 87 **Despots C**, Ding S. Using small molecules to improve generation of induced pluripotent stem cells from somatic cells. *Methods Mol Biol* 2010; **636**: 207-218 [PMID: 20336525]
  - 88 **Warren L**, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schläeger TM, Rossi DJ. Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 2010; **7**: 618-630 [PMID: 20888316 DOI: 10.1016/j.stem.2010.08.012]
  - 89 **Kim D**, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, Cha KY, Lanza R, Kim KS. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* 2009; **4**: 472-476 [PMID: 19481515 DOI: 10.1016/j.stem.2009.05.005]
  - 90 **Woltjen K**, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hämmäläinen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 2009; **458**: 766-770 [PMID: 19252478 DOI: 10.1038/nature07863]
  - 91 **Kues WA**, Herrmann D, Barg-Kues B, Haridoss S, Nowak-Imialek M, Buchholz T, Streeck M, Grebe A, Grabundzija I, Merkert S, Martin U, Hall VJ, Rasmussen MA, Ivics Z, Hyttel P, Niemann H. Derivation and characterization of sleeping beauty transposon-



- mediated porcine induced pluripotent stem cells. *Stem Cells Dev* 2013; **22**: 124-135 [PMID: 22989381 DOI: 10.1089/scd.2012.0382]
- 92 **Chabot S**, Orio J, Schmeer M, Schleef M, Golzio M, Teissie J. Minicircle DNA electrotransfer for efficient tissue-targeted gene delivery. *Gene Ther* 2013; **20**: 62-68 [PMID: 22257936 DOI: 10.1038/gt.2011.215]
- 93 **Yoshida Y**, Takahashi K, Okita K, Ichisaka T, Yamanaka S. Hypoxia enhances the generation of induced pluripotent stem cells. *Cell Stem Cell* 2009; **5**: 237-241 [PMID: 19716359 DOI: 10.1016/j.stem.2009.08.001]
- 94 **Shi Y**, Do JT, Despons C, Hahm HS, Schöler HR, Ding S. A combined chemical and genetic approach for the generation of induced pluripotent stem cells. *Cell Stem Cell* 2008; **2**: 525-528 [PMID: 18522845 DOI: 10.1016/j.stem.2008.05.011]
- 95 **Ichida JK**, Blanchard J, Lam K, Son EY, Chung JE, Egli D, Loh KM, Carter AC, Di Giorgio FP, Koszka K, Huangfu D, Akutsu H, Liu DR, Rubin LL, Eggan K. A small-molecule inhibitor of tgfbeta signaling replaces sox2 in reprogramming by inducing nanog. *Cell Stem Cell* 2009; **5**: 491-503 [PMID: 19818703 DOI: 10.1016/j.stem.2009.09.012]
- 96 **Lee CH**, Kim JH, Lee HJ, Jeon K, Lim H, Choi Hy, Lee ER, Park SH, Park JY, Hong S, Kim S, Cho SG. The generation of iPS cells using non-viral magnetic nanoparticle based transfection. *Biomaterials* 2011; **32**: 6683-6691 [PMID: 21683440 DOI: 10.1016/j.biomaterials.2011.05.070]
- 97 **Jung DW**, Kim WH, Williams DR. Reprogram or reboot: small molecule approaches for the production of induced pluripotent stem cells and direct cell reprogramming. *ACS Chem Biol* 2014; **9**: 80-95 [PMID: 24245936 DOI: 10.1021/cb400754f]
- 98 **Woltjen K**, Hämläinen R, Kibschull M, Mileikovsky M, Nagy A. Transgene-free production of pluripotent stem cells using piggyBac transposons. *Methods Mol Biol* 2011; **767**: 87-103 [PMID: 21822869 DOI: 10.1007/978-1-61779-201-4\_7]
- 99 **Grabundzija I**, Wang J, Sebe A, Erdei Z, Kajdi R, Devaraj A, Steinemann D, Suzhai K, Stein U, Cantz T, Schambach A, Baum C, Izsvák Z, Sarkadi B, Ivics Z. Sleeping Beauty transposon-based system for cellular reprogramming and targeted gene insertion in induced pluripotent stem cells. *Nucleic Acids Res* 2013; **41**: 1829-1847 [PMID: 23275558 DOI: 10.1093/nar/gks1305]
- 100 **Davis RP**, Nemes C, Varga E, Freund C, Kosmidis G, Gkatzis K, de Jong D, Szuhai K, Dinnyés A, Mummery CL. Generation of induced pluripotent stem cells from human foetal fibroblasts using the Sleeping Beauty transposon gene delivery system. *Differentiation* 2013; **86**: 30-37 [PMID: 23933400 DOI: 10.1016/j.diff.2013.06.002]
- 101 **Mo X**, Li N, Wu S. Generation and characterization of bat-induced pluripotent stem cells. *Theriogenology* 2014; **82**: 283-293 [PMID: 24853281 DOI: 10.1016/j.theriogenology.2014.04.001]
- 102 **Tsukiyama T**, Kato-Itoh M, Nakauchi H, Ohinata Y. A comprehensive system for generation and evaluation of induced pluripotent stem cells using piggyBac transposition. *PLoS One* 2014; **9**: e92973 [PMID: 24667806 DOI: 10.1371/journal.pone.0092973]
- 103 **Talluri TR**, Kumar D, Glage S, Garrels W, Ivics Z, Debowski K, Behr R, Kues WA. Non-viral reprogramming of fibroblasts into induced pluripotent stem cells by Sleeping Beauty and piggyBac transposons. *Biochem Biophys Res Commun* 2014; **450**: 581-587 [PMID: 24928388 DOI: 10.1016/j.bbrc.2014.06.014]
- 104 **Szymczak AL**, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat Biotechnol* 2004; **22**: 589-594 [PMID: 15064769 DOI: 10.1038/nbt957]
- 105 **Yusa K**, Rad R, Takeda J, Bradley A. Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods* 2009; **6**: 363-369 [PMID: 19337237 DOI: 10.1038/nmeth.1323]
- 106 **Kaji K**, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 2009; **458**: 771-775 [PMID: 19252477 DOI: 10.1038/nature07864]
- 107 **Zhou H**, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Schöler HR, Duan L, Ding S. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 2009; **4**: 381-384 [PMID: 19398399 DOI: 10.1016/j.stem.2009.04.005]
- 108 **Plews JR**, Li J, Jones M, Moore HD, Mason C, Andrews PW, Na J. Activation of pluripotency genes in human fibroblast cells by a novel mRNA based approach. *PLoS One* 2010; **5**: e14397 [PMID: 21209933 DOI: 10.1371/journal.pone.0014397]
- 109 **Yoshioka N**, Gros E, Li HR, Kumar S, Deacon DC, Maron C, Muotri AR, Chi NC, Fu XD, Yu BD, Dowdy SF. Efficient generation of human iPSCs by a synthetic self-replicative RNA. *Cell Stem Cell* 2013; **13**: 246-254 [PMID: 23910086 DOI: 10.1016/j.stem.2013.06.001]
- 110 **Card DA**, Hebbbar PB, Li L, Trotter KW, Komatsu Y, Mishina Y, Archer TK. Oct4/Sox2-regulated miR-302 targets cyclin D1 in human embryonic stem cells. *Mol Cell Biol* 2008; **28**: 6426-6438 [PMID: 18710938 DOI: 10.1128/MCB.00359-08]
- 111 **Suh MR**, Lee Y, Kim JY, Kim SK, Moon SH, Lee JY, Cha KY, Chung HM, Yoon HS, Moon SY, Kim VN, Kim KS. Human embryonic stem cells express a unique set of microRNAs. *Dev Biol* 2004; **270**: 488-498 [PMID: 15183728 DOI: 10.1016/j.ydbio.2004.02.019]
- 112 **Lin SL**, Chang DC, Chang-Lin S, Lin CH, Wu DT, Chen DT, Ying SY. Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. *RNA* 2008; **14**: 2115-2124 [PMID: 18755840 DOI: 10.1261/rna.1162708]
- 113 **Anokye-Danso F**, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y, Zhang Y, Yang W, Gruber PJ, Epstein JA, Morrissey EE. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* 2011; **8**: 376-388 [PMID: 21474102 DOI: 10.1016/j.stem.2011.03.001]
- 114 **Miyoshi N**, Ishii H, Nagano H, Haraguchi N, Dewi DL, Kano Y, Nishikawa S, Tanemura M, Mimori K, Tanaka F, Saito T, Nishimura J, Takemasa I, Mizushima T, Ikeda M, Yamamoto H, Sekimoto M, Doki Y, Mori M. Reprogramming of mouse and human cells to pluripotency using mature microRNAs. *Cell Stem Cell* 2011; **8**: 633-638 [PMID: 21620789 DOI: 10.1016/j.stem.2011.05.001]
- 115 **Gonçalves NN**, Ambrósio CE, Piedrahita JA. Stem cells and regenerative medicine in domestic and companion animals: a multispecies perspective. *Reprod Domest Anim* 2014; **49** Suppl 4: 2-10 [PMID: 25277427 DOI: 10.1111/rda.12392]
- 116 **Worringer KA**, Rand TA, Hayashi Y, Sami S, Takahashi K, Tanabe K, Narita M, Srivastava D, Yamanaka S. The let-7/LIN-41 pathway regulates reprogramming to human induced pluripotent stem cells by controlling expression of prodifferentiation genes. *Cell Stem Cell* 2014; **14**: 40-52 [PMID: 24239284 DOI: 10.1016/j.stem.2013.11.001]
- 117 **Zhang Z**, Xiang D, Heriyanto F, Gao Y, Qian Z, Wu WS. Dissecting the roles of miR-302/367 cluster in cellular reprogramming using TALE-based repressor and TALEN. *Stem Cell Reports* 2013; **1**: 218-225 [PMID: 24319658 DOI: 10.1016/j.stemcr.2013.07.002]
- 118 **Ma K**, Song G, An X, Fan A, Tan W, Tang B, Zhang X, Li Z. miRNAs promote generation of porcine-induced pluripotent stem cells. *Mol Cell Biochem* 2014; **389**: 209-218 [PMID: 24464032 DOI: 10.1007/s11010-013-1942-x]
- 119 **Heng BC**, Fussenegger M. Integration-free reprogramming of human somatic cells to induced pluripotent stem cells (iPSCs) without viral vectors, recombinant DNA, and genetic modification. *Methods Mol Biol* 2014; **1151**: 75-94 [PMID: 24838880 DOI: 10.1007/978-1-4939-0554-6\_6]
- 120 **Wang X**, Dai J. Concise review: isoforms of OCT4 contribute to the confusing diversity in stem cell biology. *Stem Cells* 2010; **28**: 885-893 [PMID: 20333750 DOI: 10.1002/stem.419]
- 121 **Chew JL**, Loh YH, Zhang W, Chen X, Tam WL, Yeap LS, Li P,



- Ang YS, Lim B, Robson P, Ng HH. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol Cell Biol* 2005; **25**: 6031-6046 [PMID: 15988017 DOI: 10.1128/MCB.25.14.6031-6046.2005]
- 122 **Masui S**, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA, Ko MS, Niwa H. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol* 2007; **9**: 625-635 [PMID: 17515932 DOI: 10.1038/ncb1589]
  - 123 **Chambers I**, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 2003; **113**: 643-655 [PMID: 12787505 DOI: 10.1016/S0092-8674(03)00392-1]
  - 124 **Kim J**, Chu J, Shen X, Wang J, Orkin SH. An extended transcriptional network for pluripotency of embryonic stem cells. *Cell* 2008; **132**: 1049-1061 [PMID: 18358816 DOI: 10.1016/j.cell.2008.02.039]
  - 125 **Niwa H**, Ogawa K, Shimosato D, Adachi K. A parallel circuit of LIF signalling pathways maintains pluripotency of mouse ES cells. *Nature* 2009; **460**: 118-122 [PMID: 19571885 DOI: 10.1038/nature08113]
  - 126 **Li Y**, McClintick J, Zhong L, Edenberg HJ, Yoder MC, Chan RJ. Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. *Blood* 2005; **105**: 635-637 [PMID: 15358627]
  - 127 **Nakagawa M**, Takizawa N, Narita M, Ichisaka T, Yamanaka S. Promotion of direct reprogramming by transformation-deficient Myc. *Proc Natl Acad Sci USA* 2010; **107**: 14152-14157 [PMID: 20660764 DOI: 10.1073/pnas.1009374107]
  - 128 **Qiu C**, Ma Y, Wang J, Peng S, Huang Y. Lin28-mediated post-transcriptional regulation of Oct4 expression in human embryonic stem cells. *Nucleic Acids Res* 2010; **38**: 1240-1248 [PMID: 19966271 DOI: 10.1093/nar/gkp1071]
  - 129 **Maekawa M**, Yamaguchi K, Nakamura T, Shibukawa R, Kodanaka I, Ichisaka T, Kawamura Y, Mochizuki H, Goshima N, Yamanaka S. Direct reprogramming of somatic cells is promoted by maternal transcription factor Glis1. *Nature* 2011; **474**: 225-229 [PMID: 21654807 DOI: 10.1038/nature10106]
  - 130 **Rossello RA**, Chen CC, Dai R, Howard JT, Hochschwender U, Jarvis ED. Mammalian genes induce partially reprogrammed pluripotent stem cells in non-mammalian vertebrate and invertebrate species. *eLife* 2013; **2**: e00036
  - 131 **Walia B**, Satija N, Tripathi RP, Gangenahalli GU. Induced pluripotent stem cells: fundamentals and applications of the reprogramming process and its ramifications on regenerative medicine. *Stem Cell Rev* 2012; **8**: 100-115 [PMID: 21671061 DOI: 10.1007/s12015-011-9279-x]
  - 132 **Wang P**, Na J. Mechanism and methods to induce pluripotency. *Protein Cell* 2011; **2**: 792-799 [PMID: 22058034 DOI: 10.1007/s13238-011-1107-1]
  - 133 **Raya A**, Rodríguez-Piñá I, Guenechea G, Vassena R, Navarro S, Barrero MJ, Consiglio A, Castellà M, Río P, Sleep E, González F, Tiscornia G, Garreta E, Aasen T, Veiga A, Verma IM, Surrallés J, Bueren J, Izpisua Belmonte JC. Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells. *Nature* 2009; **460**: 53-59 [PMID: 19483674 DOI: 10.1038/nature08129]
  - 134 **Hanna J**, Wernig M, Markoulaki S, Sun CW, Meissner A, Cassady JP, Beard C, Brambrink T, Wu LC, Townes TM, Jaenisch R. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* 2007; **318**: 1920-1923 [PMID: 18063756 DOI: 10.1126/science.1152092]
  - 135 **Park IH**, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ. Disease-specific induced pluripotent stem cells. *Cell* 2008; **134**: 877-886 [PMID: 18691744 DOI: 10.1016/j.cell.2008.07.041]
  - 136 **Soldner F**, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* 2009; **136**: 964-977 [PMID: 19269371 DOI: 10.1016/j.cell.2009.02.013]
  - 137 **Cebrian-Serrano A**, Stout T, Dinnyes A. Veterinary applications of induced pluripotent stem cells: regenerative medicine and models for disease? *Vet J* 2013; **198**: 34-42 [PMID: 24129109 DOI: 10.1016/j.tvjl.2013.03.028]
  - 138 **Kues WA**, Niemann H. Advances in farm animal transgenesis. *Prev Vet Med* 2011; **102**: 146-156 [PMID: 21601297 DOI: 10.1016/j.prevetmed.2011.04.009]
  - 139 **Ben-Nun IF**, Montague SC, Houck ML, Tran HT, Garitaonandia I, Leonardo TR, Wang YC, Charter SJ, Laurent LC, Ryder OA, Loring JF. Induced pluripotent stem cells from highly endangered species. *Nat Methods* 2011; **8**: 829-831 [PMID: 21892153 DOI: 10.1038/nmeth.1706]
  - 140 **Verma R**, Holland MK, Temple-Smith P, Verma PJ. Inducing pluripotency in somatic cells from the snow leopard (*Panthera uncia*), an endangered felid. *Theriogenology* 2012; **77**: 220-228, 228.e1-2 [PMID: 22079579 DOI: 10.1016/j.theriogenology.2011.09.022]
  - 141 **Panula S**, Medrano JV, Kee K, Bergström R, Nguyen HN, Byers B, Wilson KD, Wu JC, Simon C, Hovatta O, Reijo Pera RA. Human germ cell differentiation from fetal- and adult-derived induced pluripotent stem cells. *Hum Mol Genet* 2011; **20**: 752-762 [PMID: 21131292 DOI: 10.1093/hmg/ddq520]
  - 142 **Zhu Y**, Hu HL, Li P, Yang S, Zhang W, Ding H, Tian RH, Ning Y, Zhang LL, Guo XZ, Shi ZP, Li Z, He Z. Generation of male germ cells from induced pluripotent stem cells (iPS cells): an in vitro and in vivo study. *Asian J Androl* 2012; **14**: 574-579 [PMID: 22504877 DOI: 10.1038/aja.2012.3]
  - 143 **Gore A**, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J, Canto I, Giorgetti A, Israel MA, Kiskinis E, Lee JH, Loh YH, Manos PD, Montserrat N, Panopoulos AD, Ruiz S, Wilbert ML, Yu J, Kirkness EF, Izpisua Belmonte JC, Rossi DJ, Thomson JA, Eggan K, Daley GQ, Goldstein LS, Zhang K. Somatic coding mutations in human induced pluripotent stem cells. *Nature* 2011; **471**: 63-67 [PMID: 21368825 DOI: 10.1038/nature09805]
  - 144 **Hussein SM**, Batada NN, Vuoristo S, Ching RW, Autio R, Närvä E, Ng S, Sourour M, Hämäläinen R, Olsson C, Lundin K, Mikkola M, Trokovic R, Peitz M, Brüstle O, Bazett-Jones DP, Alitalo K, Lahesmaa R, Nagy A, Otonkoski T. Copy number variation and selection during reprogramming to pluripotency. *Nature* 2011; **471**: 58-62 [PMID: 21368824 DOI: 10.1038/nature09871]
  - 145 **Lister R**, Pelizzola M, Kida YS, Hawkins RD, Nery JR, Hon G, Antosiewicz-Bourget J, O'Malley R, Castanon R, Klugman S, Downes M, Yu R, Stewart R, Ren B, Thomson JA, Evans RM, Ecker JR. Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* 2011; **471**: 68-73 [PMID: 21289626 DOI: 10.1038/nature09798]
  - 146 **Miura K**, Okada Y, Aoi T, Okada A, Takahashi K, Okita K, Nakagawa M, Koyanagi M, Tanabe K, Ohnuki M, Ogawa D, Ikeda E, Okano H, Yamanaka S. Variation in the safety of induced pluripotent stem cell lines. *Nat Biotechnol* 2009; **27**: 743-745 [PMID: 19590502 DOI: 10.1038/nbt.1554]
  - 147 **Gün G**, Kues WA. Current progress of genetically engineered pig models for biomedical research. *BioResearch Open Access* 2014; **3**: 255-264 [DOI: 10.1089/biores.2014.0039]
  - 148 **Bassols A**, Costa C, Eckersall PD, Osada J, Sabrià J, Tibau J. The pig as an animal model for human pathologies: A proteomics perspective. *Proteomics Clin Appl* 2014; **8**: 715-731 [PMID: 25092613 DOI: 10.1002/prca.201300099]
  - 149 **Kurome M**, Geistlinger L, Kessler B, Zakhartchenko V, Klymiuk N, Wuensch A, Richter A, Baehr A, Kraeche K, Burkhardt K, Flisikowski K, Flisikowska T, Merkl C, Landmann M, Durkovic M, Tschukes A, Kraner S, Schindelbauer D, Petri T, Kind A, Nagashima H, Schnieke A, Zimmer R, Wolf E. Factors influencing the efficiency of generating genetically engineered pigs by nuclear transfer: multi-factorial analysis of a large data set. *BMC Biotechnol*



Kumar D *et al.* IPS cells from farm animals species

2013; **13**: 43 [PMID: 23688045 DOI: 10.1186/1472-6750-13-43]

150 **Duranthon V**, Beaujean N, Brunner M, Odening KE, Santos AN, Kacs Kovics I, Hiripi L, Weinstein EJ, Bosze Z. On the emerging

role of rabbit as human disease model and the instrumental role of novel transgenic tools. *Transgenic Res* 2012; **21**: 699-713 [PMID: 22382461 DOI: 10.1007/s11248-012-9599-x]

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## Cardiac disease modeling using induced pluripotent stem cell-derived human cardiomyocytes

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has been carried out by expressing the mutated proteins in *in-vitro* heterologous systems. While these studies have provided a wealth of functional details that have greatly enhanced the understanding of the pathological mechanisms, it has always been clear that heterologous expression of the mutant protein bears the intrinsic limitation of the lack of a proper intracellular environment and the lack of pathological remodeling. The results obtained from the application of the next generation sequencing technique to patients suffering from cardiac diseases have identified several loci, mostly in non-coding DNA regions, which still await functional analysis. The isolation and culture of human embryonic stem cells has initially provided a constant source of cells from which cardiomyocytes (CMs) can be obtained by differentiation. Furthermore, the possibility to reprogram cellular fate to a pluripotent state, has opened this process to the study of genetic diseases. Thus induced pluripotent stem cells (iPSCs) represent a completely new cellular model that overcomes the limitations of heterologous studies. Importantly, due to the possibility to keep spontaneously beating CMs in culture for several months, during which they show a certain degree of maturation/aging, this approach will also provide a system in which to address the effect of long-term expression of the mutated proteins or any other DNA mutation, in terms of electrophysiological remodeling. Moreover, since iPSC preserve the entire patients' genetic context, the system will help the physicians in identifying the most appropriate pharmacological intervention to correct the functional alteration. This article summarizes the current knowledge of cardiac genetic diseases modelled with iPSC.

### Abstract

Causative mutations and variants associated with cardiac diseases have been found in genes encoding cardiac ion channels, accessory proteins, cytoskeletal components, junctional proteins, and signaling molecules. In most cases the functional evaluation of the genetic alteration

**Key words:** Cardiomyopathies; Cardiac arrhythmias; Induced pluripotent stem cells; Human cardiomyocytes

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**Core tip:** This paper revises the cardiac genetic diseases that have been modeled so far using the technology that starts from patient somatic cells, reprogram their fate to a pluripotent state, and then proceed to cardiomyocyte differentiation. We will describe the main steps of this procedure, from pluripotent stem cells to mature cardiomyocytes, and we will discuss the main features linked to the different cardiac pathologies that this model recapitulate in a cell culture dish.

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## INTRODUCTION

In 19<sup>th</sup> century, the Italian physician Giulio Bizzozero, founder of the Italian school of experimental pathology, proposed the classification of tissues depending on their regeneration rate. He divided human tissues in three categories: labile, stable, and perennial, where the labile are physiologically subjected to a continuous renewal, *e.g.*, keratinocytes, or bone marrow cells; stables, that are normally in a quiescent state but still retain the capacity to proliferate, *e.g.*, hepatocytes; and perennial, the so-called post-mitotic cells, that have completely lost their regenerative potential. The most representative cells in the latter case are neurons and cardiomyocytes, highly specialized cells that, apart from their duplication inability, share also the capacity, during development, to connect to sister cells and to build an organized network capable of an electric signal transmission.

During these years we learned the methods to cultivate and to maintain labile and stable cells in culture, but neurons and cardiomyocytes are still "problematic" cells, difficult to preserve in absence of their natural support, glial cells or cardiac fibroblasts, and, moreover, almost impossible to be obtained as sample from a healthy person.

Progress in this field came from studies of developmental biologist that were able to isolate, cultivate, and differentiate adult or embryonic stem cells (ESCs). These cells basically recapitulate *in vitro* the developmental process and ESCs in particular are indeed a continuous source of terminally differentiated post-mitotic cells that can be currently easily studied. The isolation and the use of human ESCs, although obtained from surplus of *in vitro* fertilization procedures, opened a serious and profound ethical issue. The great revolution came in 2006 when Shinya Yamanaka proposed a complex protocol designed to change cellular fate by forcing transcription factor expression<sup>[1]</sup>. With this procedure, a murine

fibroblast was reprogrammed to a cell that assumed the main characteristics of an ESC: high proliferation rate as well as wide differentiation potential toward the three germ layers. Nevertheless, the search for an identical physiological counterpart has lead to the conclusion that these induced pluripotent stem cells (iPSCs), although very similar to ESCs, must be considered as artificial cells created in the lab. In 2007, the same process was applied successfully to human fibroblasts by Yamanaka's as well as Thomson's group<sup>[2,3]</sup>, thus creating cell lines that can be propagated almost indefinitely and can be used, in a close future, as a continuous source of differentiated cells for therapeutic purposes.

The application of this powerful technique to humans opened the possibility to reprogram fibroblasts isolated not only from healthy people but also from patients suffering of a genetic disease. The resulting human iPSCs (hiPSC) will keep the entire genetic information of the patient, including the mutation that has been linked to the pathology. The first examples of disease-related hiPSC involved patients with a range of human genetic diseases, whose DNA mutations, including the trisomy 21, were effectively maintained through all the reprogramming procedure<sup>[4]</sup>.

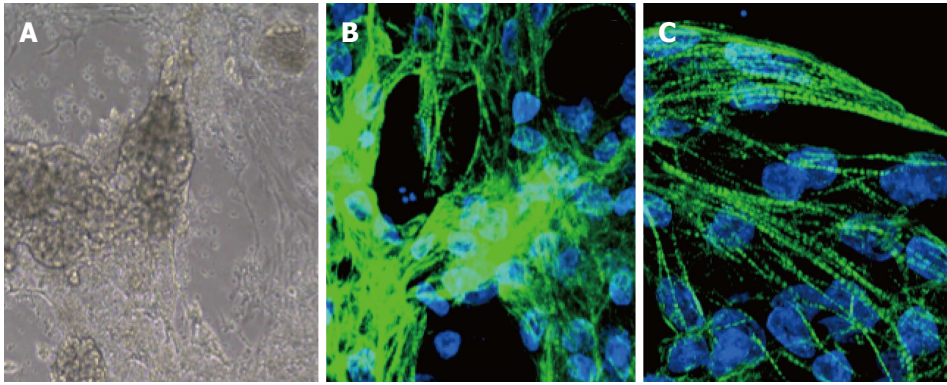
Since then, many laboratories started to model *in vitro* human genetic disease, succeeding to get a considerable number of post-mitotic cells for detailed functional studies. Indeed, this result is particularly important and useful when studying affected human cardiomyocytes or neurons, otherwise difficult to be obtained and maintained in culture. In most cases, despite the fact that hiPSC-derived cells are not exactly equal to the physiological counterpart, the system has attested successful *in vitro* replication of the main cellular characteristics already known to be associated with the modeled disease.

In this review we will discuss the important discoveries of the biological mechanisms underlying some genetic cardiomyopathies, made possible by the use of the cellular reprogramming technology.

## FROM PLURIPOTENT STEM CELLS TO SPONTANEOUSLY CONTRACTILE CELLS

*In vitro* development of ESCs has been widely studied using murine ESCs (mESCs), whose differentiation procedure in culture implies the initial leukemia inhibitory factor (LIF) removal and the formation of cellular aggregates using the "hanging drop" method. These three dimensional (3D) structures, called embryoid bodies (EBs), replicate *in vitro* the different stages of murine embryonic development<sup>[5]</sup>. Around differentiation day (dd) 8, clusters of spontaneously beating cells appear in culture; these cells express several transcriptional and structural cardiac markers and were therefore classified generically as cardiomyocytes<sup>[6]</sup>. It should be noted that mESC differentiation starts by just removing LIF also in a 2D culture, but the resulting process does not strictly





**Figure 1 Cardiomyocyte at day 16 post differentiation.** A: Microscopical view of beating areas; B: Expression of cardiac troponin T (green), nuclei (DAPI) in blue; C: Cardiomyocyte sarcomeric structures evidenced by cardiac troponin T staining (green), nuclei (DAPI) in blue.

follow embryonic development (P. Dell'Era, unpublished results).

Since their isolation, human ESCs have shown different culturing needs from the murine counterpart, and their behavior revealed also minor differentiation plasticity. Cardiac differentiation is the most glaring example of this statement. The spontaneous 3D differentiation of mESC leads to the easy appearance of spontaneous beating foci in all the considered EBs, while in the case of both hESC and hiPSC, only a modest proportion of EBs contains contracting cells. This occurrence leads to the setup of different methods aimed to increase cardiac differentiation during *in vitro* development of pluripotent stem cells. While some of these procedures retain an initial 3D EB formation, others start from a confluent 2D monolayer.

#### **Differentiation protocols**

During gastrulation, cardiomyocytes emerge from mesodermal tissue, in particular from the anterior region of the primitive streak. Bone morphogenetic protein produced by the adjacent endoderm induces the cardiomyocyte fate, whereas WNT-mediated signals from the underlying neural tube and notochord suppress cardiomyocyte specification. Therefore the timed addition of inducers and/or inhibitors of the cited pathways would guide PSC differentiation toward: mesoderm, cardiomyogenic mesoderm, cardiac precursors, and cardiomyocytes. This is conceptually the summary of several protocols. At variance the required instructive signals can be provided by a visceral endoderm-like cell line that can be used as a feeder layer during the differentiation process<sup>[7]</sup>.

Within these years, several efforts have been made to identify a protocol that would give rise to high percentage of cardiomyocytes following differentiation of either hESC or hiPSC. All the attempts lead to the creation of several tools such as defined cultivation media that, avoiding animal components, will facilitate therapeutic use of pluripotent stem cells; recombinant single protein matrices such as vitronectin or laminin;

aggregation plates to standardize the production of EBs; and the more recent commercial cardiomyocyte differentiation media (StemRD and Life Technologies). The main differentiation protocols that have been used over the years are outlined in Table 1.

We tested several of these procedures using iPSC derived from patients' fibroblasts and adapted to grow on Matrigel-coated dishes; in our experience, we observed beating areas only when cells were treated with modulators of the Wnt pathway, while the general *TNNT2* expression was achieved in most protocols (Figure 1B). Of particular interest for therapeutic purposes the recent setup by BurrIDGE *et al.*<sup>[8]</sup> that employs a chemically defined medium consisting of just three components on a dish covered by synthetic biological matrices. Indeed, using this protocol we strongly increased the number of beating cells in our culture up to 50%, but the best result, around 70%, was recently obtained using the PSC Cardiomyocyte Differentiation Kit (Life Technologies).

#### **FROM SPONTANEOUSLY CONTRACTILE CELLS TO MATURE CARDIOMYOCYTES**

In early development, the primitive heart tube is composed by small myocytes without distinct region of cells conducting and coordinating the stimulus. The contractile impulse begins with the primary pacemaker area in the primitive atrium that will later evolve in the sinoatrial node, where the fast beating pacemaker myocytes will reside. As development proceeds, also the conductive system will differentiate, generating CMs in anatomical different regions that will become the atrio-ventricular (AV) node, the AV bundles, and Purkinje fibers. Each of these CM subtype has its own electrical properties such as the presence and the form of the action potential (AP), the contraction rate, and the presence of specific currents that can be measured electrophysiologically.

However, CMs differentiated *in vitro* vary considerably from cells isolated from a mature human heart, because of the absence of humoral factors and organized



**Table 1** From pluripotent stem cells to cardiomyocyte: Details of main cardiac differentiation protocols

PSC type		Differentiation			Pre-treatment		Cardiac differentiation treatment					% Beating cells	% cTNT + cells	Ref.
h-iPSC	h-ESC	2D	3D	Others	ROCK-I	GSK-I	BMP4	Activin A	Wnt-I	FGF2	Others			
	X			END2							AA	< 40%	n.a.	[83]
X	X		X								Specific serum	5%-10% hiPSC 10%-25% hESC	n.a.	[20]
X	X		X				X			X		n.a.	> 80% hiPSC 60%-80% hESC	[84]
	X		X				X	X			AA	n.a.	50%-70%	[85]
X	X		X								p38-MAPK-I	> 23% hiPSC 50% hESC	n.a.	[86]
X		X		Sandwich	X		X	X		X		n.a.	98%	[87]
X	X	X			X	X	X	X	X		AA	n.a.	80%	[88]
X			X		X						END-2-CM	n.a.	< 10%	[89]
X		X			X		X	X		X	MEF-CM	n.a.	< 60%	
X			X				X	X			Tricho-statin A	< 50%	< 10%	[90]
X	X		X		X		X	X	X	X	VEGF	< 50% hiPSC < 40% hESC	n.a. 95% hESC	[91]
X		X				X			X		Albumin, AA	n.a.	90%	[8]
	X	X					X	X	X	X	VEGF	< 40%	< 20%	[92]
X	X		X				X	X	X		Blebbistatin	100%	90%	[93]

iPSC: Induced pluripotent stem cell; ESC: Embryonic stem cell; n.a.: Not available.

mechanical and electrical stress. In general, many of the features of hPSC-CMs are reminiscent of normal fetal cells. hPSC-CMs are spontaneously beating cells co-expressing atrial-, ventricular-, and nodal- markers, with unorganized sarcomeres, immature mitochondria, and an expression profile different from adult CMs<sup>[9]</sup>. Our data indicate that, after 16 d of *in vitro* differentiation, iPSC-derived CMs start to segregate in the various subtypes, showing pronounced sarcomeric structures that reveal a certain degree of maturation (Figure 1C).

The CMs that arise during early hESC or hiPSC *in vitro* differentiation exhibit spontaneous AP, with a relatively depolarized resting membrane potential, probably due to the temporary absence of the inward rectifier potassium current ( $I_{K1}$ )<sup>[10]</sup>.

The expression of the ion channels and, consequently, the ionic currents will undergo developmental maturation over time, as assessed by modifications in current density and property<sup>[10]</sup>. hPSC-CMs immaturity is also reflected in their excitation-contraction machinery, lacking clear T-tubuli, disorganized sarcomeric striations, and immature  $Ca^{2+}$  handling<sup>[11-13]</sup>. Unlike primary CMs that tend to undergo apoptosis or dedifferentiate, CMs derived from hPSC develop and maintain a functional phenotype in long-term culture<sup>[14]</sup>. After surviving for 80 d, late-stage hESC-CMs show pronounced multinucleation that is accompanied by an increase in cellular perimeter, and area<sup>[15]</sup>. Ultrastructural studies demonstrated that the sarcomere of hiPSC-CMs continue to mature through a 1-year culture<sup>[16]</sup>. Young hiPSC-CMs contained a low number of unaligned myofibrils and immature high-density Z-bands. Within 6 mo the myofibrils became more tightly packed and formed parallel arrays accompanied by the appearance of mature Z-, A-, H-, and I-bands. M-bands were finally detected in 360-d-old

CMs, but expression levels of M-band-specific genes remained lower in comparison with those in the adult heart<sup>[14]</sup>.

Also a different gene expression accompanies these changes: late-stage CMs show increased levels of structural filaments MYH6 and MYH7, and of other specific molecules such as connexin 43, hyperpolarization activated cyclic nucleotide-gated potassium channel 4, and sarco(endo)plasmic reticulum  $Ca^{2+}$  ATPase<sup>[14-16]</sup>. Finally, the electrophysiological profile of late-stage hESC-CMs show a significantly enhanced AP upstroke and a hyperpolarized maximum diastolic potential, and during maturation no differences were observed for AP duration (APD) to 50% or 90% (APD50, APD90) of repolarization<sup>[15]</sup>.

It must be noted that, while at early differentiation stages there are unspecified CMs that co-express different CM-subtype markers at later stages, around dd30, CMs acquire a more specific phenotype, expressing ventricular-, atrial-, and nodal-markers<sup>[8]</sup>. The relative proportion of the three CM subtypes varies among the differentiation protocols that have been used, and can be additionally modulated by supplementing chemicals, microRNAs, or biological molecules to the culture<sup>[17]</sup>.

## CARDIAC DISEASE MODELING

Despite all the discussed limitations, hPSC differentiation remains a powerful method to model *in vitro* genetic cardiac diseases, because of the capacity of these cells to give rise to terminally differentiated stable cardiac cells. The cardiac pathologies that have been modeled so far using hiPSC include some cardiomyopathies and arrhythmias, whose implicated



**Table 2** Inherited cardiac diseases modeled using Induced pluripotent stem cell

Cardiac disease	Gene	Protein/current	Chr	Mutation	Differentiation method	Cardiomyocyte subtype	Maturation days	Ref.
HCM	MYH7	Myosin heavy chain $\beta$	14q12	R663H	3D spontaneous			[19]
DCM	LMNA	Lamin A	1q22	R225X GCCA insertion	END-2 co-culture	V- and A-like	20	[25]
	TNNT2	Troponin T type 2	1q32	R173W	3D, activin, BMP4, DKK1, FGF2, VEGF	V-, A-, and N-like	> 30	[27]
	DES	Desmin	2q35	A285V	END co-culture	n.d.	> 14	[26]
BTHS	TAZ	Tafazzin	Xq28	517delG c.328T>C	2D, activin, BMP4	n.d.	> 12	[30]
LQT1	KCNQ1	Kv7.1/I(Ks)	11p15	R190Q	3D spontaneous	V-, A-, and N-like	20-30	[36]
LQT2	KCNH2	hERG/I(Kr)	7q36	R176W	3D spontaneous	V- and A-like	n.d.	[42]
				A561T		V-, A-, and N-like	25-30	[38]
				A561V		V- and A-like	21	[45]
				A614V		V-, A-, and N-like	> 30	[37]
				N996I		n.d.	20-60	[43]
LQT3	SCN5A	Nav1.5 /I(Na)	3p21	F1473C	3D, activin, BMP4, Wnt-I, FGF2	V- and A-like	25-45	[50]
				V1763M	3D spontaneous	V-, A-, and N-like	> 28	[51]
				V240M, R535Q	END-2 co-culture	V-, A-, and N-like	20-30	[52]
LQT8	CACNA1C	CaV1.2/I(Ca)	12p13	G1216A	3D + Wnt3a	V-, A-, and N-like	> 37	[55]
CPVT	RYR2	Ryanodine receptor 2/I(Ca)	1q43	F2483I	END-2 co-culture	V-, A-, and N-like	20-30	[61]
				P2328S	END-2 co-culture	Mostly V-like	n.d.	[62]
				S406L	3D spontaneous	V-, A-, and N-like	> 70	[64]
				M4109R	3D spontaneous	V-, A-, and N-like	> 30	[65]
				E2311D	3D spontaneous	V/A- and N-like	> 30	[66]
	CASQ2	Calsequestrin	1p13	D307H	3D spontaneous	n.d.	25-43	[67]
ARVC	PKP2	Plakophilin-2	12p11	L614P	3D spontaneous	V-, A-, and N-like	> 28	[72]
				A324fs335X	3D spontaneous	n.d.	> 30	[73]
				T505fsX110				
				Cryptic splicing c.2013delC	3D spontaneous	n.d.	> 60	[74]

HCM: Hypertrophic cardiomyopathy; DCM: Dilated cardiomyopathy; BTHS: Barth syndrome; LQT: Long-QT; CPVT: Catecholaminergic polymorphic ventricular tachycardia; ARVC: Arrhythmogenic right ventricular cardiomyopathy; n.d.: Not defined.

genes are listed in Table 2.

### hiPSC modeled cardiomyopathies

**Familial hypertrophic cardiomyopathy:** Hypertrophic cardiomyopathy (HCM) is a heterogeneous monogenic heart disease in which a portion of the myocardium is heavily hypertrophic. It is caused by more than 1400 mutations in at least 11 genes that encode thick and thin contractile myofilaments of the sarcomere or the adjacent Z-disc<sup>[18]</sup>. HCM patients display abnormal thickening of the left ventricular myocardium in the absence of increased hemodynamic burden. Most people with familial HCM are symptom-free or have only mild symptoms, but their risk for clinical complications such as progressive heart failure, arrhythmia, and sudden cardiac death is strongly increased.

Efforts to elucidate the mechanisms underlying development of HCM have lead to the generation of patient-specific hiPSC-CMs that recapitulate *in vitro* a number of disease characteristics including cellular hypertrophy, and contractile arrhythmia<sup>[19]</sup>. hiPSC were generated from a family cohort carrying a hereditary HCM missense mutation (Arg663His) in the MYH7 gene, and CMs were generated using the 3D spontaneous differentiation protocol<sup>[20]</sup>. Mutant hiPSC-

CMs demonstrated not only cellular enlargement and multinucleation, but also other hallmarks of HCM including expression of atrial natriuretic factor, elevation of  $\beta$ -myosin/ $\alpha$ -myosin ratio, calcineurin activation, and nuclear translocation of nuclear factor of activated T cells<sup>[19]</sup>. Using this model the authors were able to show that irregular  $\text{Ca}^{2+}$  transients and elevation of diastolic intracellular calcium  $[\text{Ca}^{2+}]_i$  precedes the presentation of other phenotypic abnormalities, strongly implicating dysregulation of  $\text{Ca}^{2+}$  cycling in the pathogenesis of the disease. Pharmaceutical drug screening of mutant hiPSC-CMs further supported elevated  $[\text{Ca}^{2+}]_i$  as a central mechanism for arrhythmia development. Indeed, only pharmaceutical blockade of  $\text{Ca}^{2+}$  and  $\text{Na}^+$  entry mitigated contractile arrhythmia in HCM-CMs<sup>[19]</sup>.

**Dilated cardiomyopathy:** Dilated cardiomyopathy (DCM) represents the final common morphological and functional consequence of various pathological conditions in which a combination of myocyte injury and necrosis associated with tissue fibrosis results in impaired heart mechanical function. Nevertheless, primary familial forms of DCM represent a genetic condition in which the pathological involvement is predominantly limited to the myocardium. The main



hallmark of primary DCM is the presence of a left or biventricular dilatation with severely impaired systolic function in the absence of abnormal loading conditions or ischemic heart disease sufficient to cause global systolic impairment<sup>[21]</sup>. Point mutations in 31 autosomal and 2 X-linked genes have been implicated in causing familial DCM (FDC) but account for only 30% to 35% of genetic causes<sup>[22]</sup>. One of the more common genes identified in FDC is LMNA, which codes for lamin A/C proteins, intermediate filament proteins of the nuclear lamina<sup>[23]</sup>.

Several animal models of LMNA mutations have been generated to provide initial insights into the pathophysiology of lamin A/C-related DCM<sup>[24]</sup>. Nevertheless the mechanism that links LMNA mutations with DCM remains uncertain.

Two different LMNA mutations have been modeled using hiPSC-CMs: an autosomal dominant non-sense mutation (R225X) in exon 4 of the lamin A/C and a GCCA insertion at base 50 in LMNA that creates a frameshift and premature stop codon, hence causing lamin A/C haploinsufficiency. LMNA<sup>R225X/WT</sup> and LMNA<sup>Frameshift/WT</sup> hiPSC-CMs showed normal phenotypes and basal electrophysiological properties as control hiPSC-CMs. However, when these CMs were subjected to electrical stimulation as in the cardiac environment, they exhibited typical nuclear abnormalities together with increased apoptosis<sup>[25]</sup>. These two properties were replicated *in vitro* by shRNA knockdown of LMNA in control hiPSC-CMs, that was ineffective when the MEK1/ERK1/2 pathway was blocked pharmacologically<sup>[25]</sup>, thus identifying this pathway as a potential therapeutic target in LMNA-related DCM.

The same group also modeled a pathogenic phenotype of DCM due to a novel A285V Desmin (DES) mutation identified by whole exome sequencing (WES) using the hiPSC-CM system<sup>[26]</sup>. Characterization of hiPSC-CMs carrying A285V-DES mutation revealed a poor co-localization of DES with several cytoskeletal proteins, including cardiac troponin-T,  $\alpha$ -actinin and F-actin, and diffuse isolated aggregations of DES-positive protein<sup>[26]</sup>. The electrophysiological analysis revealed that A285V-DES CMs exhibit significant functional abnormalities compared with the control-CMs as demonstrated by the diminished maximum rate of calcium ion re-uptake, slower spontaneous beating rate and failure to respond to the inotropic stress induced by isoproterenol<sup>[26]</sup>. When control-iPSC-CMs were transduced with a lentivirus carrying the A285V-DES mutation, the resulting CMs simulated the phenotypes of DES-DCM-CMs, thus confirming the idea that abnormal DES-positive protein aggregates due to DES mutation can cause structural and functional abnormalities in cardiomyocytes. The relevance of this study is the demonstration that patient-specific hiPSC-CMs can be used to provide confirmation of a suspected genetic basis for DCM identified by WES.

Lastly, cardiomyocytes derived from patients in a

DCM family carrying a point mutation (R173W) in the gene encoding sarcomeric protein cardiac troponin T were analyzed<sup>[27]</sup>. Compared to healthy individuals in the same family cohort, CMs from DCM patients exhibited altered regulation of Ca<sup>2+</sup>, decreased contractility, and abnormal distribution of sarcomeric  $\beta$ -actinin. When stimulated with a  $\beta$ -adrenergic agonist, CMs showed characteristics of cellular stress such as reduced beating rates, compromised contraction, and abnormal sarcomeric  $\beta$ -actinin distribution. Treatment with  $\beta$ -adrenergic blockers or overexpression of sarcoplasmic reticulum Ca<sup>2+</sup> adenosine triphosphatase rescued the pathological phenotype<sup>[27]</sup>.

**Barth syndrome:** Barth syndrome (BTHS) is a rare, metabolic, and neuromuscular genetic disorder that occurs exclusively in males. Clinical features include variable combinations of pathologies including DCM, HCM, endocardial fibroelastosis, left ventricular non-compaction, ventricular arrhythmia, sudden cardiac death<sup>[28]</sup>. The gene responsible for the disorder, tafazzin (TAZ), has recently identified: it is located on the long arm of chromosome X at Xq28, and encodes an acyltransferase that catalyzes the acylation of cardiolipin, the major phospholipid of the mitochondrial inner membrane<sup>[28,29]</sup>. So far, more than 120 mutations have been described, but no correlation with specific phenotype has been observed<sup>[28]</sup>.

hiPSCs from two unrelated individuals with BTHS were generated, BTH-H and BTH-C, carrying TAZ frameshift (c.517delG) and missense (c.328T > C) mutations, respectively. CMs were generated by treating a cell monolayer with inducers and sorted for the surface marker vascular cell adhesion molecule-1 to obtain preparations highly enriched in CMs. Purified CMs were analyzed morphologically to evaluate sarcomere organization, and to assess mitochondrial functionality. CMs were then seeded onto thin elastomers with patterned lines of fibronectin, obtaining self-organized anisotropic myocardial tissues to study contractility properties<sup>[30]</sup>. TAZ deficiency in BTHS-CMs impairs mitochondrial functionality as well as sarcomere assembly, generate contractile stress, and markedly increase reactive oxygen species (ROS) production. When linoleic acid (LA), an essential unsaturated fatty acid precursor of mature cardiolipin, was added to CM culture, the metabolic phenotype was corrected, the sarcomere organization and contractile defects were mitigated, and ROS production was strongly reduced<sup>[30]</sup>.

Using the hiPSC-derived model the authors showed that suppression of ROS, not only by LA but also by mitoTEMPO, normalized the metabolic, sarcomerogenesis and contractile phenotypes of BTHS- CMs, thus setting the basis for an effective pharmacological therapy of BTHS patients<sup>[30]</sup>.



**hiPSC modeled arrhythmias**

**Long-QT syndromes:** Long-QT syndromes (LQTS) are a group of heritable, usually autosomal dominant disorders with a estimated prevalence of 1:2500, characterized by an abnormally delayed or prolonged ventricular repolarization phase (prolongation of the QT interval on an electrocardiogram) and a propensity toward polymorphic ventricular tachycardia (often termed Torsades de pointes, TdP), syncope and sudden cardiac death in young patients<sup>[31]</sup>. Clinically LQTS present a broad range of phenotypes even among family members with identical mutations, possibly as a result of genetic modifiers<sup>[32]</sup>. To date, LQTS have been associated with over 500 different mutations in at least 13 genes encoding cardiac ion channel proteins, but the most prevalent forms are LQT1 and LQT2 caused by potassium channel mutations with a percentage of genotyped cases of > 50% and 30%-40%, respectively, and LQT3 caused by a sodium channel mutation that accounts for 10%-15%<sup>[31]</sup>.

LQT1 patients carry mutations in the *KCNQ1* gene (also known as KVLQT1 or Kv7.1), which encodes the pore-forming  $\alpha$ -subunits of the channels generating  $I_{Ks}$ , an adrenergic-sensitive, slow outward potassium current, while LQT2 implicates hERG protein (encoded by *KCNH2* gene), which constitutes pore-forming  $\alpha$  subunit of the rapidly-activating delayed rectifier potassium current ( $I_{Kr}$ )<sup>[33]</sup>. LQT3 is instead associated with gain-of-function mutations of the *SCN5A* gene, which encodes the  $\alpha$ -subunit of the  $Na^+$  ion channel  $NaV1.5$ <sup>[34]</sup>, while loss-of-function mutations in the same gene are associated with several other genetically heterogeneous disorders including Brugada syndrome, cardiac conduction disease, sick sinus syndrome sudden infant death syndrome and others<sup>[35]</sup>.

LQT1 was the first cardiac disease modeled using hiPSC and since then several hiPSC-CMs from patients carrying mutations in LQTS-associated channels have been considered<sup>[36-42]</sup>. Indeed, patient-specific hiPSC-CMs represent a platform to investigate the functionality of ion channel mutations expressed in their complex genetic backgrounds and may provide unique insight into therapeutic approaches for disease management<sup>[43]</sup>.

**LQT1:** A family affected by LQT1 was screened and an autosomal dominant missense mutation R190Q in the *KCNQ1* gene was identified<sup>[36]</sup>. hiPSC from two family members and two healthy controls were generated by retroviral vectors encoding the human transcription factors OCT3/4, SOX2, KLF4, and c-MYC (hOSKM). Using a 3D differentiation protocol, these cells were then differentiated into CMs. Spontaneously beating cells dissociated from LQT1 and control explants responded to pacing and generated three distinct types of APs, designated as "ventricular (V)", "atrial (A)", and "nodal (N)" on the basis of their similarity to the APs of human fetal heart CMs<sup>[36]</sup>.

Several disease-specific abnormalities were observed in LQT1-CMs: the duration and the rate adaptation of the AP, a 70%-80% reduction in  $I_{Ks}$ , as well as vulnerability to catecholaminergic stress<sup>[36]</sup>. R190Q-KCNQ1 is a dominant mutation and indeed in hiPSC-CMs the mutated protein was absent on cell surface, but still retained in the endoplasmic reticulum. Similar results were obtained by expressing the wild type and the mutated protein in cardiomyoblast H9c2 cell line<sup>[36]</sup>. Furthermore, electrophysiological studies confirmed the protective effect of  $\beta$ -blockade in the abnormal response to catecholamine stimulation, thus confirming the efficacy of the therapeutic approach for LQT1 patients<sup>[36]</sup>.

**LQT2:** The voltage-gated inwardly rectifying potassium channel that comprise KCNH2-encoded protein is composed of homo- or heterotetrameric complexes of pore-forming  $\alpha$  subunits, like hERG, that associate with modulating  $\beta$  subunits. hERG consists of six transmembrane  $\alpha$  helices, a pore helix, and N- and C-termini cytoplasmically located. The channel mediates the rapidly activating component of the  $I_{Kr}$  in heart<sup>[44]</sup>. A large number of natural variants have been described, most of them in association with LQT2 syndrome (see <http://www.uniprot.org/uniprot/Q12809>).

A panel of control and LQT2-related hPSC were generated and characterized by several laboratories. hiPSC-CMs showing five different hERG mutations in genetically unrelated backgrounds were intensely characterized: R176W<sup>[42]</sup>, A561T<sup>[38]</sup> and A561<sup>[45]</sup>, A614V<sup>[37]</sup>, and N996I<sup>[46]</sup>. The aminoacids 176 and 996 reside in the N- and C-terminus cytoplasmic domains respectively, while the position 561 is in a transmembrane region of the protein, and the aminoacid 614 is located in the pore-forming segment.

Heterozygous KCNH2 mutations exert a dominant-negative effect on wild-type (WT) hERG channels associated  $I_{Kr}$ , by impairing trafficking pathways or altering channel kinetics of the resulting co-assembled hERG heterotetramers<sup>[31,47]</sup>. Due to this behavior, it is possible to transfer them in a WT environment to verify their biological consequences in an unrelated genetic background.

The reprogramming and the following differentiation process were similar for all the laboratories: most of them used a retroviral transduction system, except for Mehta *et al.*<sup>[45]</sup> that choose a viral-free episomal non-integrating approach, but the following differentiation involving EB formation and spontaneous differentiation was identical for all of them.

It must be pointed out that LQT2 is a disorder with incomplete penetrance where genetic background variations can confound disease traits. For this reason, the KCNH2 N996I mutation was deeply analyzed in (1) hiPSC-CMs from LQT2 patient; (2) hiPSC-CMs from LQT2 patient, previously corrected to wild-type using an homologous recombination system; and (3) NKX2.5eGFP/w hESC-CMs where the N996I-KCNH2



mutation was introduced using the same approach as before<sup>[46]</sup>.

Several observations were commonly reported to all LQT2-CMs: intracellular patch clamp recording of APD or extracellular measurement of field potential duration (FPD) using multi electrode array (MEA) showed prolonged intervals for both A-like and V-like cells<sup>[37,38,42,45,46]</sup>. A summary of AP characteristic parameters measured by several authors in iPSC- or ESC-derived CMs is reported in Hoekstra *et al.*<sup>[48]</sup>. Nevertheless, the AP increase was more restricted when isogenic cell lines were compared<sup>[46]</sup>, thus suggesting that a different genetic background can indeed over-estimate the differences between a control versus diseased CMs.

AP was modulated in LQT2-CMs by several drugs: pinacidil, a K<sub>ATP</sub>-channel opener, significantly shortens APD<sub>90</sub>, while Na-channel blocker, ranolazine, was ineffective<sup>[37]</sup>; K channel enhancers, nicorandil and PD118057, caused AP shortening and in some cases could abolish early afterdepolarization (EAD)<sup>[38]</sup>. In most of the papers some arrhythmogenicity of LQT2-CMs was reported, as evidenced by EAD events during spontaneous recordings<sup>[37,42,45]</sup> or when challenged with the clinically used stressor, isoprenaline<sup>[38]</sup>.

As expected, the measurement of I<sub>Kr</sub>, determined by adding the specific E4031 inhibitor, showed a reduction in LQT2-derived versus control CMs<sup>[37,42,45,46]</sup>.

Definitively, the genetic correction of the N996I-*KCNH2* mutation associated with LQT2 restores I<sub>Kr</sub> density and normalizes APD in patient-specific LQT2-CMs, while the introduction of the same mutation in hESC-CMs reduced I<sub>Kr</sub> and prolonged the AP duration<sup>[46]</sup>. In addition, the molecular defects of hERG A561V and N996I mutants have been analyzed. In the NKX2.5-eGFP<sup>+</sup> hESC-N996I cells, as well as in LQT2-N996I CMs, a trafficking defect was identified. Indeed, the 155-kDa protein band, representing the form transported to the cell membrane through the Golgi, was reduced by two-fold compared to the WT protein, while the 135-kDa band, which corresponds to the protein located in the ER, was unaffected<sup>[46]</sup>.

Similarly, hERG mutation A561V causes a reduced membrane localization of glycosylated/mature protein<sup>[45]</sup>. Treatment of LQT2-A561V CMs with the calpain and proteasome inhibitor ALLN, not only increased membrane localization of mature hERG but also reduced repolarization, increased I<sub>Kr</sub> and reduced arrhythmogenic events, thus suggesting a new therapeutic approach to treat LQT2 patients<sup>[45]</sup>.

**LQT3:** The  $\alpha$ -subunit of the Na(v)1.5 cardiac sodium channel, encoded by *SCN5A* gene is composed by intracellular N- and C- terminus, and four homologous domains, attached one another by cytoplasmic linkers, forming a pore that conducts Na<sup>+</sup> ions across membrane<sup>[49]</sup>. In LQT3, the gain-of-function *SCN5A*-

mutations cause an increased persistent Na<sup>+</sup> influx during depolarization that results in an enhanced late or persistent sodium current due to defective open-state inactivation of the channel<sup>[35]</sup>. Four different *SCN5A* alterations have been modeled using hiPSC cellular system: a *de novo* heterozygous missense mutation F1473C associated with a polymorphism (K897T) in *KCNH2*, identified in a newborn patient with extreme prolonged QT interval<sup>[50]</sup>. The second *SCN5A* modification was again a *de novo* heterozygous missense V1763M mutation, found in a Chinese girl<sup>[51]</sup>, and the last two V240M and R535Q alterations were from two LQT3-diagnosed patients<sup>[52]</sup>. The F1473C mutation occurs in the channel inactivation gate of *SCN5A* while V240M and V1763M reside in transmembrane segments, and R535Q mutation occurs in the first cytoplasmic linker<sup>[53]</sup>.

In the first paper a lentiviral transduction of OSKM was used to reprogram fibroblasts, and a complex protocol rich in developmental modulators was used to achieve CMs differentiation<sup>[50]</sup>. Ma *et al.*<sup>[51]</sup> used instead an mRNA-based non viral non integrating reprogramming approach, followed by the spontaneous EB-based CM differentiation. Finally Fatima *et al.*<sup>[52]</sup> used the classical retroviral infection, followed by a differentiation protocol guided by END-2 co-culture.

The data of hiPSC-CMs from the newborn and from the Chinese girl were compared with those obtained from hiPSC-CMs of healthy parents and of a healthy sister respectively that, because of the common genetic background with the patients, strengthened the obtained results<sup>[50,51]</sup>.

Terrenoire *et al.*<sup>[50]</sup> reported a mutation-dependent increase in I<sub>NaL</sub>, a right-shifted steady-state channel availability, and faster recovery from inactivation in all clones from the proband and none of the parents' clones. By using the hiPSC-CM system, the authors easily showed that the *KCNH2* heterozygous polymorphism T897 and K897, derived from homozygous parents, had no impact on electrophysiological pathology of the proband<sup>[50]</sup>. Furthermore, the system allowed the pharmacological evaluation of the current patient's therapy, not so effective in controlling episodes of arrhythmia. Indeed, a more effective therapy has been identified as a result of the proposed study<sup>[50]</sup>.

Ma *et al.*<sup>[51]</sup> observed significantly prolonged APD in patient-derived V-like CMs compared with control cells, while Fatima *et al.*<sup>[52]</sup>, showed a tendency to prolonged APD not statistically significant. Relevant to this point Terrenoire *et al.*<sup>[50]</sup>, comment that the relatively depolarized diastolic membrane potentials at this embryonic developmental stage inactivate sodium channels and consequently minimize contributions of sodium channel activity to AP<sup>[53]</sup>. Relatively to sodium current Ma *et al.*<sup>[51]</sup> found that TTX-sensitive I<sub>NaL</sub> was significantly larger in patient-derived hiPSC-CMs compared with control hiPSC-CMs<sup>[51]</sup>, while Fatima *et al.*<sup>[52]</sup>



showed that time-to-peak for sodium current and time to 90% of inactivation of the Nav1.5 were significantly longer in the LQT3-CM<sup>[52]</sup>. In agreement with previous findings<sup>[50]</sup>, mexiletine reduced the late Na<sup>+</sup> current and shortened the APD in patient hiPSC-CMs<sup>[51]</sup>.

**LQT8/Timothy syndrome:** Timothy syndrome (TS) is a multi-system disorder characterized by cardiac, hand, facial and neurodevelopmental features that include QT prolongation, webbed fingers and toes, flattened nasal bridge, low-set ears, small upper jaw, thin upper lip, and characteristic features of autism or autistic spectrum disorders (see [www.orpha.net](http://www.orpha.net)).

TS is caused by mutations in the *CACNA1C* gene and is inherited as autosomal dominant trait. The gene codifies for Cav1.2 channel, the main L-type channel in the mammalian heart that is essential for generating the cardiac action potential and for excitation contraction coupling<sup>[54]</sup>.

To date, just one paper reported the modeling of this syndrome using hiPSC: 16 iPSC lines from two TS patients, and 10 control lines from two unrelated individuals were generated using the classic retroviruses, and CMs were then obtained spontaneously from EBs<sup>[55]</sup>.

A prolonged AP was observed in TS-V-like CMs, while no differences were observed in A-like and N-like cells<sup>[55]</sup>. The L-type Ca<sup>2+</sup> channel current had significantly reduced voltage-dependent inactivation in TS-CMs compared to control cells<sup>[55]</sup>. In addition, the TS-CMs exhibited a large number of depolarizing events that failed to trigger a full AP, similar to delayed afterdepolarizations (DADs) that arise after ectopic release of Ca<sup>2+</sup> from the sarcoplasmic reticulum and which are associated with cardiac arrhythmias<sup>[55]</sup>. Moreover, the Ca<sup>2+</sup> elevations in spontaneously contracting TS-CMs were more prolonged and more irregular than those of control CMs<sup>[55]</sup>. Finally, roscovitine rescued the electrophysiological properties of TS- CMs by increasing Cav1.2 voltage-dependent inactivation, reducing the APD, and decreasing the frequency of abnormal depolarizing events<sup>[55]</sup>.

**Catecholaminergic polymorphic ventricular tachycardia:** Another inherited cardiac disorder that was studied using hiPSC-CMs is catecholaminergic polymorphic ventricular tachycardia (CPVT) that is characterized by emotional and physical stress-induced ventricular tachyarrhythmia, syncope and sudden cardiac death in children and young adults. Two type of CPVT have been described: the autosomal dominant form (CPVT1) linked to mutations in the cardiac ryanodine receptor type 2 gene (*RYR2*) and a rare autosomal recessive form (CPVT2) caused by mutations in the calsequestrin-2 gene (*CASQ2*)<sup>[56]</sup>.

*RYR2* gene encode for the principal Ca<sup>2+</sup>-releasing channel expressed in the membrane of the sarcoplasmic reticulum (SR). Studies based on *in vitro* expression of mutant *RYR2* in heterologous cell systems and

transgenic mice carrying specific *RYR2* mutations suggested that arrhythmias in CPVT1 are due to the diastolic Ca<sup>2+</sup> leak from the SR following catecholaminergic stimulation<sup>[57]</sup>. This leakage may lead to develop DADs through activation of the membrane Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, which can eventually result in triggered activity<sup>[58]</sup>.

The *CASQ2* gene encodes for a high-capacity, low affinity Ca<sup>2+</sup> binding glycoprotein located inside the SR and involve in excitation-contraction coupling process<sup>[59]</sup>. The functional alterations in intracellular Ca<sup>2+</sup> handling resulting from the mutated *CASQ2* gene may cause DADs<sup>[60]</sup>. In 2011, Fatima and his group described the hiPSC generation from a patient with CPVT1 carrying the mutation F2483I in *RYR2* gene<sup>[61]</sup>. The authors differentiated hiPSC-CMs from fibroblasts of the CPVT patient and after 20-30 d of culture the cells were electrophysiologically analyzed. Frequent DADs and arrhythmias in CPVT-CMs exposed to adrenergic agonists were consistently observed. Furthermore, abnormal sensitivity to phosphorylation and cAMP-mediated regulation, together with the tendency for I<sub>Ca</sub>-triggered Ca<sup>2+</sup> release to continue following repolarization have been found<sup>[61]</sup>. These first results using hiPSCs-based cardiac model validated the earlier hypothesis obtained only with animal models. Different groups have obtained similar results: Kujala has demonstrated that in addition to DADs, CPVT-CMs with P2328S *RYR2* mutation displayed also EADs which may be involved in cardiac arrhythmogenesis of their patients<sup>[62]</sup>. Zhang reported specific analyses on calcium current (I<sub>Ca</sub>) and Na<sup>+</sup>-Ca<sup>2+</sup> exchanger current (I<sub>NCX</sub>): in this case RyR2 F2483I mutant CMs have aberrant unitary Ca<sup>2+</sup> signaling, smaller I<sub>NCX</sub> reflecting smaller Ca<sup>2+</sup>-stores, higher I<sub>Ca</sub>-gated Ca<sup>2+</sup>-release gains, and sensitized adrenergic regulation, consistent with functionally altered Ca<sup>2+</sup>-release profile of CPVT syndrome<sup>[63]</sup>. Jung also reported the hiPSC generation of a 24-year-old woman with a diagnosis of familial CPVT1 with S406L missense mutation in the *RYR2* gene<sup>[64]</sup>. In addition to presenting the electrophysiological properties of CPVT-CMs, the rescue capacity of dantrolene, which is believed to stabilize skeletal and cardiac RYRs by binding to a N-terminal sequence, is analyzed. Treatment with dantrolene restored normal Ca<sup>2+</sup> spark properties in CPVT-CMs under basal conditions and corrected S406L-*RYR2* hyperactivity induced by adrenergic stimulation, with minimal effects in control cells; moreover, the same drug completely abolished DADs and triggered arrhythmias<sup>[64]</sup>.

Two more studies evaluated the effects of different drugs onto CPVT-CMs: Itzhaki reported the positive effects of flecainide, an antiarrhythmic agent, and thapsigargin, a β-blocker on CMs carrying the *RYR2* M4109R mutation<sup>[65]</sup>, while Di Pasquale *et al.*<sup>[66]</sup> reported the rescue of the arrhythmic phenotype induced by catecholaminergic stress by KN-93, an antiarrhythmic



drug that inhibits  $\text{Ca}^{2+}$ /calmodulin-dependent serine-threonine protein kinase II on CMs carrying the *RYR2 E2311D* mutation.

An additional study focused on the autosomal recessive form of the disease caused by the missense mutation D307H in *CASQ2*<sup>[67]</sup>. In agreement with previous observations, isoproterenol stimulation caused DADs, oscillatory arrhythmic prepotentials and after-contractions, and diastolic intracellular calcium rising in *CASQ2*-derived CPVT model<sup>[67]</sup>. Most importantly, electron microscopy showed that CMs derived from CPVT patients present an immature morphology with less-organized myofibrils, enlarged SR cisternae, and reduced number of caveolae<sup>[67]</sup>. These data confirm previous findings derived from knock-out and knock-in *Casq2* mice that showed ultrastructural abnormalities in the SR<sup>[68,69]</sup>.

### Arrhythmogenic right ventricular cardiomyopathy:

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a heritable primary cardiac disease characterized by the replacement of CMs with fatty or fibrofatty tissue<sup>[70]</sup>. ARVC has an autosomal dominant trait with reduced penetrance; approximately 40%-50% of ARVC patients have a mutation identified in one of several genes encoding components of the desmosome, which can help confirm a diagnosis of ARVC<sup>[71]</sup>.

Also ARVC has been modeled using hiPSC by three independent groups starting from skin biopsies of five patients: retroviruses were used to transduce patient's-derived fibroblasts and CMs were differentiated with the spontaneous 3D differentiation protocol<sup>[72-74]</sup>. The five patients carried different alterations in Plakophilin-2 gene (*PKP2*), a structural component of desmosome<sup>[75]</sup>: a heterozygous L614P mutation<sup>[72]</sup>; a heterozygous insertion resulting in a frame shift from amino acid 324 to a stop codon in position 335 (A324fs335X) and a heterozygous deletion resulting in replacement of threonine by serine in position 50 and in a frame shift leading to a stop codon in position 110 (p.T50SfsX110)<sup>[73]</sup>; and, finally, a homozygous mutation that causes cryptic splicing with a 7-nucleotide deletion in exon 12, leading to frame-shift of the carboxy-terminal amino acids, whose results were further confirmed with cells carrying a heterozygous c.2013delC in exon 10 of *PKP2*<sup>[74]</sup>.

Common observations of ARVC-CMs carrying the heterozygous conditions reported specific down-regulation of *PKP2* and its interactor plakoglobin both at mRNA and at protein level<sup>[72,73]</sup>. Transmission electron microscopy (TEM) analysis displayed larger ARVC-CMs with less organized, thicker and more pleomorphic Z-bands compared with the Z-bands in control cells<sup>[72]</sup>, as well as distorted desmosomes associated with accumulation of lipid droplets, that were strongly enhanced in lipogenic media<sup>[72,73]</sup>. Relevant to this point, the addition of the GSK-3 $\beta$  inhibitor BIO strongly suppress the effects of the

lipogenic stress<sup>[73]</sup>.

The phenotypic study of CMs derived from the patient carrying the homozygous mutation led to similar conclusions: an abnormal translocation of plakoglobin proteins associated with very low  $\beta$ -catenin activity<sup>[74]</sup>. In addition, the culture of these ARVC-CMs, devoid of a correct *PKP2* carboxy terminus, in a lipogenic medium additionally containing PPAR- $\gamma$  activating drugs, demonstrated exaggerated lipogenesis and pronounced apoptosis, that can be prevented by the addition of PPAR- $\gamma$  antagonists<sup>[74]</sup>. Similar results were obtained with CMs derived from the patient carrying a heterozygous c.2013delC mutation<sup>[74]</sup>.

The rescue of the pathogenic phenotypes was achieved by introducing the wild-type *PKP2* gene back into mutant hiPSC-CMs, thus suggesting that mutation of *PKP2* is sufficient to induce the pathological features observed in ARVC-CMs<sup>[74]</sup>.

Interestingly, the same cells were used by Cerrone *et al.*<sup>[76]</sup> to verify a *PKP2*-mediated modulation of  $I_{\text{Na}}$ : indeed, as already shown using other cellular systems, ARVC-CMs showed drastically reduced  $I_{\text{Na}}$  and the deficit was restored by re-introducing the wild type *PKP2* gene.

## CONCLUSION

Since the discovery of the cellular reprogramming method followed by *in vitro* CM differentiation, several findings regarding human cardiac cells, especially those exhibiting a pathological phenotype, have been made possible. We can discuss how close or how far is the relation between hiPSC-CMs and the normal counterpart, but surely the possibility to obtain human CMs in a culture dish is adding a missing item to the scientific community. Every model has its own limitations and this is true also for hiPSC-CMs. Indeed, data from the literature reports the most evident and predictable phenotypes, leaving aside those perhaps more interesting but still unexplainable. However, as shown, many patients' features are found in these hiPSC-CMs, starting from the prolongation of the APD corresponding to the elongation of the cardiac QT interval, or EAD or DAD events equivalent to arrhythmic episodes, or the presence of cellular lipid droplets primarily found in cardiac biopsies of patients with ARVC<sup>[77]</sup>.

Human CMs have been derived also from cells of patients carrying other genetic disorders involving cardiac pathologies such as Leopard syndrome<sup>[78]</sup>, Pompe disease<sup>[79]</sup>, Duchenne muscular dystrophy<sup>[80]</sup>, Friedreich's ataxia<sup>[81]</sup>, and Fabry disease<sup>[82]</sup>.

In all of the cases, patients-derived CMs showed the main characteristic traits of the related pathology: hypertrophic cells in Leopard<sup>[78]</sup>; lower  $\beta$ -glucosidase activity, lower markers of metabolism, and higher glycogen content in Pompe<sup>[79]</sup>; a dystrophic gene expression profile in Duchenne<sup>[80]</sup>; impaired mito-



chondrial homeostasis in Friedreich's ataxia<sup>[81]</sup>; and globotriaosylceramide accumulation in Fabry disease<sup>[82]</sup>. Moreover, patients-derived CMs represent a powerful model to test type and dosage of clinically used drugs, laying the basis for a personalized therapy.

Increasing the number and diversifying the type of modeled patients will surely improve the understanding of the biological mechanism that leads to the considered disease. Nevertheless the big ongoing efforts reside in eliminating any kind of interaction of the reprogramming technology with patient's DNA and in identifying an easily reproducible protocol that leads to CM differentiation. Then, the non integrative Sendai-based reprogramming approach, followed by a chemical defined differentiation medium will surely solve the issue of reproducible cardiomyocyte differentiation.

## REFERENCES

- Takahashi K, Yamanaka S.** Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S.** Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: 18035408 DOI: 10.1016/j.cell.2007.11.019]
- Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA.** Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007; **318**: 1917-1920 [PMID: 18029452 DOI: 10.1126/science.1151526]
- Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ.** Disease-specific induced pluripotent stem cells. *Cell* 2008; **134**: 877-886 [PMID: 18691744 DOI: 10.1016/j.cell.2008.07.041]
- Wobus AM.** Potential of embryonic stem cells. *Mol Aspects Med* 2001; **22**: 149-164 [PMID: 11470141 DOI: 10.1016/S0098-2997(01)00006-1]
- Wobus AM, Rohwedel J, Maltsev V, Hescheler J.** Development of cardiomyocytes expressing cardiac-specific genes, action potentials, and ionic channels during embryonic stem cell-derived cardiogenesis. *Ann N Y Acad Sci* 1995; **752**: 460-469 [PMID: 7538739 DOI: 10.1111/j.1749-6632.1995.tb17456.x]
- Elliott DA, Braam SR, Koutsis K, Ng ES, Jenny R, Lagerqvist EL, Biben C, Hatzistavrou T, Hirst CE, Yu QC, Skelton RJ, Ward-van Oostwaard D, Lim SM, Khammy O, Li X, Hawes SM, Davis RP, Goulburn AL, Passier R, Prall OW, Haynes JM, Pouton CW, Kaye DM, Mummery CL, Elefanti AG, Stanley EG.** NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. *Nat Methods* 2011; **8**: 1037-1040 [PMID: 22020065 DOI: 10.1038/nmeth.1740]
- Burridge PW, Matsa E, Shukla P, Lin ZC, Churko JM, Ebert AD, Lan F, Diecke S, Huber B, Mordwinkin NM, Plews JR, Abilez OJ, Cui B, Gold JD, Wu JC.** Chemically defined generation of human cardiomyocytes. *Nat Methods* 2014; **11**: 855-860 [PMID: 24930130 DOI: 10.1038/nmeth.2999]
- Yang X, Pabon L, Murry CE.** Engineering adolescence: maturation of human pluripotent stem cell-derived cardiomyocytes. *Circ Res* 2014; **114**: 511-523 [PMID: 24481842 DOI: 10.1161/CIRCRESAHA.114.300558]
- Sartiani L, Bettiol E, Stillitano F, Mugelli A, Cerbai E, Jaconi ME.** Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: a molecular and electrophysiological approach. *Stem Cells* 2007; **25**: 1136-1144 [PMID: 17255522 DOI: 10.1634/stemcells.2006-0466]
- Li S, Chen G, Li RA.** Calcium signalling of human pluripotent stem cell-derived cardiomyocytes. *J Physiol* 2013; **591**: 5279-5290 [PMID: 24018947 DOI: 10.1113/jphysiol.2013.256495]
- Dolnikov K, Shilkrot M, Zeevi-Levin N, Gerech-Nir S, Amit M, Danon A, Itskovitz-Eldor J, Binah O.** Functional properties of human embryonic stem cell-derived cardiomyocytes: intracellular Ca<sup>2+</sup> handling and the role of sarcoplasmic reticulum in the contraction. *Stem Cells* 2006; **24**: 236-245 [PMID: 16322641 DOI: 10.1634/stemcells.2005-0036]
- Lieu DK, Liu J, Siu CW, McEnerney GP, Tse HF, Abu-Khalil A, Huser T, Li RA.** Absence of transverse tubules contributes to non-uniform Ca(2+) wavefronts in mouse and human embryonic stem cell-derived cardiomyocytes. *Stem Cells Dev* 2009; **18**: 1493-1500 [PMID: 19290776 DOI: 10.1089/scd.2009.0052]
- Ivashchenko CY, Pipes GC, Lozinskaya IM, Lin Z, Xiaoping X, Needle S, Grygielko ET, Hu E, Toomey JR, Lepore JJ, Willette RN.** Human-induced pluripotent stem cell-derived cardiomyocytes exhibit temporal changes in phenotype. *Am J Physiol Heart Circ Physiol* 2013; **305**: H913-H922 [PMID: 23832699 DOI: 10.1152/ajpheart.00819.2012]
- Lundy SD, Zhu WZ, Regnier M, Laflamme MA.** Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells Dev* 2013; **22**: 1991-2002 [PMID: 23461462 DOI: 10.1089/scd.2012.0490]
- Kamakura T, Makiyama T, Sasaki K, Yoshida Y, Wuriyanghai Y, Chen J, Hattori T, Ohno S, Kita T, Horie M, Yamanaka S, Kimura T.** Ultrastructural maturation of human-induced pluripotent stem cell-derived cardiomyocytes in a long-term culture. *Circ J* 2013; **77**: 1307-1314 [PMID: 23400258 DOI: 10.1253/circj.CJ-12-0987]
- Blazeski A, Zhu R, Hunter DW, Weinberg SH, Boheler KR, Zambidis ET, Tung L.** Electrophysiological and contractile function of cardiomyocytes derived from human embryonic stem cells. *Prog Biophys Mol Biol* 2012; **110**: 178-195 [PMID: 22958937 DOI: 10.1016/j.pbiomolbio.2012.07.012]
- Maron BJ, Maron MS.** Hypertrophic cardiomyopathy. *Lancet* 2013; **381**: 242-255 [PMID: 22874472 DOI: 10.1016/S0140-6736(12)60397-3]
- Lan F, Lee AS, Liang P, Sanchez-Freire V, Nguyen PK, Wang L, Han L, Yen M, Wang Y, Sun N, Abilez OJ, Hu S, Ebert AD, Navarrete EG, Simmons CS, Wheeler M, Pruitt B, Lewis R, Yamaguchi Y, Ashley EA, Bers DM, Robbins RC, Longaker MT, Wu JC.** Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells. *Cell Stem Cell* 2013; **12**: 101-113 [PMID: 23290139 DOI: 10.1016/j.stem.2012.10.010]
- Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ.** Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ Res* 2009; **104**: e30-e41 [PMID: 19213953 DOI: 10.1161/CIRCRESAHA.108.192237]
- Ku L, Feiger J, Taylor M, Mestroni L.** Cardiologist patient page. Familial dilated cardiomyopathy. *Circulation* 2003; **108**: e118-e121 [PMID: 14581388 DOI: 10.1161/01.CIR.0000097493.70422.50]
- Hershberger RE, Siegfried JD.** Update 2011: clinical and genetic issues in familial dilated cardiomyopathy. *J Am Coll Cardiol* 2011; **57**: 1641-1649 [PMID: 21492761 DOI: 10.1016/j.jacc.2011.01.015]
- Parks SB, Kushner JD, Nauman D, Burgess D, Ludwigs S, Peterson A, Li D, Jakobs P, Litt M, Porter CB, Rahko PS, Hershberger RE.** Lamin A/C mutation analysis in a cohort of 324 unrelated patients with idiopathic or familial dilated cardiomyopathy. *Am Heart J* 2008; **156**: 161-169 [PMID: 18585512 DOI: 10.1016/j.ahj.2008.01.026]
- Kubben N, Voncken JW, Konings G, van Weeghel M, van den Hoogenhof MM, Gijbels M, van Erk A, Schoonderwoerd K, van den Bosch B, Dahlmans V, Calis C, Houten SM, Misteli T, Pinto YM.** Post-natal myogenic and adipogenic developmental: defects and metabolic impairment upon loss of A-type lamins. *Nucleus* 2011; **2**: 195-207 [PMID: 21818413 DOI: 10.4161/nucl.2.3.15731]
- Siu CW, Lee YK, Ho JC, Lai WH, Chan YC, Ng KM, Wong LY, Au KW, Lau YM, Zhang J, Lay KW, Colman A, Tse HF.** Modeling of lamin A/C mutation premature cardiac aging using patient-specific induced pluripotent stem cells. *Aging (Albany NY)* 2012; **4**:



- 803-822 [PMID: 23362510]
- 26 **Tse HF**, Ho JC, Choi SW, Lee YK, Butler AW, Ng KM, Siu CW, Simpson MA, Lai WH, Chan YC, Au KW, Zhang J, Lay KW, Esteban MA, Nicholls JM, Colman A, Sham PC. Patient-specific induced-pluripotent stem cells-derived cardiomyocytes recapitulate the pathogenic phenotypes of dilated cardiomyopathy due to a novel DES mutation identified by whole exome sequencing. *Hum Mol Genet* 2013; **22**: 1395-1403 [PMID: 23300193 DOI: 10.1093/hmg/dd556]
  - 27 **Sun N**, Yazawa M, Liu J, Han L, Sanchez-Freire V, Abilez OJ, Navarrete EG, Hu S, Wang L, Lee A, Pavlovic A, Lin S, Chen R, Hajjar RJ, Snyder MP, Dolmetsch RE, Butte MJ, Ashley EA, Longaker MT, Robbins RC, Wu JC. Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy. *Sci Transl Med* 2012; **4**: 130ra47 [PMID: 22517884 DOI: 10.1126/scitranslmed.3003552]
  - 28 **Clarke SL**, Bowron A, Gonzalez IL, Groves SJ, Newbury-Ecob R, Clayton N, Martin RP, Tsai-Goodman B, Garratt V, Ashworth M, Bowen VM, McCurdy KR, Damin MK, Spencer CT, Toth MJ, Kelley RI, Steward CG. Barth syndrome. *Orphanet J Rare Dis* 2013; **8**: 23 [PMID: 23398819 DOI: 10.1186/1750-1172-8-23]
  - 29 **Bione S**, D'Adamo P, Maestrini E, Gedeon AK, Bolhuis PA, Toniolo D. A novel X-linked gene, G4.5, is responsible for Barth syndrome. *Nat Genet* 1996; **12**: 385-389 [PMID: 8630491 DOI: 10.1038/ng0496-385]
  - 30 **Wang G**, McCain ML, Yang L, He A, Pasqualini FS, Agarwal A, Yuan H, Jiang D, Zhang D, Zangi L, Geva J, Roberts AE, Ma Q, Ding J, Chen J, Wang DZ, Li K, Wang J, Wanders RJ, Kulik W, Vaz FM, Laffamme MA, Murry CE, Chien KR, Kelley RI, Church GM, Parker KK, Pu WT. Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies. *Nat Med* 2014; **20**: 616-623 [PMID: 24813252 DOI: 10.1038/nm.3545]
  - 31 **Crotti L**, Celano G, Dagradi F, Schwartz PJ. Congenital long QT syndrome. *Orphanet J Rare Dis* 2008; **3**: 18 [PMID: 18606002 DOI: 10.1186/1750-1172-3-18]
  - 32 **Giudicessi JR**, Ackerman MJ. Genotype- and phenotype-guided management of congenital long QT syndrome. *Curr Probl Cardiol* 2013; **38**: 417-455 [PMID: 24093767 DOI: 10.1016/j.cpcardiol.2013.08.001]
  - 33 **Morita H**, Wu J, Zipes DP. The QT syndromes: long and short. *Lancet* 2008; **372**: 750-763 [PMID: 18761222 DOI: 10.1016/S0140-6736(08)61307-0]
  - 34 **Wang DW**, Yazawa K, George AL, Bennett PB. Characterization of human cardiac Na<sup>+</sup> channel mutations in the congenital long QT syndrome. *Proc Natl Acad Sci USA* 1996; **93**: 13200-13205 [PMID: 8917568]
  - 35 **Liu M**, Yang KC, Dudley SC. Cardiac sodium channel mutations: why so many phenotypes? *Nat Rev Cardiol* 2014; **11**: 607-615 [PMID: 24958080 DOI: 10.1038/nrcardio.2014.85]
  - 36 **Moretti A**, Bellin M, Welling A, Jung CB, Lam JT, Bott-Flügel L, Dorn T, Goedel A, Höhnke C, Hofmann F, Seyfarth M, Sinnecker D, Schömgig A, Laugwitz KL. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. *N Engl J Med* 2014; **363**: 1397-1409 [PMID: 20660394 DOI: 10.1056/NEJMoa0908679]
  - 37 **Itzhaki I**, Maizels L, Huber I, Zwi-Dantsis L, Caspi O, Winterstern A, Feldman O, Gepstein A, Arbel G, Hammerman H, Boulous M, Gepstein L. Modelling the long QT syndrome with induced pluripotent stem cells. *Nature* 2011; **471**: 225-229 [PMID: 21240260 DOI: 10.1038/nature09747]
  - 38 **Matsa E**, Rajamohan D, Dick E, Young L, Mellor I, Staniforth A, Denning C. Drug evaluation in cardiomyocytes derived from human induced pluripotent stem cells carrying a long QT syndrome type 2 mutation. *Eur Heart J* 2011; **32**: 952-962 [PMID: 21367833 DOI: 10.1093/eurheartj/ehr073]
  - 39 **Yazawa M**, Dolmetsch RE. Modeling Timothy syndrome with iPS cells. *J Cardiovasc Transl Res* 2013; **6**: 1-9 [PMID: 23299782 DOI: 10.1007/s12265-012-9444-x]
  - 40 **Davis RP**, Casini S, van den Berg CW, Hoekstra M, Remme CA, Dambrot C, Salvatori D, Oostwaard DW, Wilde AA, Bezzina CR, Verkerk AO, Freund C, Mummery CL. Cardiomyocytes derived from pluripotent stem cells recapitulate electrophysiological characteristics of an overlap syndrome of cardiac sodium channel disease. *Circulation* 2012; **125**: 3079-3091 [PMID: 22647976 DOI: 10.1161/CIRCULATIONAHA.111.066092]
  - 41 **Egashira T**, Yuasa S, Suzuki T, Aizawa Y, Yamakawa H, Matsuhashi T, Ohno Y, Tohyama S, Okata S, Seki T, Kuroda Y, Yae K, Hashimoto H, Tanaka T, Hattori F, Sato T, Miyoshi S, Takatsuki S, Murata M, Kurokawa J, Furukawa T, Makita N, Aiba T, Shimizu W, Horie M, Kamiya K, Kodama I, Ogawa S, Fukuda K. Disease characterization using LQTS-specific induced pluripotent stem cells. *Cardiovasc Res* 2012; **95**: 419-429 [PMID: 22739119 DOI: 10.1093/cvr/cvs206]
  - 42 **Lahti AL**, Kujala VJ, Chapman H, Koivisto AP, Pekkanen-Mattila M, Kerkelä E, Hyttinen J, Kontula K, Swan H, Conklin BR, Yamanaka S, Silvennoinen O, Aalto-Setälä K. Model for long QT syndrome type 2 using human iPS cells demonstrates arrhythmogenic characteristics in cell culture. *Dis Model Mech* 2012; **5**: 220-230 [PMID: 22052944 DOI: 10.1242/dmm.008409]
  - 43 **Bellin M**, Marchetto MC, Gage FH, Mummery CL. Induced pluripotent stem cells: the new patient? *Nat Rev Mol Cell Biol* 2012; **13**: 713-726 [PMID: 23034453 DOI: 10.1038/nrm3448]
  - 44 **Snyders DJ**. Structure and function of cardiac potassium channels. *Cardiovasc Res* 1999; **42**: 377-390 [PMID: 10533574 DOI: 10.1016/S0008-6363(99)00071-1]
  - 45 **Mehta A**, Sequiera GL, Ramachandra CJ, Sudibyo Y, Chung Y, Sheng J, Wong KY, Tan TH, Wong P, Liew R, Shim W. Re-trafficking of hERG reverses long QT syndrome 2 phenotype in human iPS-derived cardiomyocytes. *Cardiovasc Res* 2014; **102**: 497-506 [PMID: 24623279 DOI: 10.1093/cvr/cvu060]
  - 46 **Bellin M**, Casini S, Davis RP, D'Aniello C, Haas J, Ward-van Oostwaard D, Tertoolen LG, Jung CB, Elliott DA, Welling A, Laugwitz KL, Moretti A, Mummery CL. Isogenic human pluripotent stem cell pairs reveal the role of a KCNH2 mutation in long-QT syndrome. *EMBO J* 2013; **32**: 3161-3175 [PMID: 24213244 DOI: 10.1038/emboj.2013.240]
  - 47 **Zhou Z**, Gong Q, Epstein ML, January CT. HERG channel dysfunction in human long QT syndrome. Intracellular transport and functional defects. *J Biol Chem* 1998; **273**: 21061-21066 [PMID: 9694858 DOI: 10.1074/jbc.273.33.21061]
  - 48 **Hoekstra M**, Mummery CL, Wilde AA, Bezzina CR, Verkerk AO. Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias. *Front Physiol* 2012; **3**: 346 [PMID: 23015789 DOI: 10.3389/fphys.2012.00346]
  - 49 **Gellens ME**, George AL, Chen LQ, Chahine M, Horn R, Barchi RL, Kallen RG. Primary structure and functional expression of the human cardiac tetrodotoxin-insensitive voltage-dependent sodium channel. *Proc Natl Acad Sci USA* 1992; **89**: 554-558 [PMID: 1309946]
  - 50 **Terrenoire C**, Wang K, Tung KW, Chung WK, Pass RH, Lu JT, Jean JC, Omari A, Sampson KJ, Kotton DN, Keller G, Kass RS. Induced pluripotent stem cells used to reveal drug actions in a long QT syndrome family with complex genetics. *J Gen Physiol* 2013; **141**: 61-72 [PMID: 23277474 DOI: 10.1085/jgp.201210899]
  - 51 **Ma D**, Wei H, Zhao Y, Lu J, Li G, Sahib NB, Tan TH, Wong KY, Shim W, Wong P, Cook SA, Liew R. Modeling type 3 long QT syndrome with cardiomyocytes derived from patient-specific induced pluripotent stem cells. *Int J Cardiol* 2013; **168**: 5277-5286 [PMID: 23998552 DOI: 10.1016/j.ijcard.2013.08.015]
  - 52 **Fatima A**, Kaifeng S, Dittmann S, Xu G, Gupta MK, Linke M, Zechner U, Nguemo F, Miltling H, Farr M, Hescheler J, Sarić T. The disease-specific phenotype in cardiomyocytes derived from induced pluripotent stem cells of two long QT syndrome type 3 patients. *PLoS One* 2013; **8**: e83005 [PMID: 24349418 DOI: 10.1371/journal.pone.0083005]
  - 53 **Ruan Y**, Liu N, Priori SG. Sodium channel mutations and arrhythmias. *Nat Rev Cardiol* 2009; **6**: 337-348 [PMID: 19377496 DOI: 10.1038/nrcardio.2009.44]
  - 54 **Shaw RM**, Colecraft HM. L-type calcium channel targeting and local signalling in cardiac myocytes. *Cardiovasc Res* 2013; **98**:



- 177-186 [PMID: 23417040 DOI: 10.1093/cvr/cvt021]
- 55 **Yazawa M**, Hsueh B, Jia X, Pasca AM, Bernstein JA, Hallmayer J, Dolmetsch RE. Using induced pluripotent stem cells to investigate cardiac phenotypes in Timothy syndrome. *Nature* 2011; **471**: 230-234 [PMID: 21307850 DOI: 10.1038/nature09855]
  - 56 **Priori SG**, Chen SR. Inherited dysfunction of sarcoplasmic reticulum Ca<sup>2+</sup> handling and arrhythmogenesis. *Circ Res* 2011; **108**: 871-883 [PMID: 21454795 DOI: 10.1161/CIRCRESAHA.110.226845]
  - 57 **Chelu MG**, Wehrens XH. Sarcoplasmic reticulum calcium leak and cardiac arrhythmias. *Biochem Soc Trans* 2007; **35**: 952-956 [PMID: 17956253 DOI: 10.1042/BST0350952]
  - 58 **Cerrone M**, Colombi B, Santoro M, di Barletta MR, Scelsi M, Villani L, Napolitano C, Priori SG. Bidirectional ventricular tachycardia and fibrillation elicited in a knock-in mouse model carrier of a mutation in the cardiac ryanodine receptor. *Circ Res* 2005; **96**: e77-e82 [PMID: 15890976 DOI: 10.1161/01.RES.0000169067.51055.72]
  - 59 **Beard NA**, Laver DR, Dulhunty AF. Calsequestrin and the calcium release channel of skeletal and cardiac muscle. *Prog Biophys Mol Biol* 2004; **85**: 33-69 [PMID: 15050380 DOI: 10.1016/j.pbiomolbio.2003.07.001]
  - 60 **Iyer V**, Aroundas AA. Unraveling the mechanisms of catecholaminergic polymorphic ventricular tachycardia. *Conf Proc IEEE Eng Med Biol Soc* 2006; **Suppl**: 6761-6764 [PMID: 17959506 DOI: 10.1109/IEMBS.2006.260941]
  - 61 **Fatima A**, Xu G, Shao K, Papadopoulos S, Lehmann M, Arnáiz-Cot JJ, Rosa AO, Nguemo F, Matzkies M, Dittmann S, Stone SL, Linke M, Zechner U, Beyer V, Hennies HC, Rosenkranz S, Klauke B, Parwani AS, Haverkamp W, Pfitzer G, Farr M, Cleemann L, Morad M, Milting H, Hescheler J, Saric T. In vitro modeling of ryanodine receptor 2 dysfunction using human induced pluripotent stem cells. *Cell Physiol Biochem* 2011; **28**: 579-592 [PMID: 22178870 DOI: 10.1159/000335753]
  - 62 **Kujala K**, Paavola J, Lahti A, Larsson K, Pekkanen-Mattila M, Viitasalo M, Lahtinen AM, Toivonen L, Kontula K, Swan H, Laine M, Silvennoinen O, Aalto-Setälä K. Cell model of catecholaminergic polymorphic ventricular tachycardia reveals early and delayed afterdepolarizations. *PLoS One* 2012; **7**: e44660 [PMID: 22962621 DOI: 10.1371/journal.pone.0044660]
  - 63 **Zhang XH**, Haviland S, Wei H, Sarić T, Fatima A, Hescheler J, Cleemann L, Morad M. Ca<sup>2+</sup> signaling in human induced pluripotent stem cell-derived cardiomyocytes (iPS-CM) from normal and catecholaminergic polymorphic ventricular tachycardia (CPVT)-afflicted subjects. *Cell Calcium* 2013; **54**: 57-70 [PMID: 23684427 DOI: 10.1016/j.ceca.2013.04.004]
  - 64 **Jung CB**, Moretti A, Mederos y Schnitzler M, Iop L, Storch U, Bellin M, Dorn T, Ruppenthal S, Pfeiffer S, Goedel A, Dirschinger RJ, Seyfarth M, Lam JT, Sinnecker D, Gudermann T, Lipp P, Laugwitz KL. Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia. *EMBO Mol Med* 2012; **4**: 180-191 [PMID: 22174035 DOI: 10.1002/emmm.201100194]
  - 65 **Itzhaki I**, Maizels L, Huber I, Gepstein A, Arbel G, Caspi O, Miller L, Belhassen B, Nof E, Glikson M, Gepstein L. Modeling of catecholaminergic polymorphic ventricular tachycardia with patient-specific human-induced pluripotent stem cells. *J Am Coll Cardiol* 2012; **60**: 990-1000 [PMID: 22749309 DOI: 10.1016/j.jacc.2012.02.066]
  - 66 **Di Pasquale E**, Lodola F, Miragoli M, Denegri M, Avelino-Cruz JE, Buonocore M, Nakahama H, Portararo P, Bloise R, Napolitano C, Condorelli G, Priori SG. CaMKII inhibition rectifies arrhythmic phenotype in a patient-specific model of catecholaminergic polymorphic ventricular tachycardia. *Cell Death Dis* 2013; **4**: e843 [PMID: 24113177 DOI: 10.1038/cddis.2013.369]
  - 67 **Novak A**, Barad L, Zeevi-Levin N, Shick R, Shtrichman R, Lorber A, Itskovitz-Eldor J, Binah O. Cardiomyocytes generated from CPVT307H patients are arrhythmogenic in response to  $\beta$ -adrenergic stimulation. *J Cell Mol Med* 2012; **16**: 468-482 [PMID: 22050625 DOI: 10.1111/j.1582-4934.2011.01476.x]
  - 68 **Rizzi N**, Liu N, Napolitano C, Nori A, Turcato F, Colombi B, Biciato S, Arcelli D, Spedito A, Scelsi M, Villani L, Esposito G, Boncompagni S, Protasi F, Volpe P, Priori SG. Unexpected structural and functional consequences of the R33Q homozygous mutation in cardiac calsequestrin: a complex arrhythmogenic cascade in a knock in mouse model. *Circ Res* 2008; **103**: 298-306 [PMID: 18583715 DOI: 10.1161/CIRCRESAHA.108.171660]
  - 69 **Knollmann BC**, Chopra N, Hlaing T, Akin B, Yang T, Etensohn K, Knollmann BE, Horton KD, Weissman NJ, Holinstat I, Zhang W, Roden DM, Jones LR, Franzini-Armstrong C, Pfeifer K. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca<sup>2+</sup> release, and catecholaminergic polymorphic ventricular tachycardia. *J Clin Invest* 2006; **116**: 2510-2520 [PMID: 16932808 DOI: 10.1172/JCI29128]
  - 70 **Basso C**, Bauce B, Corrado D, Thiene G. Pathophysiology of arrhythmogenic cardiomyopathy. *Nat Rev Cardiol* 2012; **9**: 223-233 [PMID: 22124316 DOI: 10.1038/nrcardio.2011.173]
  - 71 **Sen-Chowdhry S**, Syrris P, McKenna WJ. Role of genetic analysis in the management of patients with arrhythmogenic right ventricular dysplasia/cardiomyopathy. *J Am Coll Cardiol* 2007; **50**: 1813-1821 [PMID: 17980246 DOI: 10.1016/j.jacc.2007.08.008]
  - 72 **Ma D**, Wei H, Lu J, Ho S, Zhang G, Sun X, Oh Y, Tan SH, Ng ML, Shim W, Wong P, Liew R. Generation of patient-specific induced pluripotent stem cell-derived cardiomyocytes as a cellular model of arrhythmogenic right ventricular cardiomyopathy. *Eur Heart J* 2013; **34**: 1122-1133 [PMID: 22798562 DOI: 10.1093/eurheartj/ehs226]
  - 73 **Caspi O**, Huber I, Gepstein A, Arbel G, Maizels L, Boulos M, Gepstein L. Modeling of arrhythmogenic right ventricular cardiomyopathy with human induced pluripotent stem cells. *Circ Cardiovasc Genet* 2013; **6**: 557-568 [PMID: 24200905 DOI: 10.1161/CIRCGENETICS.113.000188]
  - 74 **Kim C**, Wong J, Wen J, Wang S, Wang C, Spiering S, Kan NG, Forcales S, Puri PL, Leone TC, Marine JE, Calkins H, Kelly DP, Judge DP, Chen HS. Studying arrhythmogenic right ventricular dysplasia with patient-specific iPSCs. *Nature* 2013; **494**: 105-110 [PMID: 23354045 DOI: 10.1038/nature11799]
  - 75 **Garrod D**, Chidgey M. Desmosome structure, composition and function. *Biochim Biophys Acta* 2008; **1778**: 572-587 [PMID: 17854763 DOI: 10.1016/j.bbamem.2007.07.014]
  - 76 **Cerrone M**, Lin X, Zhang M, Agullo-Pascual E, Pfenniger A, Chkourko Gusky H, Novelli V, Kim C, Tirasawadichai T, Judge DP, Rothenberg E, Chen HS, Napolitano C, Priori SG, Delmar M. Missense mutations in plakophilin-2 cause sodium current deficit and associate with a Brugada syndrome phenotype. *Circulation* 2014; **129**: 1092-1103 [PMID: 24352520 DOI: 10.1161/CIRCULATIONAHA.113.003077]
  - 77 **Fujita S**, Terasaki F, Otsuka K, Katashima T, Kanzaki Y, Kawamura K, Tanaka T, Kitaura Y. Markedly increased intracellular lipid droplets and disruption of intercellular junctions in biopsied myocardium from a patient with arrhythmogenic right ventricular cardiomyopathy. *Heart Vessels* 2008; **23**: 440-444 [PMID: 19037594 DOI: 10.1007/s00380-008-1079-0]
  - 78 **Carvajal-Vergara X**, Sevilla A, D'Souza SL, Ang YS, Schaniel C, Lee DF, Yang L, Kaplan AD, Adler ED, Rozov R, Ge Y, Cohen N, Edelmann LJ, Chang B, Waghay A, Su J, Pardo S, Lichtenbelt KD, Tartaglia M, Gelb BD, Lemischka IR. Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. *Nature* 2010; **465**: 808-812 [PMID: 20535210 DOI: 10.1038/nature09005]
  - 79 **Huang HP**, Chen PH, Hwu WL, Chuang CY, Chien YH, Stone L, Chien CL, Li LT, Chiang SC, Chen HF, Ho HN, Chen CH, Kuo HC. Human Pompe disease-induced pluripotent stem cells for pathogenesis modeling, drug testing and disease marker identification. *Hum Mol Genet* 2011; **20**: 4851-4864 [PMID: 21926084 DOI: 10.1093/hmg/ddr424]
  - 80 **Dick E**, Kalra S, Anderson D, George V, Ritson M, Laval S, Barresi R, Aartsma-Rus A, Lochmuller H, Denning C. Exon skipping and gene transfer restore dystrophin expression in hiPSC-cardiomyocytes harbouring DMD mutations. *Stem Cells Dev* 2013; [PMID: 23786351 DOI: 10.1089/2013.0135]
  - 81 **Hick A**, Wattenhofer-Donzé M, Chintawar S, Tropel P, Simard JP,



- Vaucamps N, Gall D, Lambot L, André C, Reutenauer L, Rai M, Teletin M, Messaddeq N, Schiffmann SN, Viville S, Pearson CE, Pandolfo M, Puccio H. Neurons and cardiomyocytes derived from induced pluripotent stem cells as a model for mitochondrial defects in Friedreich's ataxia. *Dis Model Mech* 2013; **6**: 608-621 [PMID: 23136396 DOI: 10.1242/dmm.010900]
- 82 **Itier JM**, Ret G, Viale S, Sweet L, Bangari D, Caron A, Le-Gall F, Bénichou B, Leonard J, Deleuze JF, Orsini C. Effective clearance of GL-3 in a human iPSC-derived cardiomyocyte model of Fabry disease. *J Inherit Metab Dis* 2014; **37**: 1013-1022 [PMID: 24850378 DOI: 10.1007/s10545-014-9724-5]
- 83 **Passier R**, Mummery C. Cardiomyocyte differentiation from embryonic and adult stem cells. *Curr Opin Biotechnol* 2005; **16**: 498-502 [PMID: 16099156 DOI: 10.1016/j.copbio.2005.08.003]
- 84 **Burridge PW**, Thompson S, Millrod MA, Weinberg S, Yuan X, Peters A, Mahairaki V, Koliatsos VE, Tung L, Zambidis ET. A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PLoS One* 2011; **6**: e18293 [PMID: 21494607 DOI: 10.1371/journal.pone.0018293]
- 85 **Kattman SJ**, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 2011; **8**: 228-240 [PMID: 21295278 DOI: 10.1016/j.stem.2010.12.008]
- 86 **Wei H**, Tan G, Manasi S, Kong G, Yong P, Koh C, Ooi TH, Lim SY, Wong P, Gan SU, Shim W. One-step derivation of cardiomyocytes and mesenchymal stem cells from human pluripotent stem cells. *Stem Cell Res* 2012; **9**: 87-100 [PMID: 22683798 DOI: 10.1016/j.scr.2012.04.003]
- 87 **Zhang J**, Klos M, Wilson GF, Herman AM, Lian X, Raval KK, Barron MR, Hou L, Soerens AG, Yu J, Palecek SP, Lyons GE, Thomson JA, Herron TJ, Jalife J, Kamp TJ. Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method. *Circ Res* 2012; **111**: 1125-1136 [PMID: 22912385 DOI: 10.1161/CIRCRESAHA.112.273144]
- 88 **Cao N**, Liang H, Huang J, Wang J, Chen Y, Chen Z, Yang HT. Highly efficient induction and long-term maintenance of multipotent cardiovascular progenitors from human pluripotent stem cells under defined conditions. *Cell Res* 2013; **23**: 1119-1132 [PMID: 23896987 DOI: 10.1038/cr.2013.102]
- 89 Haraguchi Y, Matsuura K, Shimizu T, Yamato M, Okano T. Simple suspension culture system of human iPS cells maintaining their pluripotency for cardiac cell sheet engineering. *J Tissue Eng Regen Med* 2013 Jun 3; Epub ahead of print [PMID: 23728860 DOI: 10.1002/term.1761]
- 90 **Lim SY**, Sivakumaran P, Crombie DE, Disting GJ, Pébay A, Dilley RJ. Trichostatin A enhances differentiation of human induced pluripotent stem cells to cardiogenic cells for cardiac tissue engineering. *Stem Cells Transl Med* 2013; **2**: 715-725 [PMID: 23884641 DOI: 10.5966/sctm.2012-0161]
- 91 **Pesl M**, Acimovic I, Pribyl J, Hezova R, Vilotic A, Fauconnier J, Vrbicky J, Kruzliak P, Skladal P, Kara T, Rotrekl V, Lacampagne A, Dvorak P, Meli AC. Forced aggregation and defined factors allow highly uniform-sized embryoid bodies and functional cardiomyocytes from human embryonic and induced pluripotent stem cells. *Heart Vessels* 2014; **29**: 834-846 [PMID: 24258387 DOI: 10.1007/s00380-013-0436-9]
- 92 **Cho SW**, Park JS, Heo HJ, Park SW, Song S, Kim I, Han YM, Yamashita JK, Youm JB, Han J, Koh GY. Dual modulation of the mitochondrial permeability transition pore and redox signaling synergistically promotes cardiomyocyte differentiation from pluripotent stem cells. *J Am Heart Assoc* 2014; **3**: e000693 [PMID: 24627421 DOI: 10.1161/JAHA.113.000693]
- 93 **Karakikes I**, Senyei GD, Hansen J, Kong CW, Azeloglu EU, Stillitano F, Lieu DK, Wang J, Ren L, Hulot JS, Iyengar R, Li RA, Hajjar RJ. Small molecule-mediated directed differentiation of human embryonic stem cells toward ventricular cardiomyocytes. *Stem Cells Transl Med* 2014; **3**: 18-31 [PMID: 24324277 DOI: 10.5966/sctm.2013-0110]

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## Stem cell therapy in inflammatory bowel disease: A promising therapeutic strategy?

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**Core tip:** Inflammatory bowel diseases are inflammatory, chronic and progressive diseases of the intestinal tract. A limited experience is available with hematopoietic and mesenchymal stem cell transplantation for the treatment of these conditions. Research is ongoing with other cell lines which have been used in conditions alike to inflammatory bowel disease and which will possibly have a therapeutic role in this condition.

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### Abstract

Inflammatory bowel diseases are inflammatory, chronic and progressive diseases of the intestinal tract for which no curative treatment is available. Research in other fields with stem cells of different sources and with immunoregulatory cells (regulatory T-lymphocytes and dendritic T-cells) opens up new expectations for their use in these diseases. The goal for stem cell-based therapy is to provide a permanent cure. To achieve this, it will be necessary to obtain a cellular product, original or genetically modified, that has a high migration capacity and homes into the intestine, has high survival after transplantation, regulates the immune reaction while not being visible to the patient's immune system, and repairs the injured tissue.

### INTRODUCTION

Inflammatory bowel disease (IBD) mainly consists of two clinical conditions, Crohn's disease (CD) and ulcerative colitis (UC). It is mainly characterized by chronic, destructive inflammation of the gastrointestinal tract for which no curative treatment is currently available.

Its etiology is unknown, but it is accepted that it could be the result of loss of tolerance to intraluminal bowel antigens<sup>[1]</sup>. Genetic, environmental, and microbiological factors are involved in its development,



together with morphological and functional changes in the intestinal barrier associated to an impaired immune response<sup>[2]</sup>. Early data supporting genetic involvement in the pathogenesis of IBD come from familial clinical studies showing a greater incidence in twins<sup>[3,4]</sup>, first-degree relatives<sup>[5,6]</sup> and given ethnic groups<sup>[7,8]</sup>. Genome-wide association scan studies have allowed for identification of more than 163 loci associated to IBD<sup>[9]</sup>, 73 genes associated to CD and 47 to UC<sup>[10]</sup>, and overlapping genes for both conditions have also been found<sup>[11]</sup>. Genetic factors would however account for less than 25% of cases<sup>[12]</sup>. The exception is represented by a monogenic disorder referred to as IBD-like diseases, which are associated with severe colitis in childhood and have at most three loci alternatives<sup>[13]</sup>. On the other hand the increase of the incidence of IBD suggests that environmental factors are more important than genetic factors in the development of IBD<sup>[14]</sup>.

Since IBD etiology is currently unknown, current treatment is intended to control the inflammatory intestinal process, thus avoiding irreversible structural damage. However, current therapeutic results are discouraging. Thirty-three percent of patients with CD do not respond to anti-TNF alfa therapy<sup>[15-18]</sup>, and one third of responders loss the response<sup>[19]</sup>. Based on all the foregoing and on advances in understanding of the pathophysiological mechanisms involved in IBD development, new biological drugs and cell therapies are being investigated.

#### ***Future of the cellular-based therapy in IBD: Lessons from preclinical and clinical studies***

Cell therapies are promising candidates for the treatment of IBD. However, inconsistent results have emerged from current clinical trials using both, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). To establish the best stem cell type, the administration route and optimal dose of cells to achieve an effective therapy and to guarantee the safety of the patient, in-depth basic research is necessary. Therefore, preclinical studies using different animal models are necessary to understand the pathogenesis of IBD. These studies will facilitate a better design of preclinical stem cell therapies that will eventually become a suitable clinical therapy for IBD patients.

## **ADULT STEM CELLS**

At present, HSCs and MSCs have been used in several clinical trials. However, up to now, the results are unpredictable. For additional information, the reader is referred to the recent review published by our group in 2014<sup>[20]</sup>.

#### ***Hematopoietic stem cells transplantation***

Use of hematopoietic stem cells transplantation (HSCT) in IBD is restricted to severe CD with few therapeutic

options. These are patients who do not respond to standard treatment in whom surgery is not an option due to extent of disease. HSCT may also be used in monogenic diseases, such as interleukin-10 deficiency, where allogenic bone marrow transplant would correct the disease by reconstituting a new immune system.

Allogenic HSCT is not currently accepted for the treatment of CD because of its high mortality rate. Current studies focus on autologous HSCT, which is intended to "reset" the immune system of the patient. Once reactive T-lymphocytes and memory cells of the patient are eliminated by chemotherapy (lymphoablation), administration of autologous HSCs generates an immunotolerant system<sup>[21-23]</sup>. Unfortunately, this effect will probably be transient until the patient cells start the inflammatory mechanisms again.

Clinical experience is limited (Table 1), with the Burt study<sup>[25]</sup> reporting complete remission in all their 24 patients. We are currently waiting for the results of the ASTIC study<sup>[28]</sup>. This is a phase III clinical trial comparing two treatment arms intended to clarify whether improvement is due to reset of the immune system or to transplantation. The first arm uses chemotherapy followed by early transplantation (30 d), and the second arm chemotherapy with late transplantation (13 mo). Results reported to date include a high adverse effect rate and appear to suggest efficacy of transplantation.

#### ***Mesenchymal stem cell transplantation***

MSCs seem to be a promising therapeutic strategy for IBD because of their ability to selectively home in to injury/inflammation areas after systemic administration, and their immunosuppressive and tissue healing properties<sup>[30,31]</sup>. However, the clinical data published about MSCs transplantation in IBD patients showed conflicting results. An explanation for these inconsistent results could be the different sources used to obtain the MSCs that could have distinct differentiation and regeneration capabilities and the variety of protocols used for their isolation and culture. A better understanding of the MSCs biology and mechanisms of action and the exploration of other sources of stem cells in preclinical models of IBD are necessary.

Bone marrow and adipose tissue are the main sources of MSCs for both experimental and clinical studies. However, these sources have several disadvantages such as the invasive procedure used for their isolation, the small number of MSCs isolated, and the low proliferation and differentiation capacities related to donor age. For this reason, in the last few years the search for alternative tissue sources for MSCs has become of vital importance. Placental tissues, both fetal (amniotic fluid, Wharton's jelly, amniotic membrane, chorionic villi) and maternal (decidua) represent an important source of MSCs with some advantages including the isolation of large number of cells in a non invasive way<sup>[32,33]</sup>. In addition, like



**Table 1** Autologous hemapoietic stem cells transplantation studies in Crohn's disease

Ref.	Patients (n)	Follow up (mo)	Deaths	Remission (number of patients, time of evaluation in months)	Recurrence (number of patients or %, time of evaluation in months)
Oyama <i>et al</i> <sup>[24]</sup>	12	18.5 mo (7-37)	No	Clinical 11 (12 mo)	1% (18.5 mo)
Burt <i>et al</i> <sup>[25]</sup>	24	60 mo	1 not related	Clinical 24 (6-12 mo)	9% (12 mo) 37% (24 mo) 43% (36 mo) 61% (48 mo) 81% (60 mo)
Cassinotti <i>et al</i> <sup>[26]</sup>	10	56 mo (23-68)	No	Clinical 10 (3 mo) Endoscopic 5 (3 mo)	20% (12 mo) 50% (24 mo) 60% (36 mo) 70% (48 mo) 70% (60 mo)
Hasselblatt <i>et al</i> <sup>[27]</sup>	12	37 mo (IQR 6-123)	No	Clinical 4/8 (6 mo) Endoscopic 5/9 (9.1 mo) 3/9 mild disease	7% (10.9 mo)
Hawkey <sup>[28]</sup>	Data from 30 out of 45 patients 16 mobilisation + HCST (A) 16 mobilisation (B)		1 death after HCST	NA A: CDAI fell 162 (IQR 0-190) B: CDAI fell 82 (IQR 41-137)	NA
Jauregui-Amezaga <i>et al</i> <sup>[29]</sup>	21 evaluable	12 mo	1 after CMV infection and multiorganic failure	NA	NA

NA: Not available; CDAI: Crohn's disease activity index; IQR: Interquartile range; CMV: Cytomegalovirus.

bone marrow MSCs, placenta-derived MSCs are non-immunogenic and immunomodulatory stem cells with high expansion and differentiation capacity<sup>[32-35]</sup>.

An important issue in using MSCs is their safety. Although some studies supported that there is a risk of MSCs malignant transformation<sup>[36,37]</sup>, several recent studies using different types of MSCs supported that there is neither *in vitro* risk of development chromosomal aberrations after long term culture nor *in vivo* induction of tumors<sup>[30,32,38,39]</sup>.

For IBD treatment it is essential to increase the number of cells that migrate and home in to the intestine. A preclinical model of radiation enteritis treated with MSCs genetically modified to express the CXCR-4 receptor showed an increase of MSCs migration to intestinal site of injury and an improvement of symptoms<sup>[40]</sup>. In the same way, MSCs coated with antibodies against vascular cell adhesion molecule VCAM-1 showed an increased cell migration of MSCs to inflamed colon and thereby an increased tissue repair capacity<sup>[41]</sup>. A different strategy is to select a subpopulation of MSCs within the bone marrow that expresses high levels of EphrinB2. This subpopulation has an increased migration capacity to intestinal injury areas, and as a consequence, these MSCs would help to improve healing of intestinal injury<sup>[42]</sup>. Once MSCs engraft in the intestinal damaged tissue they can proliferate and transdifferentiate into intestinal stem cells, or secrete cytokines and growth factors that will promote the proliferation and differentiation of intestinal stem cells in order to repair the injured areas of the intestinal tissue<sup>[43]</sup>.

Besides the migration, homing and tissue repair capabilities of MSCs, they also have an important function in modulating the inflammation and high

immune response within the injured tissues. These immunomodulatory properties of MSCs are of special importance in the treatment of IBD. Systemic administration of bone marrow MSCs in a mouse model of chemical-induced colitis<sup>[43]</sup> and in a pig model of radiation-induced proctitis<sup>[44]</sup>, down-regulated autoimmune and inflammatory responses, and as a consequence, facilitated tissue regeneration.

The experience in luminal CD is limited (Table 2). Experience in UC is even smaller, and was mainly obtained in Russian studies about response of clinical activity<sup>[50]</sup>, changes in the pattern of systemic cytokines<sup>[51]</sup> and elimination of cytomegalovirus after Mesenchymal stem cell transplantation (MSCT)<sup>[52]</sup>. The most important work in this field is a phase III study<sup>[48]</sup> that plans to include 330 patients who will be treated with MSCs at different doses, but final results are not expected until 2018. According to data reported to date, the safety profile appears to be favorable, and formation of aberrant tissue has not been detected.

As regards local treatment for perianal CD (Table 3), a single study using bone marrow cells is available<sup>[55]</sup>, and there is an 11-year experience of the Spanish group with MSCs taken from fat tissue (ASCs)<sup>[53,54,56]</sup>, initially autologous, except for a phase I / II trial using donor cells<sup>[56]</sup>. We are currently waiting for completion of a phase III trial using donor cells which is planned to recruit a large patient sample. Two Korean studies using autologous ASCs have more recently been published. The first was carried out to evaluate the safety of the treatment<sup>[57]</sup>. The second is a phase II study<sup>[58]</sup>. A total of 43 patients were injected with ASCs. Among these, 33 were included in the modified per protocol analysis. The results showed complete sealing of 27 patients 8 wk after the final injection of



**Table 2 Mesenchymal stem cell transplantation studies in luminal inflammatory bowel diseases**

Ref.	Patient (n)	Procedence	Follow up (d, mo or wk)	SAEs	Response/remission (number of patients, time of evaluation)	Recurrence (number of patients, time of evaluation)
Onken <i>et al</i> <sup>[45]</sup>	10 CD (9 evaluable)	BM Allogenic	28 d	No SAEs	Clinical 3/1 (28 d)	NA
Duijvestein <i>et al</i> <sup>[46]</sup>	10 CD (9 evaluable)	BM Autologous	14 wk	No SAEs	Clinical 3/0 (6 wk) Endoscopic 0/2 (6 wk)	NA
Liang <i>et al</i> <sup>[47]</sup>	7 (4 CD/3UC)	BM/umbilical cord Allogenic	19 mo (range 6-32)	No SAEs	Clinical 7/3 (12 wk) Endoscopic 3/0 (3-5 mo)	1-3
Osiris Therapeutics <sup>[48]</sup>	Estimated 330 CD	BM Allogenic	NA	NA	NA	NA
Forbes <i>et al</i> <sup>[49]</sup>	16 CD (15 evaluable)	BM Allogenic	42 d	1 SAE probably not related	Clinical 12/8 (42 d) Endoscopic 7/0 (42 d)	NA
Lazebnik <i>et al</i> <sup>[50]</sup>	39 UC 11 CD	BM Allogenic	4-8 mo	NA	Clinical response UC 39/39 CD 11/11	NA

BM: Bone marrow; SAEs: Serious adverse events; NA: Not available; CD: Crohn's disease; UC: Ulcerative colitis.

**Table 3 Mesenchymal stem cell transplantation studies in perianal Crohn's disease**

Ref.	Patients (n)	Procedence	Follow up (mo)	SAEs related	Response/closure (number of patients or fistulas, time of evaluation)	Recurrence (number of patients, time of evaluation in months)
García-Olmo <i>et al</i> <sup>[53]</sup>	4 patients (8 fistulas)	Adipose Autologous	22 mo (range 12-30)	No	2/6 (2 mo)	NA
García-Olmo <i>et al</i> <sup>[54]</sup>	49 (14 CD) 25 (7 CD) fibrin glue (group A) 24 (7CD) ASCs (group B)	Adipose Autologous	12 mo	No	Group A: NA/1 (7 CD) Group B: 2/5 (7 CD)	3/17 global recurrence in group B (12 mo) Data for CD NA
Ciccocioppo <i>et al</i> <sup>[55]</sup>	10	BM Autologous	12 mo	No	3/7 (12 mo)	0/7 (12 mo)
de la Portilla <i>et al</i> <sup>[56]</sup>	22 per protocol	Adipose Allogenic	6 mo	2 SAEs possibly related Pyrexia Abscess	Closure: 5/18 fistulas (6 mo)	NA
Cho <i>et al</i> <sup>[57]</sup>	10	Adipose Autologous	6 mo	No SAEs	1/3 (2 mo)	0/3 (8 mo)
Lee <i>et al</i> <sup>[58]</sup>	33 per protocol	Adipose Autologous	12 mo	No SAEs	5/27 (2 mo) Per protocol	3/26 (12 mo)

BM: Bone marrow; CD: Crohn's disease; NA: Not available.

ASCs. No serious adverse effects were reported.

It is obvious that MSCs are a promising tool in the treatment of IBD. However, a large amount of work remains to be done to understand the mechanisms through which MSCs regulate the immune system, homeostasis and tissue repair. This knowledge will provide us with new tools to implement an effective MSCs-based treatment for IBD.

## AMNIOTIC FLUID STEM CELLS

Amniotic fluid stem cells (AFSCs) are isolated from the excess of second-trimester amniotic fluid obtained during routine amniocentesis for prenatal diagnosis.

Recently, AFSCs were used in a neonatal rat model of necrotizing enterocolitis, one of the primary causes of morbidity and mortality in neonates, showed a decrease in intestinal damage, an increase in gut tissue repair and a higher survival<sup>[59,60]</sup>. A better understanding of the AFSCs biology and mechanisms of action may help to develop strategies for their use in other IBD.

## INDUCED PLURIPOTENT STEM CELLS

These are pluripotent cells derived from somatic cells by the introduction of reprogramming factors (Oct-4, Sox2, Klf4, c-Myc, Nanog and Lin28). These pluripotent cells can be differentiated to any



tissue specific cells to generate autologous cells for cell-replacement therapy<sup>[61]</sup>. Human intestinal organoids have recently been generated from these cell lines<sup>[62]</sup>. This will allow in the future for studying the pathophysiology of the disease and for testing new therapies, including generation of potentially viable tissues. Induced pluripotent stem cells (iPSCs) have been derived from somatic cells obtained from patients suffering a variety of diseases and important progress has been made in establishing preclinical iPSC-based disease models including IBD<sup>[63]</sup>. Although iPSCs do not have the ethical problems of embryonic stem cells, there are many similarities between them and, as a consequence, iPSCs could develop teratomas following transplantation, hindering their use in clinical trials.

## INTESTINAL STEM CELLS

Intestinal stem cells (ISCs) are a rare population of fast-cycling Lgr5<sup>+</sup> cells and slow-cycling Tert<sup>+</sup>/Bmi1<sup>+</sup> cells situated above them at the crypt base. ISCs are in charge of the renewal of the intestinal epithelium which is changed every 4-5 d and in the regeneration of the intestinal epithelium after injury or inflammation<sup>[64]</sup>. It has been suggested that Lgr5<sup>+</sup> cells and Tert<sup>+</sup>/Bmi1<sup>+</sup> cells are two functionally different populations of ISCs<sup>[65]</sup>. Lgr5<sup>+</sup> cells are responsible for the maintenance of the normal homeostatis conditions, whereas Tert<sup>+</sup>/Bmi1<sup>+</sup> cells are more quiescent cells responsible for the intestinal epithelium regeneration under injury or inflammation conditions<sup>[66,67]</sup>. ISCs have proliferation and multipotency capabilities, *i.e.*, they are able to divide and later differentiate into all intestinal subtypes (enterocytes, goblet cells, Paneth cells and neuroendocrine cells). Recently, research in the ISCs field has advanced greatly and many ISCs markers have been identified<sup>[68]</sup>. However, an exhaustive characterization of ISCs as well as the identification of specific markers still remains elusive<sup>[64]</sup>.

Transplantation of fetal and adult ISCs expanded *in vitro* presented a strong engraftment and healing potential in a colonic injury model in mice<sup>[69,70]</sup>. However, ISCs in culture maintained as single cells have a very limited use in the study of the development of IBD and as a method for drug screening. Recently, intestinal organoids have been obtained from adult mouse and human ISCs<sup>[66,71,72]</sup>. These organoids were able to engraft and repair murine and human epithelium and represent an important step forward in the treatment of IBD<sup>[73]</sup>. Human organoids will be a very useful tool to study the pathological mechanisms of the disease from a specific patient and to test which is the best treatment to repair the intestinal epithelium for that patient. These organoids will be an important way to reach a more personalized medicine for IBD. These results highlight that those intestinal stem cells are a very promising source of stem cells for future patient-specific regeneration of the digestive tract<sup>[68]</sup>.

## ENDOTHELIAL PROGENITOR CELLS

Besides local inflammation, IBD is characterized by anomalous angiogenesis/vasculogenesis and severe damage in epithelial cells<sup>[74]</sup>. Important results have been obtained using endothelial progenitor cells (EPCs) for the treatment of hindlimb ischemia and myocardial ischemia. Recently, EPCs transplantation into fetal sheep showed an efficient migration and homing within the mucosal layer and a contribution to the vasculogenesis of the intestine<sup>[75]</sup>. These results suggest that EPCs could represent an additional source of cells for IBD cellular therapy, on their own or in combination with other stem cells such as MSCs.

## TOLEROGENIC IMMUNE CELL THERAPIES

In inflammatory disorders, special interest has been given to therapeutic strategies that could enhance the patient's tolerance response to intraluminal antigens. T-regulatory cells (Tregs) suppress immune responses of other cells and maintain tolerance to self-antigens. Tregs can be generated *ex vivo* by activation of both, murine and human CD4 T cells, suggesting that they could be an extra source of cells for cellular therapies in IBD. Intraperitoneal injection of induced Tregs in a mouse model of chronic colitis showed an attenuation of the preexisting gut inflammation response<sup>[76,77]</sup>.

Dendritic cells (DCs) are antigen-presenting cells involved in immunity and tolerance. DCs seem to be the most important regulators of immune tolerance in the gastrointestinal system, however, extensive studies are necessary to understand their role in this tissue and their mechanisms of action<sup>[78]</sup>. Like MSCs, tolerogenic-DCs (tol-DCs) do not express neither MHCII nor the T-cell co-stimulatory molecules, and will not activate an immune response in the host. *Ex vivo* generated tolerogenic-DCs are available as a clinical grade product and used as therapeutic vaccines to restore antigen-specific tolerance in autoimmune diseases<sup>[78]</sup>. Tol-DCs have been used in very few recent clinical studies such as rheumatoid arthritis and other not inflammatory diseases, and as a result conclusions about their clinical efficiency are still elusive. Several mouse models of colitis showed an important effect of tol-DCs in the prevention and reduction of symptoms of IBD<sup>[79-83]</sup>. However, several questions must be resolved before tol-DCs can be used in IBD cellular therapy in humans, mostly due to the differences in IBD and tol-DCs between mice and humans<sup>[84]</sup>.

## CONCLUSION

The goal for stem cell-based therapy is to provide a permanent cure for IBD. To achieve this, it will be necessary to obtain a cellular product (original or genetically modified) that has a high migration and homes into the intestine, has high survival after



transplantation, regulates the immune reaction which is not detectable to the patient's immune system, and will repair the injured tissue. Intestinal tissue is composed of several cell types and IBD are characterized by widespread damage. Cell-based therapies will probably be designed as a combination of several cell types that will produce a synergic therapeutic response.

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## REFERENCES

- 1 **Martínez-Montiel MP**, Muñoz-Yagüe MT. Biologic therapies for chronic inflammatory bowel disease. *Rev Esp Enferm Dig* 2006; **98**: 265-291 [PMID: 16792456]
- 2 **Neurath MF**, Travis SP. Mucosal healing in inflammatory bowel diseases: a systematic review. *Gut* 2012; **61**: 1619-1635 [PMID: 22842618]
- 3 **Tysk C**, Lindberg E, Järnerot G, Flodérus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut* 1988; **29**: 990-996 [PMID: 3396969]
- 4 **Orholm M**, Binder V, Sørensen TI, Rasmussen LP, Kyvik KO. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. *Scand J Gastroenterol* 2000; **35**: 1075-1081 [PMID: 11099061]
- 5 **Orholm M**, Munkholm P, Langholz E, Nielsen OH, Sørensen TI, Binder V. Familial occurrence of inflammatory bowel disease. *N Engl J Med* 1991; **324**: 84-88 [PMID: 1984188]
- 6 **Peeters M**, Nevens H, Baert F, Hiele M, de Meyer AM, Vlietinck R, Rutgeerts P. Familial aggregation in Crohn's disease: increased age-adjusted risk and concordance in clinical characteristics. *Gastroenterology* 1996; **111**: 597-603 [PMID: 8780562]
- 7 **Roth MP**, Petersen GM, McElree C, Feldman E, Rotter JI. Geographic origins of Jewish patients with inflammatory bowel disease. *Gastroenterology* 1989; **97**: 900-904 [PMID: 2777043]
- 8 **Yang H**, McElree C, Roth MP, Shanahan F, Targan SR, Rotter JI. Familial empirical risks for inflammatory bowel disease: differences between Jews and non-Jews. *Gut* 1993; **34**: 517-524 [PMID: 8491401]
- 9 **Jostins L**, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, Lee JC, Schumm LP, Sharma Y, Anderson CA, Essers J, Mitrovic M, Ning K, Cleynen I, Theatre E, Spain SL, Raychaudhuri S, Goyette P, Wei Z, Abraham C, Achkar JP, Ahmad T, Amininejad L, Ananthakrishnan AN, Andersen V, Andrews JM, Baidoo L, Balschun T, Bampton PA, Bitton A, Boucher G, Brand S, Büning C, Cohain A, Cichon S, D'Amato M, De Jong D, Devaney KL, Dubinsky M, Edwards C, Ellinghaus D, Ferguson LR, Franchimont D, Fransen K, Geary R, Georges M, Gieger C, Glas J, Haritunians T, Hart A, Hawkey C, Hedl M, Hu X, Karlsten TH, Kupcinskas L, Kugathasan S, Latiano A, Laukens D, Lawrance IC, Lees CW, Louis E, Mahy G, Mansfield J, Morgan AR, Mowat C, Newman W, Palmieri O, Ponsioen CY, Potocnik U, Prescott NJ, Regueiro M, Rotter JI, Russell RK, Sanderson JD, Sans M, Satsangi J, Schreiber S, Simms LA, Sventoraityte J, Targan SR, Taylor KD, Tremelling M, Verspaget HW, De Vos M, Wijmenga C, Wilson DC, Winkelmann J, Xavier RJ, Zeissig S, Zhang B, Zhang CK, Zhao H, Silverberg MS, Annesse V, Hakonarson H, Brant SR, Radford-Smith G, Mathew CG, Rioux JD, Schadt EE, Daly MJ, Franke A, Parkes M, Vermeire S, Barrett JC, Cho JH. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 2012; **491**: 119-124 [PMID: 23128233 DOI: 10.1038/nature11582]
- 10 **Fiocchi C**. Genes and 'in-vironment': how will our concepts on the pathophysiology of inflammatory bowel disease develop in the future? *Dig Dis* 2012; **30** Suppl 3: 2-11 [PMID: 23295686 DOI: 10.1159/000342585]
- 11 **Franke A**, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, Lees CW, Balschun T, Lee J, Roberts R, Anderson CA, Bis JC, Bumpstead S, Ellinghaus D, Festen EM, Georges M, Green T, Haritunians T, Jostins L, Latiano A, Mathew CG, Montgomery GW, Prescott NJ, Raychaudhuri S, Rotter JI, Schumm P, Sharma Y, Simms LA, Taylor KD, Whiteman D, Wijmenga C, Baldassano RN, Barclay M, Bayless TM, Brand S, Büning C, Cohen A, Colombel JF, Cottone M, Stronati L, Denson T, De Vos M, D'Inca R, Dubinsky M, Edwards C, Florin T, Franchimont D, Gearry R, Glas J, Van Gossom A, Guthery SL, Halfvarson J, Verspaget HW, Hugot JP, Karban A, Laukens D, Lawrance I, Lemann M, Levine A, Libioulle C, Louis E, Mowat C, Newman W, Panés J, Phillips A, Proctor DD, Regueiro M, Russell R, Rutgeerts P, Sanderson J, Sans M, Seibold F, Steinhardt AH, Stokkers PC, Torkvist L, Kullak-Ublick G, Wilson D, Walters T, Targan SR, Brant SR, Rioux JD, D'Amato M, Weersma RK, Kugathasan S, Griffiths AM, Mansfield JC, Vermeire S, Duerr RH, Silverberg MS, Satsangi J, Schreiber S, Cho JH, Annesse V, Hakonarson H, Daly MJ, Parkes M. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010; **42**: 1118-1125 [PMID: 21102463 DOI: 10.1038/ng.717]
- 12 **Park JH**, Wacholder S, Gail MH, Peters U, Jacobs KB, Chanock SJ, Chatterjee N. Estimation of effect size distribution from genome-wide association studies and implications for future discoveries. *Nat Genet* 2010; **42**: 570-575 [PMID: 20562874 DOI: 10.1038/ng.610]
- 13 **Uhlig HH**. Monogenic diseases associated with intestinal inflammation: implications for the understanding of inflammatory bowel disease. *Gut* 2013; **62**: 1795-1805 [PMID: 24203055 DOI: 10.1136/gutjnl-2012-303956]
- 14 **Cho JH**. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 2008; **8**: 458-466 [PMID: 18500230 DOI: 10.1038/nri2340]
- 15 **Hanauer SB**, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 2002; **359**: 1541-1549 [PMID: 12047962 DOI: 10.1016/S0140-6736(02)08512-4]
- 16 **Colombel JF**, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52-65 [PMID: 17241859 DOI: 10.1053/j.gastro.2006.11.041]
- 17 **Sandborn WJ**, Feagan BG, Stoinov S, Honiball PJ, Rutgeerts P, Mason D, Bloomfield R, Schreiber S. Certolizumab pegol for the treatment of Crohn's disease. *N Engl J Med* 2007; **357**: 228-238 [PMID: 17634458 DOI: 10.1056/NEJMoa067594]
- 18 **Schreiber S**, Khaliq-Kareemi M, Lawrance IC, Thomsen OØ, Hanauer SB, McColm J, Bloomfield R, Sandborn WJ. Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med* 2007; **357**: 239-250 [PMID: 17634459 DOI: 10.1056/NEJMoa062897]
- 19 **Yanai H**, Hanauer SB. Assessing response and loss of response to biological therapies in IBD. *Am J Gastroenterol* 2011; **106**: 685-698 [PMID: 21427713 DOI: 10.1038/ajg.2011.103]
- 20 **Martínez-Montiel Mdel P**, Gómez-Gómez GJ, Flores AI. Therapy with stem cells in inflammatory bowel disease. *World J Gastroenterol* 2014; **20**: 1211-1227 [PMID: 24574796 DOI: 10.3748/wjg.v20.i5.1211]
- 21 **Clerici M**, Cassinotti A, Onida F, Trabattini D, Annaloro C, Della Volpe A, Rainone V, Lissoni F, Duca P, Sampietro G, Fociani P, Vago G, Foschi D, Arduzzone S, Delilieri GL, Porro GB. Immunomodulatory effects of unselected haematopoietic stem cells autotransplantation in refractory Crohn's disease. *Dig Liver Dis* 2011; **43**: 946-952 [PMID: 21907652 DOI: 10.1016/j.dld.2011.07.021]



- 22 **García-Bosch O**, Ricart E, Panés J. Review article: stem cell therapies for inflammatory bowel disease - efficacy and safety. *Aliment Pharmacol Ther* 2010; **32**: 939-952 [PMID: 20804451 DOI: 10.1111/j.1365-2036.2010.04439.x]
- 23 **van Deen WK**, Oikonomopoulos A, Hommes DW. Stem cell therapy in inflammatory bowel disease: which, when and how? *Curr Opin Gastroenterol* 2013; **29**: 384-390 [PMID: 23666365 DOI: 10.1097/MOG.0b013e328361f763]
- 24 **Oyama Y**, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjak N, Kletzel M, Whittington PF, Burt RK. Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. *Gastroenterology* 2005; **128**: 552-563 [PMID: 15765390 DOI: 10.1053/j.gastro.2004.11.051]
- 25 **Burt RK**, Craig RM, Milanetti F, Quigley K, Gozdzia P, Bucha J, Testori A, Halverson A, Verda L, de Villiers WJ, Jovanovic B, Oyama Y. Autologous nonmyeloablative hematopoietic stem cell transplantation in patients with severe anti-TNF refractory Crohn disease: long-term follow-up. *Blood* 2010; **116**: 6123-6132 [PMID: 20837778 DOI: 10.1182/blood-2010-06-292391]
- 26 **Cassinotti A**, Annaloro C, Sampietro G, Fociani P, Fichera M, Maconi G, Lombardini I, Bezzio C, Foschi D, Lambertenghi Deliliers G, Bianchi Porro G, De Franchis I, Ardizzone S. Autologous haematopoietic stem cell transplantation without CD34 cell selection for refractory Crohn's disease: The Milan experience after 5 years. *J Crohns Colitis* 2012; **6**: S153-S154 [DOI: 10.1016/S1873-9946(12)60381-X]
- 27 **Hasselblatt P**, Drogitz K, Potthoff K, Bertz H, Kruis W, Schmidt C, Stallmach A, Schmitt-Graeff A, Finke J, Kreisel W. Remission of refractory Crohn's disease by high-dose cyclophosphamide and autologous peripheral blood stem cell transplantation. *Aliment Pharmacol Ther* 2012; **36**: 725-735 [PMID: 22937722 DOI: 10.1111/apt.12032]
- 28 **Hawkey C**, Allez M, Ardizzone S, Clark M, Clark L, Colombel J-F, Danese S, Farge-Bancel D, Labopin M, Lindsay J, Norman A, Onida F, Ricart E, Rogler G, Rovira M, Russell N, Satsangi J, Travis S, Tyndall A, Vermeire S. Clinical and endoscopic improvement following hemopoietic stem cell transplantation in the ASTIC trial. *J Crohns Colitis* 2013; **7**: S4 [DOI: 10.1016/S1873-9946(13)60010-0]
- 29 **Jauregui-Amezaga A**, Rovira M, Pin'o Donnay S, Marin PJ, Feu F, Elizalde JJ, Fernández-Avilés F, Martínez C, Rosiñol L, Suarez-Lledó M, Masamunt MC, Ramírez-Morros A, Gallego M, Ordás I, Panés J, Ricart E. Hematopoietic stem cell transplantation in refractory Crohn's disease: Feasibility and toxicity. *J Crohns Coliti* 2014; **8**: S263 [DOI: 10.1016/S1873-9946(14)60591-2]
- 30 **Vegh I**, Grau M, Gracia M, Grande J, de la Torre P, Flores AI. Decidua mesenchymal stem cells migrated toward mammary tumors in vitro and in vivo affecting tumor growth and tumor development. *Cancer Gene Ther* 2013; **20**: 8-16 [PMID: 23037810 DOI: 10.1038/cgt.2012.71]
- 31 **Griffin MD**, Elliman SJ, Cahill E, English K, Ceredig R, Ritter T. Concise review: adult mesenchymal stromal cell therapy for inflammatory diseases: how well are we joining the dots? *Stem Cells* 2013; **31**: 2033-2041 [PMID: 23766124 DOI: 10.1002/stem.1452]
- 32 **Macías MI**, Grande J, Moreno A, Domínguez I, Bornstein R, Flores AI. Isolation and characterization of true mesenchymal stem cells derived from human term decidua capable of multilineage differentiation into all 3 embryonic layers. *Am J Obstet Gynecol* 2010; **203**: 495.e9-495.e23 [PMID: 20692642 DOI: 10.1016/j.ajog.2010.06.045]
- 33 **Parolini O**, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA, Redl H, Sakuragawa N, Wolbank S, Zeisberger S, Zisch A, Strom SC. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* 2008; **26**: 300-311 [PMID: 17975221]
- 34 **Haddad R**, Saldanha-Araujo F. Mechanisms of T-cell immunosuppression by mesenchymal stromal cells: what do we know so far? *Biomed Res Int* 2014; **2014**: 216806 [PMID: 25025040]
- 35 **Bornstein R**, Macías MI, de la Torre P, Grande J, Flores AI. Human decidua-derived mesenchymal stromal cells differentiate into hepatic-like cells and form functional three-dimensional structures. *Cytotherapy* 2012; **14**: 1182-1192 [PMID: 22900961 DOI: 10.3109/14653249.2012.706706]
- 36 **Rubio D**, Garcia-Castro J, Martín MC, de la Fuente R, Cigudosa JC, Lloyd AC, Bernad A. Spontaneous human adult stem cell transformation. *Cancer Res* 2005; **65**: 3035-3039 [PMID: 15833829]
- 37 **Rosland GV**, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwicz J, Tonn JC, Goldbrunner R, Lønning PE, Bjerkvig R, Schichor C. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. *Cancer Res* 2009; **69**: 5331-5339 [PMID: 19509230 DOI: 10.1158/0008-5472.CAN-08-4630]
- 38 **Tarte K**, Gaillard J, Lataillade JJ, Fouillard L, Becker M, Mossafa H, Tchirkov A, Rouard H, Henry C, Splingard M, Dulong J, Monnier D, Gourmelon P, Gorin NC, Sensebé L. Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. *Blood* 2010; **115**: 1549-1553 [PMID: 20032501 DOI: 10.1182/blood-2009-05-219907]
- 39 **Bernardo ME**, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, Montagna D, Maccario R, Villa R, Daidone MG, Zuffardi O, Locatelli F. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* 2007; **67**: 9142-9149 [PMID: 17909019]
- 40 **Zhang J**, Gong JF, Zhang W, Zhu WM, Li JS. Effects of transplanted bone marrow mesenchymal stem cells on the irradiated intestine of mice. *J Biomed Sci* 2008; **15**: 585-594 [PMID: 18763056 DOI: 10.1007/s11373-008-9256-9]
- 41 **Ko IK**, Kim BG, Awadallah A, Mikulan J, Lin P, Letterio JJ, Dennis JE. Targeting improves MSC treatment of inflammatory bowel disease. *Mol Ther* 2010; **18**: 1365-1372 [PMID: 20389289 DOI: 10.1038/mt.2010.54]
- 42 **Colletti E**, El Shabrawy D, Soland M, Yamagami T, Mokhtari S, Osborne C, Schlauch K, Zanjani ED, Porada CD, Almeida-Porada G. EphB2 isolates a human marrow stromal cell subpopulation with enhanced ability to contribute to the resident intestinal cellular pool. *FASEB J* 2013; **27**: 2111-2121 [PMID: 23413357]
- 43 **Chen QQ**, Yan L, Wang CZ, Wang WH, Shi H, Su BB, Zeng QH, Du HT, Wan J. Mesenchymal stem cells alleviate TNBS-induced colitis by modulating inflammatory and autoimmune responses. *World J Gastroenterol* 2013; **19**: 4702-4717 [PMID: 23922467 DOI: 10.3748/wjg.v19.i29.4702]
- 44 **Linard C**, Busson E, Holler V, Strup-Perrot C, Lacave-Lapalun JV, Lhomme B, Prat M, Devauchelle P, Sabourin JC, Simon JM, Bonneau M, Lataillade JJ, Benderitter M. Repeated autologous bone marrow-derived mesenchymal stem cell injections improve radiation-induced proctitis in pigs. *Stem Cells Transl Med* 2013; **2**: 916-927 [PMID: 24068742]
- 45 **Onken J**, Gallup D, Hanson J, Pandak M, Custer L. Successful outpatient treatment of refractory Crohn's disease using adult mesenchymal stem cells. American College of Gastroenterology Annual Meeting. Las Vegas, 2006. Available from: URL: <http://universe.gi.org/contentitem.asp?c=1047>
- 46 **Duijvestein M**, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH, Verhaar AP, Fibbe WE, van den Brink GR, Hommes DW. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut* 2010; **59**: 1662-1669 [PMID: 20921206 DOI: 10.1136/gut.2010.215152]
- 47 **Liang J**, Zhang H, Wang D, Feng X, Wang H, Hua B, Liu B, Sun L. Allogeneic mesenchymal stem cell transplantation in seven patients with refractory inflammatory bowel disease. *Gut* 2012; **61**: 468-469 [PMID: 21617158 DOI: 10.1136/gutjnl-2011-300083]
- 48 **Osiris Therapeutics**. A phase III, multicenter, placebo-controlled,



- randomized, double-blind study to evaluate the safety and efficacy of Prochimaltm (ex vivo cultured adult human mesenchymal stem cells) intravenous infusion for induction of remission in subjects experiencing treatment-refractory moderate-to-severe Crohn's disease. *ClinicalTrials.gov* 2010; NCT00482092. Available from: URL: <http://www.clinicaltrials.gov/ct2/show/NCT00482092>
- 49 **Forbes GM**, Sturm MJ, Leong RW, Sparrow MP, Segarajasingam D, Cummins AG, Phillips M, Herrmann RP. A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol* 2014; **12**: 64-71 [PMID: 23872668 DOI: 10.1016/j.cgh.2013.06.021]
- 50 **Lazebnik LB**, Konopliannikov AG, Kniazev OV, Parfenov AI, Tsaregorodtseva TM, Ruchkina IN, Khomeriki SG, Rogozina VA, Konopliannikova OA. [Use of allogeneic mesenchymal stem cells in the treatment of intestinal inflammatory diseases]. *Ter Arkh* 2010; **82**: 38-43 [PMID: 20387674]
- 51 **Kniazev OV**, Parfenov AI, Ruchkina IN, Lazebnik LB, Sagynbaeva VÉ. [Immune response to biological therapy of inflammatory bowel diseases]. *Ter Arkh* 2013; **85**: 55-59 [PMID: 24640669]
- 52 **Knyazev O**, Ruchkina I, Konoplyannikov A, Schakhpazyan N, Parfenov A. The elimination of cytomegalovirus in patients with ulcerative colitis without antiviral therapy. *J Crohns Colitis* 2014; **8**: S202-S203 [DOI: 10.1016/S1873-9946(14)60451-7]
- 53 **García-Olmo D**, García-Arranz M, Herreros D, Pascual I, Peiro C, Rodríguez-Montes JA. A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. *Dis Colon Rectum* 2005; **48**: 1416-1423 [PMID: 15933795 DOI: 10.1007/s10350-005-0052-6]
- 54 **García-Olmo D**, Herreros D, Pascual I, Pascual JA, Del-Valle E, Zorrilla J, De-La-Quintana P, García-Arranz M, Pascual M. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. *Dis Colon Rectum* 2009; **52**: 79-86 [PMID: 19273960 DOI: 10.1007/DCR.0b013e3181973487]
- 55 **Ciccocioppo R**, Bernardo ME, Sgarrella A, Maccario R, Avanzini MA, Ubezio C, Minelli A, Alvisi C, Vanoli A, Calliada F, Dionigi P, Perotti C, Locatelli F, Corazza GR. Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. *Gut* 2011; **60**: 788-798 [PMID: 21257987 DOI: 10.1136/gut.2010.214841]
- 56 **de la Portilla F**, Alba F, García-Olmo D, Herreras JM, González FX, Galindo A. Expanded allogeneic adipose-derived stem cells (eASCs) for the treatment of complex perianal fistula in Crohn's disease: results from a multicenter phase I/IIa clinical trial. *Int J Colorectal Dis* 2013; **28**: 313-323 [PMID: 23053677 DOI: 10.1007/s00384-012-1581-9]
- 57 **Cho YB**, Lee WY, Park KJ, Kim M, Yoo HW, Yu CS. Autologous adipose tissue-derived stem cells for the treatment of Crohn's fistula: a phase I clinical study. *Cell Transplant* 2013; **22**: 279-285 [PMID: 23006344 DOI: 10.3727/096368912X656045]
- 58 **Lee WY**, Park KJ, Cho YB, Yoon SN, Song KH, Kim do S, Jung SH, Kim M, Yoo HW, Kim I, Ha H, Yu CS. Autologous adipose tissue-derived stem cells treatment demonstrated favorable and sustainable therapeutic effect for Crohn's fistula. *Stem Cells* 2013; **31**: 2575-2581 [PMID: 23404825 DOI: 10.1002/stem.1357]
- 59 **Zani A**, Cananzi M, Lauriti G, Fascetti-Leon F, Wells J, Siow B, Lythgoe MF, Pierro A, Eaton S, De Coppi P. Amniotic fluid stem cells prevent development of ascites in a neonatal rat model of necrotizing enterocolitis. *Eur J Pediatr Surg* 2014; **24**: 57-60 [PMID: 23852724 DOI: 10.1055/s-0033-1350059]
- 60 **Zani A**, Cananzi M, Fascetti-Leon F, Lauriti G, Smith VV, Bollini S, Ghionzoli M, D'Arrigo A, Pozzobon M, Piccoli M, Hicks A, Wells J, Siow B, Sebire NJ, Bishop C, Leon A, Atala A, Lythgoe MF, Pierro A, Eaton S, De Coppi P. Amniotic fluid stem cells improve survival and enhance repair of damaged intestine in necrotising enterocolitis via a COX-2 dependent mechanism. *Gut* 2014; **63**: 300-309 [PMID: 23525603 DOI: 10.1136/gutjnl-2012-303735]
- 61 **Wu SM**, Hochedlinger K. Harnessing the potential of induced pluripotent stem cells for regenerative medicine. *Nat Cell Biol* 2011; **13**: 497-505 [PMID: 21540845 DOI: 10.1038/ncb0511-497]
- 62 **Watson CL**, Mahe MM, Múnera J, Howell JC, Sundaram N, Poling HM, Schweitzer JI, Vallance JE, Mayhew CN, Sun Y, Grabowski G, Finkbeiner SR, Spence JR, Shroyer NF, Wells JM, Helmrath MA. An in vivo model of human small intestine using pluripotent stem cells. *Nat Med* 2014; **20**: 1310-1314 [PMID: 25326803 DOI: 10.1038/nm.3737]
- 63 **Wagnerova A**, Gardlik R. In vivo reprogramming in inflammatory bowel disease. *Gene Ther* 2013; **20**: 1111-1118 [PMID: 24025994 DOI: 10.1038/gt.2013.43]
- 64 **Moossavi S**, Zhang H, Sun J, Rezaei N. Host-microbiota interaction and intestinal stem cells in chronic inflammation and colorectal cancer. *Expert Rev Clin Immunol* 2013; **9**: 409-422 [PMID: 23634736 DOI: 10.1586/eci.13.27]
- 65 **Yan KS**, Chia LA, Li X, Ootani A, Su J, Lee JY, Su N, Luo Y, Heilshorn SC, Amieva MR, Sangiorgi E, Capecchi MR, Kuo CJ. The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. *Proc Natl Acad Sci USA* 2012; **109**: 466-471 [PMID: 22190486 DOI: 10.1073/pnas.1118857109]
- 66 **Basak O**, van de Born M, Korving J, Beumer J, van der Elst S, van Es JH, Clevers H. Mapping early fate determination in Lgr5+ crypt stem cells using a novel Ki67-RFP allele. *EMBO J* 2014; **33**: 2057-2068 [PMID: 25092767]
- 67 **Philpott A**, Winton DJ. Lineage selection and plasticity in the intestinal crypt. *Curr Opin Cell Biol* 2014; **31C**: 39-45 [PMID: 25083805 DOI: 10.1016/j.ceb.2014.07.002]
- 68 **Mohamed MS**, Chen Y, Yao CL. Intestinal stem cells and stem cell-based therapy for intestinal diseases. *Cytotechnology* 2015; **67**: 177-189 [PMID: 24981313]
- 69 **Yui S**, Nakamura T, Sato T, Nemoto Y, Mizutani T, Zheng X, Ichinose S, Nagaishi T, Okamoto R, Tsuchiya K, Clevers H, Watanabe M. Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5+ stem cell. *Nat Med* 2012; **18**: 618-623 [PMID: 22406745 DOI: 10.1038/nm.2695]
- 70 **Fordham RP**, Yui S, Hannan NR, Soendergaard C, Madgwick A, Schweiger PJ, Nielsen OH, Vallier L, Pedersen RA, Nakamura T, Watanabe M, Jensen KB. Transplantation of expanded fetal intestinal progenitors contributes to colon regeneration after injury. *Cell Stem Cell* 2013; **13**: 734-744 [PMID: 24139758 DOI: 10.1016/j.stem.2013.09.015]
- 71 **Kuratnik A**, Giardina C. Intestinal organoids as tissue surrogates for toxicological and pharmacological studies. *Biochem Pharmacol* 2013; **85**: 1721-1726 [PMID: 23623789 DOI: 10.1016/j.bcp.2013.04.016]
- 72 **Sato T**, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, Clevers H. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 2009; **459**: 262-265 [PMID: 19329995 DOI: 10.1038/nature07935]
- 73 **Belchior GG**, Sogayar MC, Grikscheit TC. Stem cells and biopharmaceuticals: vital roles in the growth of tissue-engineered small intestine. *Semin Pediatr Surg* 2014; **23**: 141-149 [PMID: 24994528 DOI: 10.1053/j.sempedsurg.2014.06.011]
- 74 **Costa C**, Incio J, Soares R. Angiogenesis and chronic inflammation: cause or consequence? *Angiogenesis* 2007; **10**: 149-166 [PMID: 17457680]
- 75 **Wood JA**, Colletti E, Mead LE, Ingram D, Porada CD, Zanjani ED, Yoder MC, Almeida-Porada G. Distinct contribution of human cord blood-derived endothelial colony forming cells to liver and gut in a fetal sheep model. *Hepatology* 2012; **56**: 1086-1096 [PMID: 22488442 DOI: 10.1002/hep.25753]
- 76 **Foussat A**, Cottrez F, Brun V, Fournier N, Breittmayer JP, Groux H. A comparative study between T regulatory type 1 and CD4+CD25+ T cells in the control of inflammation. *J Immunol* 2003; **171**: 5018-5026 [PMID: 14607898]
- 77 **Karlsson F**, Martinez NE, Gray L, Zhang S, Tsunoda I, Grisham MB. Therapeutic evaluation of ex vivo-generated versus natural regulatory T-cells in a mouse model of chronic gut inflammation. *Inflamm Bowel Dis* 2013; **19**: 2282-2294 [PMID: 23893082 DOI: 10.1097/MIB.0b013e31829c32dd]
- 78 **Cabezón R**, Benítez-Ribas D. Therapeutic potential of tolerogenic dendritic cells in IBD: from animal models to clinical application. *Clin Dev Immunol* 2013; **2013**: 789814 [PMID: 24319468 DOI: 10.1155/2013/789814]



- 79 **Pedersen AE**, Schmidt EG, Gad M, Poulsen SS, Claesson MH. Dexamethasone/ $\alpha$ -25-dihydroxyvitamin D<sub>3</sub>-treated dendritic cells suppress colitis in the SCID T-cell transfer model. *Immunology* 2009; **127**: 354-364 [PMID: 19019085 DOI: 10.1111/j.1365-2567.2008.02996.x]
- 80 **Pedersen AE**, Gad M, Kristensen NN, Haase C, Nielsen CH, Claesson MH. Tolerogenic dendritic cells pulsed with enterobacterial extract suppress development of colitis in the severe combined immunodeficiency transfer model. *Immunology* 2007; **121**: 526-532 [PMID: 17428312]
- 81 **Yamanishi H**, Murakami H, Ikeda Y, Abe M, Kumagi T, Hiasa Y, Matsuura B, Onji M. Regulatory dendritic cells pulsed with carbonic anhydrase I protect mice from colitis induced by CD4+CD25- T cells. *J Immunol* 2012; **188**: 2164-2172 [PMID: 22291189 DOI: 10.4049/jimmunol.1100559]
- 82 **Gonzalez-Rey E**, Delgado M. Therapeutic treatment of experimental colitis with regulatory dendritic cells generated with vasoactive intestinal peptide. *Gastroenterology* 2006; **131**: 1799-1811 [PMID: 17087944]
- 83 **Sakuraba A**, Sato T, Kamada N, Kitazume M, Sugita A, Hibi T. Th1/Th17 immune response is induced by mesenteric lymph node dendritic cells in Crohn's disease. *Gastroenterology* 2009; **137**: 1736-1745 [PMID: 19632232 DOI: 10.1053/j.gastro.2009.07.049]
- 84 **Neurath MF**. Animal models of inflammatory bowel diseases: illuminating the pathogenesis of colitis, ileitis and cancer. *Dig Dis* 2012; **30** Suppl 1: 91-94 [PMID: 23075875 DOI: 10.1159/000341131]

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## "Second-generation" stem cells for cardiac repair

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an inspiring alternative to restore cardiac function after myocardial infarction. A large body of evidence has been obtained in this field but there is no conclusive data on the efficacy of these treatments. Preclinical studies and early reports in humans have been encouraging and have fostered a rapid clinical translation, but positive results have not been uniformly observed and when present, they have been modest. Several types of stem cells, manufacturing methods and delivery routes have been tested in different clinical settings but direct comparison between them is challenging and hinders further research. Despite enormous achievements, major barriers have been found and many fundamental issues remain to be resolved. A better knowledge of the molecular mechanisms implicated in cardiac development and myocardial regeneration is critically needed to overcome some of these hurdles. Genetic and pharmacological priming together with the discovery of new sources of cells have led to a "second generation" of cell products that holds an encouraging promise in cardiovascular regenerative medicine. In this report, we review recent advances in this field focusing on the new types of stem cells that are currently being tested in human beings and on the novel strategies employed to boost cell performance in order to improve cardiac function and outcomes after myocardial infarction.

**Key words:** Stem cells; Cardiac repair; Myocardial infarction; Heart failure; Second generation

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**Core tip:** Myocardial infarction and heart failure represent two of the most prevalent and fatal diseases. Stem cell therapies represent a novel approach capable of restoring the cellular loss observed in these conditions. Data from initial human studies have been encouraging but inconclusive. However, refinements in cell populations as well as new stem cell sources are currently being tested in large phase III clinical trials after showing positive results in preclinical models and early clinical reports, thus holding a promise for the achievement of a true

### Abstract

Over the last years, stem cell therapy has emerged as



myocardial regeneration after myocardial infarction. We review here recent developments in this field.

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## INTRODUCTION

Cardiac heart disease is a leading cause of mortality and morbidity worldwide. Coronary heart disease (CHD) is estimated to affect near 16 million people in the United States and accounts for about 1 of every 6 deaths<sup>[1]</sup>. CHD and myocardial infarction (MI) are also a major cause of heart failure (HF), which is an important public health issue with an estimated prevalence of 5 million in the United States<sup>[1]</sup> and 23 million worldwide<sup>[2]</sup>.

Risk factor modification, advances in medical, device-based therapies and interventional management have improved cardiovascular outcomes<sup>[3]</sup>, but even when mortality has shown a decline in most developed countries since mid 70s<sup>[1,4-6]</sup>, CHD is the leading cause of death in the United States<sup>[7]</sup>. Its main consequence - HF - carries a poor prognosis, even worse than many cancers<sup>[8]</sup>. Moreover, the decline in MI acute mortality achieved with primary percutaneous coronary interventions (PCI), has augmented the prevalence of HF among survivors<sup>[9,10]</sup>, because of the development of substantial scarring despite reperfusion strategies<sup>[11]</sup>.

Current treatment of systolic HF is mainly focused on counterweighting the neurohormonal activation cascade, improving contractile function and reducing the incidence of sudden cardiac death<sup>[3]</sup>. None of these approaches is capable of restoring the cellular loss produced after MI, which is the cornerstone of the adverse ventricular remodeling process that leads to HF. Heart transplantation is a not viable alternative in most cases, and is hampered by limited donor hearts.

Since the beginning of the past decade, regenerative cardiac therapies have emerged as an option to satisfy these unmet needs<sup>[12-14]</sup>. Until the end of the last century, the human heart was believed to be a terminally differentiated post mitotic organ, unable to be repaired after an injury such acute MI. This dogma was challenged in 2001 by the evidence of mitosis in cardiomyocytes after MI<sup>[15]</sup>. Beyond that, and in contrast to some lower vertebrates<sup>[16]</sup>, cardiac self-repair ability is limited in human adults<sup>[17]</sup>, and unable to replace the massive cellular loss that occurs after MI, but its discovery opened up the possibility to be externally enhanced. In order to achieve this objective, two approaches can be adopted: (1) the addition of

progenitor cells for the repopulation of the damaged heart with contractile cells and vascular structures; and (2) the enhancement of cardiac self-repair ability. Most times both mechanisms are concomitant. The former, represented by stem cell therapy, has been the most extensively investigated, being tested in several studies enrolling thousands of patients worldwide and evaluating different cell types and delivery routes. Promising results were reported in preclinical studies, leading to a quick translation into clinical trials. Initial reports in human beings were encouraging and established the safety of these therapies. But their efficacy has been subject of continuous debate, since robust evidence is lacking due to inconsistency in benefits observed in clinical trials. Differences in methodologies have been invoked to explain these discrepancies. Notwithstanding, this initial preclinical and clinical data have provided important insights into the selection and manufacturing of cell products, their delivery and the mechanisms of action that drive myocardial regeneration. The initial concept of achieving cardiac repair by differentiation of stem cells into functional cardiomyocytes and vascular structures has shifted towards a paracrine paradigm, where stem cells exert their beneficial effects by promoting myocardial salvage and self-repair mechanisms.

So far, the optimal source for stem cell therapy as well as its processing, delivery route and dosage, remains unknown and many fundamental issues need to be addressed. Ideal stem cell therapy must accomplish with several requirements including: (1) the ability to regenerate damaged myocardium; (2) the ease of obtaining, storing and delivering; (3) no tumorigenesis, immunogenicity or ongoing ethical issues; and (4) cost-effectiveness. The heterogeneous cell products employed in the early years of regenerative cardiac medicine have been replaced by more purified stem cell populations with a greater reparative potential, and *in vitro* priming of stem cells to enhance their engraftment, survival, plasticity and paracrine activity, has also been extensively investigated. All of these advances have lead to a new generation of stem cells ("second-generation" stem cells) that should overcome the hurdles found with first-generation ones. In this review we summarize recent research and novel strategies in this field, focusing on priming of "first-generation" cells and on the new cell products that are being tested for cardiac regeneration after MI.

## GENETICALLY ENGINEERED SKELETAL MYOBLASTS

The first type of stem cell thought to be useful for cardiac regenerative purposes were autologous skeletal myoblasts. Their muscular phenotype and many other advantageous features including ease of isolation through muscle biopsy, rapid expansion *in vitro* and lack of ethical or immunological issues made them



an attractive option<sup>[18]</sup>. In fact, their use in animal models<sup>[19-21]</sup> and phase I non-randomized human trials<sup>[22-26]</sup> described their ability to form some cardiac structures and yielded promising results regarding improvement in cardiac performance after MI.

Nevertheless, subsequent studies documented that myoblasts differentiate into skeletal myocytes instead of cardiomyocytes<sup>[27]</sup>, and the first and larger randomized controlled trial in humans, the MAGIC trial, showed no benefits on cardiac function<sup>[28]</sup>.

More worrisome is the lack of electro-mechanical coupling of these cells, that made them prone to generate ventricular arrhythmias due to their inability to express certain cardiac-specific genes codifying important proteins of the gap junctions, as N-cadherin and connexin-4<sup>[25,28,29]</sup>. Down-regulation of these genes is induced by the transdifferentiation process<sup>[29]</sup>. However, improved electrical coupling as well as a reduction in the arrhythmogenic potential of the transplanted cells was demonstrated by the enhancement of connexin-43 expression *via* genetic manipulation<sup>[30-32]</sup>.

Another drawback of skeletal myoblasts in their application for cardiac repair is massive apoptosis and their low survival rate when applied to the ischemic myocardium<sup>[33]</sup>. Pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF), have showed their ability to induce angiogenesis<sup>[34,35]</sup>. Indeed, transfected skeletal myoblasts with augmented VEGF and FGF expression exhibit increased survival, promoted by an anti-inflammatory and angiogenic effect<sup>[36-38]</sup>. Cell survival after transplantation can also be improved using myoblasts lacking the *MyoD* gene. These myoblasts induce angiogenesis *via* secretion of stromal cell-derived factor-1 (SDF-1) and placental growth factor<sup>[39]</sup>, and are less sensitive to apoptosis by up-regulation of a number of anti-apoptotic genes (*Pax3*, *Bcl-2*, *Bcl-xl*)<sup>[40]</sup>.

Despite of these advances, skeletal myoblasts seem far from being the optimal cell source, with safer and more efficacious novel cell types displacing its role in cardiac regenerative medicine.

## BONE MARROW DERIVED STEM CELLS

### **Bone marrow derived mononuclear stem cells**

Bone marrow derived mononuclear stem cells (BMMNCs) are the most extensively used type of cells for cardiac regeneration. They represent a heterogeneous mixture of mononuclear stem cells including hematopoietic and endothelial progenitors and mesenchymal stem cells. The landmark study of Orlic *et al.*<sup>[41]</sup> was the proof-of-concept that a population of Lin<sup>-</sup>/c-kit<sup>+</sup> BMMNCs was capable of transdifferentiating into myocytes and vascular structures and restore a large amount of the damaged myocardium after MI in a mice model. This finding generated great interest in this type of cells, spurring

an intense research and rapid clinical translation.

The exact mechanisms by which BMMNCs are thought to work remain poorly understood. Different explanations have been advocated including paracrine effects, transdifferentiation into cardiomyocytes and activation of resident cardiac stem cells<sup>[42-44]</sup>.

Many studies have shown a positive effect of BMMNCs in cardiac function after MI, both in the acute and chronic phases<sup>[45-51]</sup>, although some other trials reported a less evident or even absent benefit<sup>[52-62]</sup>. The different clinical settings where cells have been tested, as well as methodology differences in these trials may explain, at least in part, the contradictory observed results. Five systematic reviews and meta-analyses concluded that BMMNCs transplantation after MI resulted in a better left ventricular function and remodeling with a modest but significant improvement in left ventricular ejection fraction (LVEF), ranging from 2.55% to 3.96%. No safety concerns have been observed and what is more provocative, better clinical outcomes have been documented in treated patients, although these trials did not include hard clinical endpoints as primary objectives<sup>[63-67]</sup>.

BMMNCs-based therapies face many limitations: (1) homing, engraftment and maintenance of cells in the transplanted tissue<sup>[68]</sup>; (2) limited plasticity when using adult stem cells<sup>[69,70]</sup>; and (3) depletion and impairment of their functionality associated with advancing age and comorbidities<sup>[71,72]</sup>. Many different strategies for cell enhancement have been studied in the last years in order to overcome these problems.

MicroRNA (miRs) are small non-coding RNAs controlling gene expression, either by inducing mRNA degradation or by blocking mRNA translation<sup>[73]</sup>. They are involved in the maintenance of the pluripotent state of stem cells and its over-expression is able to induce such a state in somatic cells<sup>[73,74]</sup>. They also participate in the differentiation and lineage commitment processes<sup>[73,74]</sup>. Thus, miRNAs have been investigated as a target for stem cell enhancement. For instance, cardiovascular diseases and ageing promote up-regulation of the pro-apoptotic and anti-proliferative miR-34a in human BMMNCs. Its inhibition *in vitro* has shown to improve cell survival and their therapeutic benefit in a mice model of MI<sup>[75]</sup>. Furthermore, BMMNCs seem to regulate the expression of miRs in cardiomyocytes *in vivo*. In a mice model, intramyocardial delivery of BMMNCs mediated a paracrine effect by releasing insulin-like growth factor 1 (IGF-1) which inhibited the expression of the pro-apoptotic miR-34, thereby exerting a cardioprotective effect<sup>[76]</sup>.

But as in the case of skeletal myoblasts, new and more refined cell products are supplanting BMMNCs. In the meanwhile, definitive conclusions about the efficacy of BMMNCs after MI are expected to be elicited by the ongoing BAMi trial (NCT01569178). This large scaled, multinational, multicenter randomized and controlled trial, will test for the first time, the effect of



intracoronary infusion of BMMNCs three to six days after acute MI with LVEF < 45% on all-cause mortality as its primary endpoint. Over 3000 patients in 11 European countries are planned to be enrolled. This trial will help to trace the future directions that should be followed with BMMNCs therapies, supporting its administration, warranting further investigations or definitively leaving apart their use to focus on new generations of cells.

### Mesenchymal stem cells

Mesenchymal stem cells (MSCs), also known as multipotent stromal cells, represent a subset of BMMNCs discovered more than 40 years ago<sup>[77]</sup>. They can be found within connective tissue in other organs, from which they can be easily isolated and cultured. They have awoken a big interest in recent years because they display a number of traits that made them an attractive cell product for cardiac repair<sup>[78]</sup>. MSCs are multipotent and have the capability to transdifferentiate into lineages of mesodermal tissues<sup>[79,80]</sup>, including cardiomyocytes<sup>[81]</sup>. *In vivo* studies have also proved the ability of human MSCs to differentiate into cardiomyocytes in adult mice hearts<sup>[82]</sup>. They also display a great paracrine potential, secreting growth factors that promote endogenous healing<sup>[83]</sup>.

A number of preclinical studies have shown the benefit of these cells in cardiac function after MI<sup>[84-86]</sup>. Clinical trials have also elicited promising results<sup>[87,88]</sup> and a small and recent randomized phase I and II placebo-controlled trial suggested that transendocardial injection of MSCs is superior to BMMNCs and placebo in reducing scar size in chronic ischemic cardiomyopathy<sup>[89]</sup>.

But similarly to BMMNCs, autologous use of MSCs is hampered by their loss of functionality associated with ageing and comorbidities<sup>[71,72]</sup>, and their heterogeneous phenotype compromises their therapeutic effect<sup>[90]</sup>. A variety of different strategies have been developed in order to improve MSCs regenerative potential. One of the most promising is the so-called "guided cardiopoiesis" of MSCs. This term defines the process by which a stem cell is engaged towards a cardiac differentiation program while its proliferative and self-renewal capacities remain intact<sup>[91]</sup>. This can be achieved by mimicking the cardiogenic instructive signals that drive the embryonic development of the heart<sup>[92]</sup>. The up-regulation of certain cardiac transcription factors such as Nkx-2.5, MEF2C, FOG-2, TBX5, MESP1 and GATA-4 is responsible of the adoption of a cardiogenic phenotype in MSCs, preserving their proliferative ability before the final differentiation step towards sarcomerogenesis begins<sup>[92,93]</sup>. The up-regulation of these cardiac transcription factors is feasible when MSCs are cultured within a cardiogenic cocktail containing transforming growth factor beta (TGF- $\beta$ ), bone morphogenic protein (BMP)-4, activin A, FGF-2, IGF-1, interleukin (IL)-6, factor IIa (h-alpha-thrombin) and retinoic acid<sup>[93]</sup>. Differentiation beyond this intermediate state is possible by exposing the cells to a 1% human

platelet lysate medium<sup>[93]</sup>. In a murine model of ischemic cardiomyopathy, the priming of MSCs with these growth factors led to a cardiopoietic phenotype that improved their therapeutic benefit (by yielding sarcomere-containing myocytes with electromechanical response, and with mitochondrial structures closer to that seen in adult cardiomyocytes)<sup>[93]</sup>. In another rat model of MI, transplantation of pretreated MSCs with IGF-1, BMP-2 and FGF-2 led to increased tolerance to hypoxic conditions of the MSCs and augmented expression of connexin-43 and gap junctions linking MSCs to cardiomyocytes. Indeed, MSCs exerted a protective effect over cardiomyocytes against hypoxia mediated by these gap junctions, showing a superior myocardial salvage of the infarcted heart and leading to an improvement in left ventricular function<sup>[94]</sup>. Recently, the C-CURE clinical trial (Table 1) addressed the feasibility and the safety of cardiopoietic autologous MSCs in a randomized trial involving 48 patients with chronic ischemic HF<sup>[95]</sup>. In this phase II study, the lineage-guided therapy with primed MSCs resulted in a total improvement in LVEF of 7% over baseline, compared to 0.2% in the standard of care group. Reductions in left ventricular volumes and increases in the 6-min walk distance were also observed. No adverse events related with the cells were documented<sup>[95]</sup>. This study has supported the initiation of two ongoing large phase III trials, CHART-1 (NCT01768702) and CHART-2, where autologous cardiopoietic MSCs will be tested in patients with chronic HF secondary to IM.

A drawback of MSCs therapy is the poor survival and the low retention rate of transplanted cells that limit their regenerative potential. *Ex vivo* genetic modification may enhance viability of the transplanted cells by over-expression of anti-apoptotic genes. MSCs transduced with vectors encoding for genes such as heat shock protein (Hsp27)<sup>[96]</sup>, microRNA-1<sup>[97]</sup>, and protein kinase type 1 $\alpha$ <sup>[98]</sup> have shown increased survival and a more effective and efficient performance restoring cardiac function compared to conventional MSCs in rodent models of MI. Other pharmacological approaches to improve cell retention have also been evaluated. Thymosin  $\beta$ 4 is a protein implicated in cytoskeletal homeostasis that has been shown to protect MSCs against hypoxic injury and to increase cell retention in a rat model of MI<sup>[99]</sup>.

In summary, MSCs are among the most promising cells for cardiac reparative medicine. Novel approaches that prime cell functionality have shown encouraging results that have led to ongoing large-scaled randomized phase III clinical trials.

## CARDIAC STEM CELLS

The classic concept stating that the adult mammalian heart is a post-mitotic organ without self-renewal capacity was challenged in the beginning of the past decade. The first evidence came out with the finding of mitosis among cardiomyocytes in human hearts after MI by Beltrami *et al.*<sup>[15]</sup>. Shortly after, the same group



**Table 1** Clinical trials evaluating new stem cells for cardiac repair following myocardial infarction

Study	n	Design	Type of cells	Delivery route	Clinical setting	Follow-up	Outcomes
Bartunek <i>et al</i> <sup>[95]</sup> (C-CURE)	47	Multicenter, randomized 2:1 (cells <i>vs</i> standard of care)	Autolo-gous bone marrow derived cardiopietic MSCs	Endo- myocardial injection	Chronic ischemic heart failure (LVEF 15%-40%)	Safety 2 yr Efficacy 6 mo	Feasible and safe ↑ LVEF ↓ LVESV ↑ 6-min walk distance and improvements in QoL and NYHA
Bolli <i>et al</i> <sup>[113]</sup> (SCIPIO)	23	Unicenter, randomized 2:1 (cells <i>vs</i> standard of care)	Autolo-gous c-kit+/lin- CSCs	Intra-coronary infusion	Chronic ischemic heart failure (LVEF ≤ 40% four months post CABG)	12 mo	Feasible and safe ↑ LVEF ↓ Infarct size
Malliaras <i>et al</i> <sup>[122]</sup> (CADUCEUS)	25	Two centers, randomized 2:1 (cells <i>vs</i> standard of care)	Autolo-gous CDCs	Intra-coronary infusion	Chronic ischemic heart failure (1.5-3 mo after MI)	12 mo	Feasible and safe ↓ Infarct size ↑ Viable myocardium and regional contractility ≈ LVEF and ventricular volumes
Hare <i>et al</i> <sup>[144]</sup> (POSEIDON)	30	Multicenter, randomized 1:1 (autologous <i>vs</i> allogeneic cells)	Three different doses of autologous or allogeneic bone marrow derived MSCs	Endo-myocardial injection	Chronic ischemic heart failure (LVEF ≤ 50%)	12 mo	Feasible and safe ≈ LVEF Autologous ↑ 6-min walk distance and QoL Allogeneic ↓ LVEDV

↑: Indicates increased; ↓: Indicates decreased; ≈: Indicates no change; MI: Myocardial infarction; MSCs: Mesenchymal stem cells; LVEF: Left ventricular ejection fraction; LVESV: Left ventricular end-systolic volume; QoL: Quality of life; CSCs: Cardiac stem cells; CABG: Coronary artery by-pass graft; CDCs: Cardiosphere-derived cells; LVEDV: Left ventricular end-systolic volume.

described the existence of a population of multipotent and clonogenic cells that expressed the tyrosine kinase receptor in their surface (c-kit<sup>+</sup>) and that were able to differentiate into the three cardiac lineages (cardiomyocytes, smooth muscle cells and endothelial cells)<sup>[100]</sup>. Since then, strong evidence supported the concept that turnover in the adult cardiomyocyte population is provided by cardiac stem cells (CSCs)<sup>[17,101]</sup>, but the magnitude of this turnover and the exact underlying mechanisms remain unknown<sup>[102]</sup>. CSCs are rapidly activated after myocardial injuries or physiological stimuli<sup>[103-105]</sup>. Recently, Ellison *et al*<sup>[106]</sup> have demonstrated in a murine model that some of the new cardiomyocytes generated after a myocardial injury are c-kit<sup>+</sup> CSCs descendants. Nevertheless, this finding does not rule out other concomitant mechanisms that could be implicated in myocardial regeneration, and a recent report by van Berlo *et al*<sup>[107]</sup> suggests that the new cardiomyocytes generated from CSCs are functionally insignificant. In fact, it is evident that self-renewal capacity of the adult human heart is unable to restore the large amount of cellular loss after MI.

Stem cell therapy with CSCs may offer some advantages over other extra-cardiac cells, because they are believed to be more prone to differentiate towards cardiac lineages. Most research has focused on transplanting CSCs in the infarcted area and various preclinical studies using this approach in different animal models have demonstrated the ability of CSCs to alleviate left ventricular dysfunction in both acute and chronic MI<sup>[100,108-112]</sup>.

These promising results warranted translation into

clinical research and led to the conduction of the first phase I clinical trial using c-kit<sup>+</sup> CSCs, the SCIPIO trial<sup>[113]</sup> (Table 1). This study reported that isolation and expansion of CSCs from cardiac tissue, harvested during coronary artery by-pass graft surgery, was feasible. Intracoronary injection of these cells in patients with left ventricular dysfunction (LVEF < 40%) after surgical revascularization improved LVEF by 12.3% compared with baseline, reduced scar size and was associated with improvements in quality of life and New York Heart Association functional class. These are preliminary data of the first eight patients enrolled in the study, which is still ongoing.

However the number of CSCs in adult hearts is small, and albeit their *ex vivo* isolation and expansion are feasible, their availability represents a major challenge. In 2004, Messina *et al*<sup>[114]</sup> first described the *in vitro* formation of self-adherent multicellular spherical clusters from human surgical cardiac biopsies in culture, termed cardiospheres (CSs). These CSs were clonogenic and able to yield the three cardiac lineages *in vitro* and *in vivo* when transplanted in an animal model of MI, resulting in improvements in fractional shortening<sup>[114]</sup>. CSs have a core of c-kit<sup>+</sup> cells surrounded by progenitors in distinct stages of the differentiation process towards the three major cardiac lineages. They can also be obtained through percutaneous endomyocardial biopsy and used as source for cardiospheres-derived cells (CDCs), a mixed cell population that is clonogenic, express surface markers typical of stem cells (c-kit<sup>+</sup>, CD105<sup>+</sup>), negligible hematopoietic markers, and that can be expanded on fibronectin<sup>[115]</sup>. This approach, described by Marbán *et al*<sup>[115]</sup>, has some advantages, *i.e.*,



the larger number of cells that can be obtained and the shorter time of manufacturing, in comparison to other methods to produce CSCs<sup>[111,116,117]</sup>. In animal models of MI, human CDCs have been shown to promote cardiac regeneration and to improve LVEF<sup>[115,118,119]</sup>. The same group conducted a direct comparison between CDCs and other extracardiac stem cells including BMMNCs, MSCs and adipose derived stem cells (ADSCs) in order to address their potency for myocardial repair. *In vitro*, CDCs showed a greater myogenic differentiation potency and higher angiogenic and paracrine potential. *In vivo*, injection of CDCs in infarcted mice provided the greatest functional benefit<sup>[120]</sup>.

This preclinical evidence led to the first phase I clinical trial using CDCs, the CADUCEUS trial (Table 1). This study randomized 25 patients with LVEF 25%-45%, two to four weeks after MI, to receive intracoronary autologous CDCs obtained from endomyocardial biopsies or standard of care. The primary safety endpoint was the proportion of patients who died due to ventricular tachycardia, ventricular fibrillation, or sudden unexpected death, or had MI after cell infusion, new cardiac tumor formation on MRI, or a major adverse cardiac event (MACE: Composite of death and hospital admission for HF or non-fatal recurrent MI). Preliminary efficacy endpoints on MRI parameters were also analyzed. Autologous CDCs treatment was safe and associated with reductions in scar size, increased viable myocardium and regional contractility. Nonetheless, ventricular volumes and LVEF did not differ between groups<sup>[121,122]</sup>, casting doubts about the true regenerative effect of CDCs.

Notwithstanding, both SCPIO and CADUCEUS trials included a small number of patients and there are some issues concerning their results, such as the low expression of cardiac markers in CSCs in the former, and the lack of benefit in terms of LVEF in the latter. Moreover, and although in both studies a true regeneration of damaged myocardium was invoked, the real underlying mechanism of action of these cells remains unproved.

Similarly to other extra-cardiac cell types, engraftment and retention rates of the transplanted CSCs and CDCs within the harsh environment of the infarcted myocardium are low, regardless of the administration route<sup>[112,123]</sup>. Although direct differentiation of cardiac progenitor cells in cardiac lineages has been observed, this observation of low CSCs retention rates into the myocardium further supports the hypothesis of a paracrine effect, as another pathway responsible of the positive effect of the cells<sup>[124]</sup>.

In order to overcome the limited survival and retention of the transplanted cells, different strategies have been tested in animal models. Mohsin *et al.*<sup>[125]</sup> used genetic manipulation to deliver the pro-survival gene Pim-1 kinase into human CSCs, resulting in a superior cellular engraftment and differentiation, that led to a superior reparative potential compared with conventional CSCs in an immuno-compromised

mice model of MI. Interestingly, no oncogenic issues were observed because telomere lengthening induced by Pim-1 overexpression was transitory<sup>[126]</sup>. Another option for priming CSCs and make them less prone to apoptosis under hypoxic conditions is the preconditioning with cytoprotective pharmacological agents. For example, treatment of CSCs with cobalt protoporphyrin reduces oxidative stress-induced apoptosis, by up-regulation of heme oxygenase 1, COX-2, and anti-apoptotic proteins (BCL2, BCL2-A1, and MCL-1) and increased phosphorylation of NRF2<sup>[127]</sup>. Hypoxic protection can also be achieved by means of  $\beta$ -O-linkage of N-acetylglucosamine to certain pathway proteins. Increases in this pro-survival signaling system by thiamet-G significantly improved CDCs survival after hypoxic injury<sup>[128]</sup>. In addition, exposure of CSCs to H<sub>2</sub>O<sub>2</sub> for 2 d before transplantation stimulates neoangiogenesis in the peri-infarct area following ischemic-reperfusion injury and could be a viable therapeutic option to prevent HF<sup>[129]</sup>. CDCs engraftment and differentiation can also be enhanced by controlled release of FGF in ischemic myocardium in a pig model of MI, resulting in a significant functional improvement<sup>[130]</sup>.

Finally, previous reports suggest that one of the mechanisms by which MSCs promote cardiac repair is the stimulation of endogenous CSCs *via* paracrine effects<sup>[131]</sup>. This finding raised the question whether combining both MSCs and CSCs could amplify the response to cell therapy. This hypothesis has been recently addressed by Williams *et al.*<sup>[132]</sup>, they found that intramyocardial delivery of human CSCs and bone marrow MSCs in a swine model of MI resulted in a 2-fold greater scar reduction and a 7-fold enhancement of engraftment of stem cells compared with each cell therapy alone, leading to improvements in hemodynamics and LVEF.

The discovery of CSC has revolutionized regenerative medicine. As they are derived from the target organ, they are supposed to be more committed with a cardiac fate, and the understanding of the molecular mechanisms that steer their mode of action is providing new insights that extend application of cell-based therapies. Early clinical experience with CSCs/CDCs warrants further investigation in larger phase II studies that will address the unresolved issues of SCPIO and CADUCEUS. At present, three clinical trials are evaluating the effect of CSCs and CSs in myocardial regeneration after MI: the RECONSTRUCT (NCT01496209), the ALLSTAR (NCT01458405) and the ALCADIA trials.

## ALLOGENEIC STEM CELL THERAPY FOR CARDIAC REPAIR

Allogeneic stem cell therapy may offer many advantages in cardiac regenerative medicine. Firstly, allogeneic cells could be used as a scalable and reproducible cell product readily available "off-the-shelf"



that could be administered in the setting of primary PCI avoiding delays inherent to harvest and culture of autologous stem cells. Secondly, this type of cells can be obtained from young and healthy donors, thereby avoiding the aforementioned impairment in autologous stem cells functionality observed with advancing age and comorbidities.

Most research in allogeneic cardiac regeneration has been done with MSCs due to their immunoprivileged profile and immunosuppressive properties. MSCs lack expression of major histocompatibility class II antigens<sup>[133]</sup>, down-regulate T cells response through direct contact and secretion of anti-inflammatory cytokines<sup>[134]</sup> and significantly affect the ability of dendritic cells to prime T-cell responses<sup>[135]</sup>. These findings raised interest in MSCs for allogeneic stem cell transplantation. Nonetheless, recent data have challenged this assumption suggesting that under certain inflammatory environment and during their differentiation process, MSCs can switch their immune phenotype towards an immune-enhancing pattern, limiting their long term survival and benefits<sup>[136,137]</sup>. However, and as pointed out before, autologous stem cell therapy faces the same problem, and if the advocated paracrine effect is responsible of stem cell regenerative capacity, survival and maintenance of cells in the transplanted heart could not be indispensable<sup>[138]</sup>.

There is a large body of preclinical work supporting the safety and the efficacy of allogeneic MSCs therapy<sup>[84,139-142]</sup>. In a large animal model of MI in pigs, Amado *et al.*<sup>[139]</sup> demonstrated that allogeneic MSCs injections in the necrotic area were feasible with no evidence of rejection and provided near-normalization of cardiac function and Quevedo *et al.*<sup>[84]</sup> refrained these results in a swine model of chronic ischemic cardiomyopathy, where allogeneic MSCs showed to be able to differentiate in the three cardiac lineages and to improve LVEF. These findings supported the first-in-man clinical trial using allogeneic bone marrow-derived MSCs by Hare *et al.*<sup>[143]</sup>. They conducted a randomized, double-blinded, placebo-controlled phase I trial in which 53 patients with reperfused MI were allocated to intravenous administration of allogeneic human MSCs or placebo. The study met its primary safety objective with no evidence of tumor formation or immunogenicity. It also suggested a greater benefit in cardiac function in larger MIs and improved outcomes regarding arrhythmias and pulmonary performance<sup>[143]</sup>. The same group has recently conducted a head-to-head comparison between autologous and allogeneic MSCs in a phase I / II randomized pilot study involving 30 patients with chronic ischemic left ventricular dysfunction secondary to MI, the POSEIDON trial<sup>[144]</sup> (Table 1). In this case, cell therapy was delivered by transendocardial injection. Both therapies were safe, with no or negligible allogeneic sensitization in the allogeneic group and no evidence of ectopic tissue formation. Both types of cells reduced infarct

size as measured by early enhancement defects in multidetector computed tomography, but no significant improvements in ejection fraction were documented. Intriguingly, allogeneic MSCs reduced LV end-diastolic volumes while autologous did not. Furthermore, improvements in quality of life, NYHA and 6-min walk test were more evident with autologous therapy. However, the small number of patients and the absence of a placebo group make these observations difficult to interpret. An interesting finding was the greater benefit regarding LV volumes and LVEF obtained with low-dose concentrations of MSCs vs high doses (20 million vs 200 million), which is consistent with preclinical data<sup>[145]</sup> and warrants further investigation of the optimum dose before undertaking large clinical trials.

Mesenchymal precursor cells (MPCs) represent an immature subpopulation of bone marrow-derived cells that express the Stro3<sup>+</sup> marker<sup>[146]</sup>. MPCs are multipotent cells with an extensive proliferative and differentiation capacity as well as a great paracrine potential that outperforms the one of MSCs<sup>[147,148]</sup>. They also display an immunoprivileged phenotype, appropriate for allogeneic transplantation. Previous animal studies have provided evidence on the positive effect of these cells in the setting of acute MI when delivered intramyocardially<sup>[145,148,149]</sup> or by intracoronary infusion<sup>[150]</sup>. Houtgraaf *et al.*<sup>[150]</sup> have recently reported the benefit of the intracoronary infusion of different doses of allogeneic MPCs, when delivered few minutes after reperfusion in a sheep model of MI. The therapy was safe and provided reductions in infarct size *via* cardiomyocyte salvation and proliferation, and induced angiogenesis. It was also suggested that the smaller size of MPCs, compared to MSCs, prevents microvascular obstruction due to cellular aggregation observed in previous studies. Similarly to the findings of the POSEIDON trial, the benefit was more evident in the low-dose group (< 75 million vs 200 million), an observation which may be related to the better coronary flow observed after infusion with lower number of cells.

Recently, Penn *et al.*<sup>[151]</sup> have tested a different bone marrow-derived cell product, named MultiStem, in 19 patients with acute MI in an allogeneic setting and using a novel delivery approach. Three different doses of cells were injected through a microneedle in the culprit vessel adventitia two to five days after primary PCI. The procedure was safe and LVEF was increased when compared to registry controls.

Another milestone in the field of allogeneic cell therapy is being carried out in the CAREMI clinical trial. This study represents the first-in-man experience with allogeneic CSCs in the acute setting of CHD. It is a phase I / II clinical trial that has been designed with a first dose-escalation phase and a second part with a randomized and controlled design. In this trial, 55 patients with acute reperfused MI and LVEF < 45% will be treated with intracoronary infusion of human



allogeneic CSCs, being the primary objective the rate of death from any cause at 30 d (dose-escalation phase) and MACE and magnetic resonance parameters at 12 mo (randomized phase).

In summary, allogeneic cell therapy represent a novel approach in cardiac regeneration that could overcome some of the strong barriers found by autologous cell products. Before large scaled trials are performed, some concerning issues as immunogenicity and rejection must be thoroughly examined.

## OTHER SOURCES OF STEM CELLS

In contrast to adult stem cells, embryonic stem cells (ESCs) are pluripotent, *i.e.*, they are capable of differentiate into cells of the three germ layers. ESCs isolated from blastocysts demonstrate a great potential to generate functional cardiomyocytes and their transplantation into infarcted hearts in animal models have been evaluated showing improvements in LV function<sup>[152,153]</sup>. Despite these findings, ESCs-based therapies face strong barriers that go beyond biological aspects, such as immune rejection or tumor formation. Ethical issues also remain a major limitation to the widespread use of these cells. So far, no clinical trial has tested ESCs in humans and is unlikely to be done.

Given these constraints, Takahashi and Yamanaka reported in 2006 the feasibility of generating induced pluripotent stem cells (iPSCs) from mouse adult fibroblasts by the transduction of four transcription factors that led to a phenotype similar to that of ESCs<sup>[154]</sup>. This achievement could prevent the immunogenicity issues inherent to allogeneic ESCs application, but do not avoid oncogenic risks<sup>[155]</sup>. On top of this, current reprogramming of somatic cells is a low efficient process that should be refined before translation to clinical trials. Therefore, nowadays, iPSCs represent a new source of cells that holds a great promise for reparative medicine but is not ready for their application in human beings yet.

ADSCs represent a population of stem cells located in the adipose tissue that are able to differentiate into multiple cell lineages including cardiomyocytes and vascular cells<sup>[156]</sup>. They offer two major advantages over some previously mentioned types of cells: firstly, the easy and repeatable access that makes it possible to harvest large amounts of adipose tissue by a minimally invasive method and, secondly, their increased proliferative potential in culture<sup>[157]</sup>. Preclinical reports have documented that administration of ADSCs after MI improves cardiac function<sup>[158,159]</sup>, in a range similar to BMMNCs<sup>[159]</sup>. They have also been tested in 2 clinical trials, the APOLLO<sup>[160]</sup> and the PRECISE trials<sup>[161]</sup>. The APOLLO trial was a small randomized, double-blind, placebo controlled, phase I / II study designed to assess the safety and the feasibility of intracoronary infusion of ADSCs in patients with large ST-segment elevation acute MIs. The study proved the feasibility and the safety of both liposuction and ADSCs

intracoronary infusion after MI, and showed a trend towards improved cardiac function and reductions in scar formation<sup>[160]</sup>. The PRECISE trial reported that intramyocardial injection of ADSCs in patients with refractory angina not amenable for revascularization ("no-option" patients) improved exercise capacity and myocardial perfusion, reduced scar size and preserved maximal oxygen consumption<sup>[161]</sup>. However, the discovery of CSCs and new developments with MSCs and allogeneic therapies have somehow waned the initial interest in ADSCs.

## STEMLESS APPROACHES

To conclude, stemless therapies to restore cardiac function after MI haven also been proposed. As outlined above, if stem cell benefits are mediated by a paracrine effect and by activation of self-repair mechanisms, exogenous administration of the cytokines and growth factors implicated in the regeneration process could theoretically replace cell transplantation with a readily and "off-the-self" available product, similarly to other current biological treatments. Nadal-Ginard *et al.*<sup>[102]</sup> advocate this cell-free therapies. They have reported that intracoronary administration of IGF-1 and hepatocyte growth factor after coronary reperfusion reduced cardiac remodeling, induced myocardial regeneration and improved ventricular function in a pig model of MI. This was achieved by activation of c-kit<sup>+</sup> endogenous CSCs, which expanded and generated new cardiomyocytes and microvessels<sup>[162,163]</sup>. Other acellular approaches have tried to promote stem cell recruitment by the infarcted myocardium. Modulation of the CXC-chemokine receptor 4 and SDF-1 axis *via* gene therapy has shown to exert beneficial effects in preclinical studies and in human phase I trials<sup>[164,165]</sup>.

In spite of that, molecular, cellular and myocardial tissue regeneration mechanisms are highly complex and driven by the interplay of several factors, not yet completely understood. Therefore, it seems unlikely that the simple administration of one or two growth factors could be capable of inducing a full cardiac regeneration. Nevertheless, further research in this field is warranted in order to shed light on these mechanisms and to elucidate if stemless approaches are a feasible option in the future.

## CONCLUSION

Regenerative therapies represent a novel paradigm in cardiovascular medicine, and have grown up over infancy and into adolescence. Translation of basic research and animal studies into the clinical scenario has never been so quick, driven by the enormous enthusiasm raised by the possibility of achieving heart regeneration. This initial euphoria has been dampened by some contradictory results and by the modest benefits observed in cardiac function, leading



to skepticism in a part of the scientific community. But these pioneering results should not be interpreted as the definite evidence in favor or against cell-based reparative therapies. Instead, they have established their safety and have shown the strong barriers that should be overcome, providing important insights about the source of cells to be used as well as about manufacturing processes and delivery routes. With all this background, the state-of-the-art in the field of stem cell therapy can be summarized as follows: (1) The molecular mechanisms that rule myocardial repair are highly intricate and still remain poorly understood. It is unlikely that the simple administration of "first-generation" stem cells or growth factors to the failing heart will be able to achieve a complete restoration of cardiac function; (2) As a result of a better knowledge of the molecular and genetic mechanisms that induce progenitor cell proliferation and differentiation into cardiac structures, a new generation of cell products ("second-generation" stem cells) are now being evaluated in large clinical trials after promising and encouraging results in phase I and II studies; and (3) Combining cellular, molecular and genetic basic research with preclinical studies and well-designed clinical trials, together with more collaborative research networks and with the definitive help of bioengineering, will be the keys for the definitive development of cardiac regenerative medicine.

Keeping all this in mind, the future of stem cell-based therapies is more promising than ever, and the goal of a true regeneration or repair of the damaged myocardium stands awaiting in the years to come.

## REFERENCES

- Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation* 2014; **129**: e28-e292 [PMID: 24352519 DOI: 10.1161/01.cir.0000441139.02102.80]
- McMurray JJ, Petrie MC, Murdoch DR, Davie AP. Clinical epidemiology of heart failure: public and private health burden. *Eur Heart J* 1998; **19** Suppl P: P9-16 [PMID: 9886707]
- McMurray JJ. Clinical practice. Systolic heart failure. *N Engl J Med* 2010; **362**: 228-238 [PMID: 20089973 DOI: 10.1056/NEJMc0909392]
- Ford ES, Ajani UA, Croft JB, Critchley JA, Labarthe DR, Kottke TE, Giles WH, Capewell S. Explaining the decrease in U.S. deaths from coronary disease, 1980-2000. *N Engl J Med* 2007; **356**: 2388-2398 [PMID: 17554120 DOI: 10.1056/NEJMs053935]
- Kuulasmaa K, Tunstall-Pedoe H, Dobson A, Fortmann S, Sans S, Tolonen H, Evans A, Ferrario M, Tuomilehto J. Estimation of contribution of changes in classic risk factors to trends in coronary-event rates across the WHO MONICA Project populations. *Lancet* 2000; **355**: 675-687 [PMID: 10703799]
- Preis SR, Hwang SJ, Coady S, Pencina MJ, D'Agostino RB, Savage PJ, Levy D, Fox CS. Trends in all-cause and cardiovascular disease mortality among women and men with and without diabetes mellitus in the Framingham Heart Study, 1950 to 2005. *Circulation* 2009; **119**: 1728-1735 [PMID: 19307472 DOI: 10.1161/circulationaha.108.829176]
- Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Stafford R, Thom T, Wasserthiel-Smoller S, Wong ND, Wylie-Rosett J. Executive summary: heart disease and stroke statistics--2010 update: a report from the American Heart Association. *Circulation* 2010; **121**: 948-954 [PMID: 20177011 DOI: 10.1161/circulationaha.109.192666]
- Stewart S, MacIntyre K, Hole DJ, Capewell S, McMurray JJ. More 'malignant' than cancer? Five-year survival following a first admission for heart failure. *Eur J Heart Fail* 2001; **3**: 315-322 [PMID: 11378002 DOI: 10.1016/S1388-9842(00)00141-0]
- Kovacic JC, Fuster V. From treating complex coronary artery disease to promoting cardiovascular health: therapeutic transitions and challenges, 2010-2020. *Clin Pharmacol Ther* 2011; **90**: 509-518 [PMID: 21900892 DOI: 10.1038/clpt.2011.173]
- Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. Heart disease and stroke statistics--2012 update: a report from the American Heart Association. *Circulation* 2012; **125**: e2-e220 [PMID: 22179539 DOI: 10.1161/CIR.0b013e31823ac046]
- Yeh RW, Sidney S, Chandra M, Sorel M, Selby JV, Go AS. Population trends in the incidence and outcomes of acute myocardial infarction. *N Engl J Med* 2010; **362**: 2155-2165 [PMID: 20558366 DOI: 10.1056/NEJMoa0908610]
- Ptaszek LM, Mansour M, Ruskin JN, Chien KR. Towards regenerative therapy for cardiac disease. *Lancet* 2012; **379**: 933-942 [PMID: 22405796 DOI: 10.1016/s0140-6736(12)60075-0]
- Sánchez PL, Villa A, Sanz R, Domínguez M, Ludwig I, Fernández ME, Fernández-Avilés F. Present and future of stem cells for cardiovascular therapy. *Ann Med* 2007; **39**: 412-427 [PMID: 17852037 DOI: 10.1080/07853890701513746]
- Wollert KC, Drexler H. Cell therapy for the treatment of coronary heart disease: a critical appraisal. *Nat Rev Cardiol* 2010; **7**: 204-215 [PMID: 20177405 DOI: 10.1038/nrcardio.2010.1]
- Beltrami AP, Urbancsek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, Anversa P. Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med* 2001; **344**: 1750-1757 [PMID: 11396441 DOI: 10.1056/nejm200106073442303]
- Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. *Science* 2002; **298**: 2188-2190 [PMID: 12481136 DOI: 10.1126/science.1077857]
- Hsieh PC, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat Med* 2007; **13**: 970-974 [PMID: 17660827 DOI: 10.1038/nm1618]
- Ciecierska A, Chodkowska K, Motyl T, Sadkowski T. Myogenic cells applications in regeneration of post-infarction cardiac tissue. *J Physiol Pharmacol* 2013; **64**: 401-408 [PMID: 24101386]
- Farahmand P, Lai TY, Weisel RD, Fazel S, Yau T, Menasché P, Li RK. Skeletal myoblasts preserve remote matrix architecture and global function when implanted early or late after coronary ligation into infarcted or remote myocardium. *Circulation* 2008; **118**: S130-S137 [PMID: 18824744 DOI: 10.1161/circulationaha.107.757617]
- Gavira JJ, Perez-Illarbe M, Abizanda G, García-Rodríguez A,



- Orbe J, Páramo JA, Belzunce M, Rábago G, Barba J, Herreros J, Panizo A, de Jalón JA, Martínez-Caro D, Prósper F. A comparison between percutaneous and surgical transplantation of autologous skeletal myoblasts in a swine model of chronic myocardial infarction. *Cardiovasc Res* 2006; **71**: 744-753 [PMID: 16843451 DOI: 10.1016/j.cardiores.2006.06.018]
- 21 **Taylor DA**, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, Glower DD, Kraus WE. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med* 1998; **4**: 929-933 [PMID: 9701245 DOI: 10.1038/nm0898-929]
  - 22 **Dib N**, McCarthy P, Campbell A, Yeager M, Pagani FD, Wright S, MacLellan WR, Fonarow G, Eisen HJ, Michler RE, Binkley P, Buchele D, Korn R, Ghazoul M, Dinsmore J, Opie SR, Diethrich E. Feasibility and safety of autologous myoblast transplantation in patients with ischemic cardiomyopathy. *Cell Transplant* 2005; **14**: 11-19 [PMID: 15789658 DOI: 10.3727/000000005783983296]
  - 23 **Hagège AA**, Marolleau JP, Vilquin JT, Alhérière A, Peyrard S, Duboc D, Abergel E, Messas E, Mousseaux E, Schwartz K, Desnos M, Menasché P. Skeletal myoblast transplantation in ischemic heart failure: long-term follow-up of the first phase I cohort of patients. *Circulation* 2006; **114**: 1108-1113 [PMID: 16820558 DOI: 10.1161/circulationaha.105.000521]
  - 24 **Menasché P**, Hagège AA, Scorsin M, Pouzet B, Desnos M, Duboc D, Schwartz K, Vilquin JT, Marolleau JP. Myoblast transplantation for heart failure. *Lancet* 2001; **357**: 279-280 [PMID: 11214133 DOI: 10.1016/S0140-6736(00)03617-5]
  - 25 **Menasché P**, Hagège AA, Vilquin JT, Desnos M, Abergel E, Pouzet B, Bel A, Sarateanu S, Scorsin M, Schwartz K, Bruneval P, Benbunan M, Marolleau JP, Duboc D. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *J Am Coll Cardiol* 2003; **41**: 1078-1083 [PMID: 12679204 DOI: 10.1016/S0735-1097(03)00092-5]
  - 26 **Siminiak T**, Kalawski R, Fiszer D, Jerzykowska O, Rzeźniczak J, Rozwadowska N, Kurpisz M. Autologous skeletal myoblast transplantation for the treatment of postinfarction myocardial injury: phase I clinical study with 12 months of follow-up. *Am Heart J* 2004; **148**: 531-537 [PMID: 15389244 DOI: 10.1016/j.ahj.2004.03.043]
  - 27 **Reinecke H**, Poppa V, Murry CE. Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting. *J Mol Cell Cardiol* 2002; **34**: 241-249 [PMID: 11851363 DOI: 10.1006/jmcc.2001.1507]
  - 28 **Menasché P**, Alfieri O, Janssens S, McKenna W, Reichenspurner H, Trinquart L, Vilquin JT, Marolleau JP, Seymour B, Larghero J, Lake S, Chatellier G, Solomon S, Desnos M, Hagège AA. The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation. *Circulation* 2008; **117**: 1189-1200 [PMID: 18285565 DOI: 10.1161/circulationaha.107.734103]
  - 29 **Fernandes S**, Amirault JC, Lande G, Nguyen JM, Forest V, Bignolais O, Lamirault G, Heudes D, Orsonneau JL, Heymann MF, Charpentier F, Lemarchand P. Autologous myoblast transplantation after myocardial infarction increases the inducibility of ventricular arrhythmias. *Cardiovasc Res* 2006; **69**: 348-358 [PMID: 16376327 DOI: 10.1016/j.cardiores.2005.10.003]
  - 30 **Abraham MR**, Henrikson CA, Tung L, Chang MG, Aon M, Xue T, Li RA, O' Rourke B, Marbán E. Antiarrhythmic engineering of skeletal myoblasts for cardiac transplantation. *Circ Res* 2005; **97**: 159-167 [PMID: 15976318 DOI: 10.1161/01.RES.0000174794.22491.a0]
  - 31 **Fernandes S**, van Rijen HV, Forest V, Evain S, Leblond AL, Mérot J, Charpentier F, de Bakker JM, Lemarchand P. Cardiac cell therapy: overexpression of connexin43 in skeletal myoblasts and prevention of ventricular arrhythmias. *J Cell Mol Med* 2009; **13**: 3703-3712 [PMID: 19438811 DOI: 10.1111/j.1582-4934.2009.00740.x]
  - 32 **Roell W**, Lewalter T, Sasse P, Tallini YN, Choi BR, Breitbach M, Doran R, Becher UM, Hwang SM, Bostani T, von Maltzahn J, Hofmann A, Reining S, Eiberger B, Gabris B, Pfeifer A, Welz A, Willecke K, Salama G, Schrickel JW, Kotlikoff MI, Fleischmann BK. Engraftment of connexin 43-expressing cells prevents post-infarct arrhythmia. *Nature* 2007; **450**: 819-824 [PMID: 18064002 DOI: 10.1038/nature06321]
  - 33 **Maurel A**, Azarnoush K, Sabbah L, Vignier N, Le Lore'h M, Mandet C, Bissery A, Garcin I, Carrion C, Fiszman M, Bruneval P, Hagege A, Carpentier A, Vilquin JT, Menasché P. Can cold or heat shock improve skeletal myoblast engraftment in infarcted myocardium? *Transplantation* 2005; **80**: 660-665 [PMID: 16177642 DOI: 10.1097/01.tp.0000172178.35488.31]
  - 34 **Rissanen TT**, Korpisalo P, Markkanen JE, Liimatainen T, Ordén MR, Kholová I, de Goede A, Heikura T, Gröhn OH, Ylä-Herttuala S. Blood flow remodels growing vasculature during vascular endothelial growth factor gene therapy and determines between capillary arterIALIZATION and sprouting angiogenesis. *Circulation* 2005; **112**: 3937-3946 [PMID: 16344386 DOI: 10.1161/circulationaha.105.543124]
  - 35 **Rissanen TT**, Markkanen JE, Arve K, Rutanen J, Kettunen MI, Vajanto I, Jauhainen S, Cashion L, Gruchala M, Näränen O, Taipale P, Kauppinen RA, Rubanyi GM, Ylä-Herttuala S. Fibroblast growth factor 4 induces vascular permeability, angiogenesis and arteriogenesis in a rabbit hindlimb ischemia model. *FASEB J* 2003; **17**: 100-102 [PMID: 12475908 DOI: 10.1096/fj.02-0377fje]
  - 36 **Yau TM**, Fung K, Weisel RD, Fujii T, Mickle DA, Li RK. Enhanced myocardial angiogenesis by gene transfer with transplanted cells. *Circulation* 2001; **104**: 1218-1222 [PMID: 11568059 DOI: 10.1161/hc37t1.094896]
  - 37 **Yau TM**, Li G, Weisel RD, Reheman A, Jia ZQ, Mickle DA, Li RK. Vascular endothelial growth factor transgene expression in cell-transplanted hearts. *J Thorac Cardiovasc Surg* 2004; **127**: 1180-1187 [PMID: 15052220 DOI: 10.1016/j.jtcvs.2003.09.052]
  - 38 **Zimna A**, Janeczka A, Rozwadowska N, Fraczek M, Kucharzewska P, Rucinski M, Mietkiewski T, Kurpisz M. Biological properties of human skeletal myoblasts genetically modified to simultaneously overexpress the pro-angiogenic factors vascular endothelial growth factor-A and fibroblast growth factor-4. *J Physiol Pharmacol* 2014; **65**: 193-207 [PMID: 24781729]
  - 39 **Nakamura Y**, Asakura Y, Piras BA, Hirai H, Tastad CT, Verma M, Christ AJ, Zhang J, Yamazaki T, Yoshiyama M, Asakura A. Increased angiogenesis and improved left ventricular function after transplantation of myoblasts lacking the MyoD gene into infarcted myocardium. *PLoS One* 2012; **7**: e41736 [PMID: 22848585 DOI: 10.1371/journal.pone.0041736]
  - 40 **Hirai H**, Verma M, Watanabe S, Tastad C, Asakura Y, Asakura A. MyoD regulates apoptosis of myoblasts through microRNA-mediated down-regulation of Pax3. *J Cell Biol* 2010; **191**: 347-365 [PMID: 20956382 DOI: 10.1083/jcb.201006025]
  - 41 **Orlic D**, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. *Nature* 2001; **410**: 701-705 [PMID: 11287958 DOI: 10.1038/35070587]
  - 42 **Behfar A**, Crespo-Diaz R, Terzic A, Gersh BJ. Cell therapy for cardiac repair--lessons from clinical trials. *Nat Rev Cardiol* 2014; **11**: 232-246 [PMID: 24594893 DOI: 10.1038/nrcardio.2014.9]
  - 43 **Dimmeler S**, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest* 2005; **115**: 572-583 [PMID: 15765139 DOI: 10.1172/jci24283]
  - 44 **Segers VF**, Lee RT. Stem-cell therapy for cardiac disease. *Nature* 2008; **451**: 937-942 [PMID: 18288183 DOI: 10.1038/nature06800]
  - 45 **Assmus B**, Honold J, Schächinger V, Britten MB, Fischer-Rasokat U, Lehmann R, Teupe C, Pistorius K, Martin H, Abolmaali ND, Tonn T, Dimmeler S, Zeiher AM. Transcatheter transplantation of progenitor cells after myocardial infarction. *N Engl J Med* 2006; **355**: 1222-1232 [PMID: 16990385 DOI: 10.1056/NEJMoa051779]
  - 46 **Assmus B**, Schächinger V, Teupe C, Britten M, Lehmann R, Döbert N, Grünwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation* 2002; **106**: 3009-3017 [PMID: 12473544 DOI: 10.1161/01.CIR.0000043246.74879.CD]
  - 47 **Fernández-Avilés F**, San Román JA, García-Frade J,



- Fernández ME, Peñarrubia MJ, de la Fuente L, Gómez-Bueno M, Cantalapiedra A, Fernández J, Gutierrez O, Sánchez PL, Hernández C, Sanz R, García-Sancho J, Sánchez A. Experimental and clinical regenerative capability of human bone marrow cells after myocardial infarction. *Circ Res* 2004; **95**: 742-748 [PMID: 15358665 DOI: 10.1161/01.RES.0000144798.54040.ed]
- 48 **Schächinger V**, Erbs S, Elsässer A, Haberbosch W, Hambrecht R, Hölschermann H, Yu J, Corti R, Mathey DG, Hamm CW, Süselbeck T, Assmus B, Tonn T, Dimmeler S, Zeiher AM. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med* 2006; **355**: 1210-1221 [PMID: 16990384 DOI: 10.1056/NEJMoa060186]
- 49 **Stamm C**, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schümichen C, Nienaber CA, Freund M, Steinhoff G. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet* 2003; **361**: 45-46 [PMID: 12517467 DOI: 10.1016/S0140-6736(03)12110-1]
- 50 **Strauer BE**, Brehm M, Zeus T, Köstering M, Hernandez A, Sorg RV, Kögler G, Wernet P. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* 2002; **106**: 1913-1918 [PMID: 12370212 DOI: 10.1161/01.CIR.0000034046.87607.1C]
- 51 **Wollert KC**, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A, Drexler H. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet* 2004; **364**: 141-148 [PMID: 15246726 DOI: 10.1016/S0140-6736(04)16626-9]
- 52 **Grajek S**, Popiel M, Gil L, Breborowicz P, Lesiak M, Czepczyński R, Sawiński K, Straburzyńska-Migaj E, Araszkiewicz A, Czyż A, Kozłowska-Skrzypczak M, Komarnicki M. Influence of bone marrow stem cells on left ventricle perfusion and ejection fraction in patients with acute myocardial infarction of anterior wall: randomized clinical trial: Impact of bone marrow stem cell intracoronary infusion on improvement of microcirculation. *Eur Heart J* 2010; **31**: 691-702 [PMID: 20022872 DOI: 10.1093/eurheartj/ehp536]
- 53 **Janssens S**, Dubois C, Bogaert J, Theunissen K, Deroose C, Desmet W, Kalantzi M, Herbots L, Sinnaeve P, Dens J, Maertens J, Rademakers F, Dymarkowski S, Gheysens O, Van Cleemput J, Bormans G, Nuyts J, Belmans A, Mortelmans L, Boogaerts M, Van de Werf F. Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. *Lancet* 2006; **367**: 113-121 [PMID: 16413875 DOI: 10.1016/S0140-6736(05)67861-0]
- 54 **Lunde K**, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, Endresen K, Ilebakk A, Mangschau A, Fjeld JG, Smith HJ, Taraldsrud E, Grøgaard HK, Bjørnerheim R, Brekke M, Müller C, Hopp E, Ragnarsson A, Brinchmann JE, Forfang K. Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med* 2006; **355**: 1199-1209 [PMID: 16990383 DOI: 10.1056/NEJMoa055706]
- 55 **Meyer GP**, Wollert KC, Lotz J, Steffens J, Lippolt P, Fichtner S, Hecker H, Schaefer A, Arseniev L, Hertenstein B, Ganser A, Drexler H. Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. *Circulation* 2006; **113**: 1287-1294 [PMID: 16520413 DOI: 10.1161/circulationaha.105.575118]
- 56 **Penicka M**, Horak J, Kobylka P, Pytlík R, Kozák T, Belohlavek O, Lang O, Skalická H, Simek S, Paleček T, Linhart A, Aschermann M, Widimsky P. Intracoronary injection of autologous bone marrow-derived mononuclear cells in patients with large anterior acute myocardial infarction: a prematurely terminated randomized study. *J Am Coll Cardiol* 2007; **49**: 2373-2374 [PMID: 17572255 DOI: 10.1016/j.jacc.2007.04.009]
- 57 **Perin EC**, Willerson JT, Pepine CJ, Henry TD, Ellis SG, Zhao DX, Silva GV, Lai D, Thomas JD, Kronenberg MW, Martin AD, Anderson RD, Traverse JH, Penn MS, Anwaruddin S, Hatzopoulos AK, Gee AP, Taylor DA, Cogle CR, Smith D, Westbrook L, Chen J, Handberg E, Olson RE, Geither C, Bowman S, Francescon J, Baraniuk S, Piller LB, Simpson LM, Loghin C, Aguilar D, Richman S, Zierold C, Bettencourt J, Sayre SL, Vojvodic RW, Skarlatos SI, Gordon DJ, Ebert RF, Kwak M, Moyé LA, Simari RD. Effect of transendocardial delivery of autologous bone marrow mononuclear cells on functional capacity, left ventricular function, and perfusion in chronic heart failure: the FOCUS-CCTRN trial. *JAMA* 2012; **307**: 1717-1726 [PMID: 22447880 DOI: 10.1001/jama.2012.418]
- 58 **Sürder D**, Manka R, Lo Cicero V, Moccetti T, Rufibach K, Soncin S, Turchetto L, Radrizzani M, Astori G, Schwitter J, Erne P, Zuber M, Auf der Maur C, Jamshidi P, Gaemperli O, Windecker S, Moschovitis A, Wahl A, Bühler I, Wyss C, Kozzerke S, Landmesser U, Lüscher TF, Corti R. Intracoronary injection of bone marrow-derived mononuclear cells early or late after acute myocardial infarction: effects on global left ventricular function. *Circulation* 2013; **127**: 1968-1979 [PMID: 23596006 DOI: 10.1161/circulationaha.112.001035]
- 59 **Traverse JH**, Henry TD, Ellis SG, Pepine CJ, Willerson JT, Zhao DX, Forder JR, Byrne BJ, Hatzopoulos AK, Penn MS, Perin EC, Baran KW, Chambers J, Lambert C, Raveendran G, Simon DI, Vaughan DE, Simpson LM, Gee AP, Taylor DA, Cogle CR, Thomas JD, Silva GV, Jorgenson BC, Olson RE, Bowman S, Francescon J, Geither C, Handberg E, Smith DX, Baraniuk S, Piller LB, Loghin C, Aguilar D, Richman S, Zierold C, Bettencourt J, Sayre SL, Vojvodic RW, Skarlatos SI, Gordon DJ, Ebert RF, Kwak M, Moyé LA, Simari RD. Effect of intracoronary delivery of autologous bone marrow mononuclear cells 2 to 3 weeks following acute myocardial infarction on left ventricular function: the LateTIME randomized trial. *JAMA* 2011; **306**: 2110-2119 [PMID: 22084195 DOI: 10.1001/jama.2011.1670]
- 60 **Traverse JH**, Henry TD, Pepine CJ, Willerson JT, Zhao DX, Ellis SG, Forder JR, Anderson RD, Hatzopoulos AK, Penn MS, Perin EC, Chambers J, Baran KW, Raveendran G, Lambert C, Lerman A, Simon DI, Vaughan DE, Lai D, Gee AP, Taylor DA, Cogle CR, Thomas JD, Olson RE, Bowman S, Francescon J, Geither C, Handberg E, Kappenman C, Westbrook L, Piller LB, Simpson LM, Baraniuk S, Loghin C, Aguilar D, Richman S, Zierold C, Spoon DB, Bettencourt J, Sayre SL, Vojvodic RW, Skarlatos SI, Gordon DJ, Ebert RF, Kwak M, Moyé LA, Simari RD. Effect of the use and timing of bone marrow mononuclear cell delivery on left ventricular function after acute myocardial infarction: the TIME randomized trial. *JAMA* 2012; **308**: 2380-2389 [PMID: 23129008 DOI: 10.1001/jama.2012.28726]
- 61 **Traverse JH**, McKenna DH, Harvey K, Jorgenson BC, Olson RE, Bostrom N, Kadidlo D, Lesser JR, Jagadeesan V, Garberich R, Henry TD. Results of a phase I, randomized, double-blind, placebo-controlled trial of bone marrow mononuclear stem cell administration in patients following ST-elevation myocardial infarction. *Am Heart J* 2010; **160**: 428-434 [PMID: 20826249 DOI: 10.1016/j.ahj.2010.06.009]
- 62 **Wöhrle J**, Merkle N, Mailänder V, Nusser T, Schauwecker P, von Scheidt F, Schwarz K, Bommer M, Wiesneth M, Schrezenmeier H, Hombach V. Results of intracoronary stem cell therapy after acute myocardial infarction. *Am J Cardiol* 2010; **105**: 804-812 [PMID: 20211323 DOI: 10.1016/j.amjcard.2009.10.060]
- 63 **Abdel-Latif A**, Bolli R, Tleyjeh IM, Montori VM, Perin EC, Hornung CA, Zuba-Surma EK, Al-Mallah M, Dawn B. Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. *Arch Intern Med* 2007; **167**: 989-997 [PMID: 17533201 DOI: 10.1001/archinte.167.10.989]
- 64 **Delewi R**, Hirsch A, Tijssen JG, Schächinger V, Wojakowski W, Roncalli J, Aakhus S, Erbs S, Assmus B, Tendera M, Goekmen Turan R, Corti R, Henry T, Lemarchand P, Lunde K, Cao F, Huikuri HV, Sürder D, Simari RD, Janssens S, Wollert KC, Plewka M, Grajek S, Traverse JH, Zijlstra F, Piek JJ. Impact of intracoronary bone marrow cell therapy on left ventricular function in the setting of ST-segment elevation myocardial infarction: a collaborative meta-analysis. *Eur Heart J* 2014; **35**: 989-998 [PMID: 24026778 DOI: 10.1093/eurheartj/eh372]
- 65 **Jeevanantham V**, Butler M, Saad A, Abdel-Latif A, Zuba-Surma



- EK, Dawn B. Adult bone marrow cell therapy improves survival and induces long-term improvement in cardiac parameters: a systematic review and meta-analysis. *Circulation* 2012; **126**: 551-568 [PMID: 22730444 DOI: 10.1161/circulationaha.111.086074]
- 66 **Martin-Rendon E**, Brunskill SJ, Hyde CJ, Stanworth SJ, Mathur A, Watt SM. Autologous bone marrow stem cells to treat acute myocardial infarction: a systematic review. *Eur Heart J* 2008; **29**: 1807-1818 [PMID: 18523058 DOI: 10.1093/eurheartj/ehn220]
  - 67 **Zimmet H**, Porapakham P, Porapakham P, Sata Y, Haas SJ, Itescu S, Forbes A, Krum H. Short- and long-term outcomes of intracoronary and endogenously mobilized bone marrow stem cells in the treatment of ST-segment elevation myocardial infarction: a meta-analysis of randomized control trials. *Eur J Heart Fail* 2012; **14**: 91-105 [PMID: 22065869 DOI: 10.1093/eurjhf/hfr148]
  - 68 **Lafamme MA**, Murry CE. Regenerating the heart. *Nat Biotechnol* 2005; **23**: 845-856 [PMID: 16003373 DOI: 10.1038/nbt1117]
  - 69 **Murry CE**, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JJ, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004; **428**: 664-668 [PMID: 15034593 DOI: 10.1038/nature02446]
  - 70 **Balsam LB**, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* 2004; **428**: 668-673 [PMID: 15034594 DOI: 10.1038/nature02460]
  - 71 **Stolz A**, Jones E, McGonagle D, Scutt A. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech Ageing Dev* 2008; **129**: 163-173 [PMID: 18241911 DOI: 10.1016/j.mad.2007.12.002]
  - 72 **Dimmeler S**, Leri A. Aging and disease as modifiers of efficacy of cell therapy. *Circ Res* 2008; **102**: 1319-1330 [PMID: 18535269 DOI: 10.1161/circresaha.108.175943]
  - 73 **Heinrich EM**, Dimmeler S. MicroRNAs and stem cells: control of pluripotency, reprogramming, and lineage commitment. *Circ Res* 2012; **110**: 1014-1022 [PMID: 22461365 DOI: 10.1161/circresaha.111.243394]
  - 74 **Leonardo TR**, Schultheisz HL, Loring JF, Laurent LC. The functions of microRNAs in pluripotency and reprogramming. *Nat Cell Biol* 2012; **14**: 1114-1121 [PMID: 23131918 DOI: 10.1038/ncb2613]
  - 75 **Xu Q**, Seeger FH, Castillo J, Iekushi K, Boon RA, Farcas R, Manavski Y, Li YG, Assmus B, Zeiher AM, Dimmeler S. MicroRNA-34a contributes to the impaired function of bone marrow-derived mononuclear cells from patients with cardiovascular disease. *J Am Coll Cardiol* 2012; **59**: 2107-2117 [PMID: 22651868 DOI: 10.1016/j.jacc.2012.02.033]
  - 76 **Iekushi K**, Seeger F, Assmus B, Zeiher AM, Dimmeler S. Regulation of cardiac microRNAs by bone marrow mononuclear cell therapy in myocardial infarction. *Circulation* 2012; **125**: 1765-1773 [PMID: 22403243 DOI: 10.1161/circulationaha.111.079699]
  - 77 **Friedenstein AJ**, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970; **3**: 393-403 [PMID: 5523063]
  - 78 **Schuleri KH**, Boyle AJ, Hare JM. Mesenchymal stem cells for cardiac regenerative therapy. *Handb Exp Pharmacol* 2007; **(180)**: 195-218 [PMID: 17554510 DOI: 10.1007/978-3-540-68976-8\_9]
  - 79 **Pittenger MF**, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-147 [PMID: 10102814 DOI: 10.1126/science.284.5411.143]
  - 80 **Phinney DG**, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells* 2007; **25**: 2896-2902 [PMID: 17901396 DOI: 10.1634/stemcells.2007-0637]
  - 81 **Makino S**, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest* 1999; **103**: 697-705 [PMID: 10074487 DOI: 10.1172/jci5298]
  - 82 **Toma C**, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 2002; **105**: 93-98 [PMID: 11772882 DOI: 10.1161/hc0102.101442]
  - 83 **Williams AR**, Hare JM. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. *Circ Res* 2011; **109**: 923-940 [PMID: 21960725 DOI: 10.1161/circresaha.111.243147]
  - 84 **Quevedo HC**, Hatzistergos KE, Oskouei BN, Feigenbaum GS, Rodriguez JE, Valdes D, Pattany PM, Zambrano JP, Hu Q, McNiece I, Heldman AW, Hare JM. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proc Natl Acad Sci USA* 2009; **106**: 14022-14027 [PMID: 19666564 DOI: 10.1073/pnas.0903201106]
  - 85 **Schuleri KH**, Feigenbaum GS, Centola M, Weiss ES, Zimmet JM, Turney J, Kellner J, Zviman MM, Hatzistergos KE, Detrick B, Conte JV, McNiece I, Steenbergen C, Lardo AC, Hare JM. Autologous mesenchymal stem cells produce reverse remodelling in chronic ischaemic cardiomyopathy. *Eur Heart J* 2009; **30**: 2722-2732 [PMID: 19586959 DOI: 10.1093/eurheartj/ehp265]
  - 86 **Shake JG**, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, Pittenger MF, Martin BJ. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg* 2002; **73**: 1919-1925; discussion 1926 [PMID: 12078791]
  - 87 **Katritsis DG**, Sotiropoulou PA, Karvouni E, Karabinos I, Korovesis S, Perez SA, Voridis EM, Papamichail M. Transcatheter transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium. *Catheter Cardiovasc Interv* 2005; **65**: 321-329 [PMID: 15954106 DOI: 10.1002/ccd.20406]
  - 88 **Williams AR**, Trachtenberg B, Velazquez DL, McNiece I, Altman P, Rouy D, Mendizabal AM, Pattany PM, Lopera GA, Fishman J, Zambrano JP, Heldman AW, Hare JM. Intramyocardial stem cell injection in patients with ischemic cardiomyopathy: functional recovery and reverse remodeling. *Circ Res* 2011; **108**: 792-796 [PMID: 21415390 DOI: 10.1161/circresaha.111.242610]
  - 89 **Heldman AW**, DiFede DL, Fishman JE, Zambrano JP, Trachtenberg BH, Karantalis V, Mushtaq M, Williams AR, Suncion VY, McNiece IK, Ghersin E, Soto V, Lopera G, Miki R, Willens H, Hendel R, Mitrani R, Pattany P, Feigenbaum G, Oskouei B, Byrnes J, Lowery MH, Sierra J, Pujol MV, Delgado C, Gonzalez PJ, Rodriguez JE, Bagno LL, Rouy D, Altman P, Foo CW, da Silva J, Anderson E, Schwarz R, Mendizabal A, Hare JM. Transendocardial mesenchymal stem cells and mononuclear bone marrow cells for ischemic cardiomyopathy: the TAC-HFT randomized trial. *JAMA* 2014; **311**: 62-73 [PMID: 24247587 DOI: 10.1001/jama.2013.282909]
  - 90 **Psaltis PJ**, Zannettino AC, Worthley SG, Gronthos S. Concise review: mesenchymal stromal cells: potential for cardiovascular repair. *Stem Cells* 2008; **26**: 2201-2210 [PMID: 18599808 DOI: 10.1634/stemcells.2008-0428]
  - 91 **Behfar A**, Terzic A. Derivation of a cardiopoietic population from human mesenchymal stem cells yields cardiac progeny. *Nat Clin Pract Cardiovasc Med* 2006; **3** Suppl 1: S78-S82 [PMID: 16501637 DOI: 10.1038/ncpcardio0429]
  - 92 **Behfar A**, Faustino RS, Arrell DK, Dzeja PP, Perez-Terzic C, Terzic A. Guided stem cell cardiopoiesis: discovery and translation. *J Mol Cell Cardiol* 2008; **45**: 523-529 [PMID: 18835562 DOI: 10.1016/j.yjmcc.2008.09.122]
  - 93 **Behfar A**, Yamada S, Crespo-Diaz R, Nesbitt JJ, Rowe LA, Perez-Terzic C, Gaussin V, Homsy C, Bartunek J, Terzic A. Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem cells in chronic myocardial infarction. *J Am Coll Cardiol* 2010; **56**: 721-734 [PMID: 20723802 DOI: 10.1016/j.jacc.2010.03.066]
  - 94 **Hahn JY**, Cho HJ, Kang HJ, Kim TS, Kim MH, Chung JH, Bae



- JW, Oh BH, Park YB, Kim HS. Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. *J Am Coll Cardiol* 2008; **51**: 933-943 [PMID: 18308163 DOI: 10.1016/j.jacc.2007.11.040]
- 95 **Bartunek J**, Behfar A, Dolatabadi D, Vanderheyden M, Ostojic M, Dens J, El Nakadi B, Banovic M, Beleslin B, Vrolix M, Legrand V, Vrints C, Vanoverschelde JL, Crespo-Diaz R, Homys C, Tendera M, Waldman S, Wijns W, Terzic A. Cardiopoietic stem cell therapy in heart failure: the C-CURE (Cardiopoietic stem Cell therapy in heart failURE) multicenter randomized trial with lineage-specified biologics. *J Am Coll Cardiol* 2013; **61**: 2329-2338 [PMID: 23583246 DOI: 10.1016/j.jacc.2013.02.071]
- 96 **McGinley LM**, McMahon J, Stocca A, Duffy A, Flynn A, O'Toole D, O'Brien T. Mesenchymal stem cell survival in the infarcted heart is enhanced by lentivirus vector-mediated heat shock protein 27 expression. *Hum Gene Ther* 2013; **24**: 840-851 [PMID: 23987185 DOI: 10.1089/hum.2011.009]
- 97 **Huang F**, Li ML, Fang ZF, Hu XQ, Liu QM, Liu ZJ, Tang L, Zhao YS, Zhou SH. Overexpression of MicroRNA-1 improves the efficacy of mesenchymal stem cell transplantation after myocardial infarction. *Cardiology* 2013; **125**: 18-30 [PMID: 23615185 DOI: 10.1159/000347081]
- 98 **Wang L**, Pasha Z, Wang S, Li N, Feng Y, Lu G, Millard RW, Ashraf M. Protein kinase G1  $\alpha$  overexpression increases stem cell survival and cardiac function after myocardial infarction. *PLoS One* 2013; **8**: e60087 [PMID: 23536905 DOI: 10.1371/journal.pone.0060087]
- 99 **Ye L**, Zhang P, Duval S, Su L, Xiong Q, Zhang J. Thymosin  $\beta$ 4 increases the potency of transplanted mesenchymal stem cells for myocardial repair. *Circulation* 2013; **128**: S32-S41 [PMID: 24030419 DOI: 10.1161/circulationaha.112.000025]
- 100 **Beltrami AP**, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 2003; **114**: 763-776 [PMID: 14505575 DOI: 10.1016/S0092-8674(03)00687-1]
- 101 **Bergmann O**, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. Evidence for cardiomyocyte renewal in humans. *Science* 2009; **324**: 98-102 [PMID: 19342590 DOI: 10.1126/science.1164680]
- 102 **Nadal-Ginard B**, Ellison GM, Torella D. The cardiac stem cell compartment is indispensable for myocardial cell homeostasis, repair and regeneration in the adult. *Stem Cell Res* 2014; **13**: 615-630 [PMID: 24838077 DOI: 10.1016/j.scr.2014.04.008]
- 103 **Angert D**, Berretta RM, Kubo H, Zhang H, Chen X, Wang W, Ogorek B, Barbe M, Houser SR. Repair of the injured adult heart involves new myocytes potentially derived from resident cardiac stem cells. *Circ Res* 2011; **108**: 1226-1237 [PMID: 21454756 DOI: 10.1161/circresaha.110.239046]
- 104 **Ellison GM**, Torella D, Karakikes I, Nadal-Ginard B. Myocyte death and renewal: modern concepts of cardiac cellular homeostasis. *Nat Clin Pract Cardiovasc Med* 2007; **4** Suppl 1: S52-S59 [PMID: 17230216 DOI: 10.1038/ncpcardio0773]
- 105 **Ellison GM**, Waring CD, Vicinanza C, Torella D. Physiological cardiac remodelling in response to endurance exercise training: cellular and molecular mechanisms. *Heart* 2012; **98**: 5-10 [PMID: 21880653 DOI: 10.1136/heartjnl-2011-300639]
- 106 **Ellison GM**, Vicinanza C, Smith AJ, Aquila I, Leone A, Waring CD, Henning BJ, Stirparo GG, Papait R, Scarfò M, Agosti V, Viglietto G, Condorelli G, Indolfi C, Ottolenghi S, Torella D, Nadal-Ginard B. Adult c-kit(pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. *Cell* 2013; **154**: 827-842 [PMID: 23953114 DOI: 10.1016/j.cell.2013.07.039]
- 107 **van Berlo JH**, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marbán E, Molkentin JD. c-kit+ cells minimally contribute cardiomyocytes to the heart. *Nature* 2014; **509**: 337-341 [PMID: 24805242 DOI: 10.1038/nature13309]
- 108 **Bolli R**, Tang XL, Sanganalmath SK, Rimoldi O, Mosna F, Abdel-Latif A, Jneid H, Rota M, Leri A, Kajstura J. Intracoronary delivery of autologous cardiac stem cells improves cardiac function in a porcine model of chronic ischemic cardiomyopathy. *Circulation* 2013; **128**: 122-131 [PMID: 23757309 DOI: 10.1161/circulationaha.112.001075]
- 109 **Dawn B**, Stein AB, Urbanek K, Rota M, Whang B, Rastaldo R, Torella D, Tang XL, Rezazadeh A, Kajstura J, Leri A, Hunt G, Varma J, Prabhu SD, Anversa P, Bolli R. Cardiac stem cells delivered intravascularly traverse the vessel barrier, regenerate infarcted myocardium, and improve cardiac function. *Proc Natl Acad Sci USA* 2005; **102**: 3766-3771 [PMID: 15734798 DOI: 10.1073/pnas.0405957102]
- 110 **Linke A**, Müller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A, Castaldo C, Cascapera S, Böhm M, Quaini F, Urbanek K, Leri A, Hintze TH, Kajstura J, Anversa P. Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. *Proc Natl Acad Sci USA* 2005; **102**: 8966-8971 [PMID: 15951423 DOI: 10.1073/pnas.0502678102]
- 111 **Rota M**, Padin-Iruegas ME, Misao Y, De Angelis A, Maestroni S, Ferreira-Martins J, Fiumana E, Rastaldo R, Arcarese ML, Mitchell TS, Boni A, Bolli R, Urbanek K, Hosoda T, Anversa P, Leri A, Kajstura J. Local activation or implantation of cardiac progenitor cells rescues scarred infarcted myocardium improving cardiac function. *Circ Res* 2008; **103**: 107-116 [PMID: 18556576 DOI: 10.1161/circresaha.108.178525]
- 112 **Tang XL**, Rokosh G, Sanganalmath SK, Yuan F, Sato H, Mu J, Dai S, Li C, Chen N, Peng Y, Dawn B, Hunt G, Leri A, Kajstura J, Tiwari S, Shirk G, Anversa P, Bolli R. Intracoronary administration of cardiac progenitor cells alleviates left ventricular dysfunction in rats with a 30-day-old infarction. *Circulation* 2010; **121**: 293-305 [PMID: 20048209 DOI: 10.1161/circulationaha.109.871905]
- 113 **Bolli R**, Chugh AR, D'Amario D, Loughran JH, Stoddard MF, Ikram S, Beache GM, Wagner SG, Leri A, Hosoda T, Sanada F, Elmore JB, Goichberg P, Cappetta D, Solankhi NK, Fahsah I, Rokosh DG, Slaughter MS, Kajstura J, Anversa P. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. *Lancet* 2011; **378**: 1847-1857 [PMID: 22088800 DOI: 10.1016/s0140-6736(11)61590-0]
- 114 **Messina E**, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A. Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* 2004; **95**: 911-921 [PMID: 15472116 DOI: 10.1161/01.res.0000147315.71699.51]
- 115 **Smith RR**, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marbán E. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation* 2007; **115**: 896-908 [PMID: 17283259 DOI: 10.1161/circulationaha.106.655209]
- 116 **Beazri C**, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. Human cardiac stem cells. *Proc Natl Acad Sci USA* 2007; **104**: 14068-14073 [PMID: 17709737 DOI: 10.1073/pnas.0706760104]
- 117 **Oh H**, Bradfute SB, Gallardo TD, Nakamura T, Gausson V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci USA* 2003; **100**: 12313-12318 [PMID: 14530411 DOI: 10.1073/pnas.2132126100]
- 118 **Johnston PV**, Sasano T, Mills K, Evers R, Lee ST, Smith RR, Lardo AC, Lai S, Steenbergen C, Gerstenblith G, Lange R, Marbán E. Engraftment, differentiation, and functional benefits of autologous cardiosphere-derived cells in porcine ischemic cardiomyopathy. *Circulation* 2009; **120**: 1075-1083 [PMID: 19738142 DOI: 10.1161/circulationaha.108.816058]
- 119 **Lee ST**, White AJ, Matsushita S, Malliaras K, Steenbergen C, Zhang Y, Li TS, Terrovitis J, Yee K, Simsir S, Makkar R, Marbán



- E. Intramyocardial injection of autologous cardiospheres or cardiosphere-derived cells preserves function and minimizes adverse ventricular remodeling in pigs with heart failure post-myocardial infarction. *J Am Coll Cardiol* 2011; **57**: 455-465 [PMID: 21251587 DOI: 10.1016/j.jacc.2010.07.049]
- 120 **Li TS**, Cheng K, Malliaras K, Smith RR, Zhang Y, Sun B, Matsushita N, Blusztajn A, Terrovitis J, Kusuoka H, Marbán L, Marbán E. Direct comparison of different stem cell types and subpopulations reveals superior paracrine potency and myocardial repair efficacy with cardiosphere-derived cells. *J Am Coll Cardiol* 2012; **59**: 942-953 [PMID: 22381431 DOI: 10.1016/j.jacc.2011.11.029]
  - 121 **Makkar RR**, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marbán L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G, Marbán E. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. *Lancet* 2012; **379**: 895-904 [PMID: 22336189 DOI: 10.1016/S0140-6736(12)60195-0]
  - 122 **Malliaras K**, Makkar RR, Smith RR, Cheng K, Wu E, Bonow RO, Marbán L, Mendizabal A, Cingolani E, Johnston PV, Gerstenblith G, Schuleri KH, Lardo AC, Marbán E. Intracoronary cardiosphere-derived cells after myocardial infarction: evidence of therapeutic regeneration in the final 1-year results of the CADUCEUS trial (Cardiosphere-Derived autologous stem Cells to reverse ventricular dysfunction). *J Am Coll Cardiol* 2014; **63**: 110-122 [PMID: 24036024 DOI: 10.1016/j.jacc.2013.08.724]
  - 123 **Hong KU**, Guo Y, Li QH, Cao P, Al-Maqtari T, Vajravelu BN, Du J, Book MJ, Zhu X, Nong Y, Bhatnagar A, Bolli R. c-kit<sup>+</sup> Cardiac stem cells alleviate post-myocardial infarction left ventricular dysfunction despite poor engraftment and negligible retention in the recipient heart. *PLoS One* 2014; **9**: e96725 [PMID: 24806457 DOI: 10.1371/journal.pone.0096725]
  - 124 **Chimenti I**, Smith RR, Li TS, Gerstenblith G, Messina E, Giacomello A, Marbán E. Relative roles of direct regeneration versus paracrine effects of human cardiosphere-derived cells transplanted into infarcted mice. *Circ Res* 2010; **106**: 971-980 [PMID: 20110532 DOI: 10.1161/circresaha.109.210682]
  - 125 **Mohsin S**, Khan M, Toko H, Bailey B, Cottage CT, Wallach K, Nag D, Lee A, Siddiqi S, Lan F, Fischer KM, Gude N, Quijada P, Avitabile D, Truffa S, Collins B, Dembitsky W, Wu JC, Sussman MA. Human cardiac progenitor cells engineered with Pim-1 kinase enhance myocardial repair. *J Am Coll Cardiol* 2012; **60**: 1278-1287 [PMID: 22841153 DOI: 10.1016/j.jacc.2012.04.047]
  - 126 **Cottage CT**, Neidig L, Sundararaman B, Din S, Joyo AY, Bailey B, Gude N, Hariharan N, Sussman MA. Increased mitotic rate coincident with transient telomere lengthening resulting from pim-1 overexpression in cardiac progenitor cells. *Stem Cells* 2012; **30**: 2512-2522 [PMID: 22915504 DOI: 10.1002/stem.1211]
  - 127 **Cai C**, Teng L, Vu D, He JQ, Guo Y, Li Q, Tang XL, Rokosh G, Bhatnagar A, Bolli R. The heme oxygenase 1 inducer (CoPP) protects human cardiac stem cells against apoptosis through activation of the extracellular signal-regulated kinase (ERK)/NRF2 signaling pathway and cytokine release. *J Biol Chem* 2012; **287**: 33720-33732 [PMID: 22879597 DOI: 10.1074/jbc.M112.385542]
  - 128 **Zafir A**, Readnower R, Long BW, McCracken J, Aird A, Alvarez A, Cummins TD, Li Q, Hill BG, Bhatnagar A, Prabhu SD, Bolli R, Jones SP. Protein O-GlcNAcylation is a novel cytoprotective signal in cardiac stem cells. *Stem Cells* 2013; **31**: 765-775 [PMID: 23335157 DOI: 10.1002/stem.1325]
  - 129 **Pendergrass KD**, Boopathy AV, Seshadri G, Maiellaro-Rafferty K, Che PL, Brown ME, Davis ME. Acute preconditioning of cardiac progenitor cells with hydrogen peroxide enhances angiogenic pathways following ischemia-reperfusion injury. *Stem Cells Dev* 2013; **22**: 2414-2424 [PMID: 23544670 DOI: 10.1089/scd.2012.0673]
  - 130 **Takehara N**, Tsutsumi Y, Tateishi K, Ogata T, Tanaka H, Ueyama T, Takahashi T, Takamatsu T, Fukushima M, Komeda M, Yamagishi M, Yaku H, Tabata Y, Matsubara H, Oh H. Controlled delivery of basic fibroblast growth factor promotes human cardiosphere-derived cell engraftment to enhance cardiac repair for chronic myocardial infarction. *J Am Coll Cardiol* 2008; **52**: 1858-1865 [PMID: 19038683 DOI: 10.1016/j.jacc.2008.06.052]
  - 131 **Hatzistergos KE**, Quevedo H, Oskouei BN, Hu Q, Feigenbaum GS, Margitich IS, Mazhari R, Boyle AJ, Zambrano JP, Rodriguez JE, Dulce R, Pattany PM, Valdes D, Revilla C, Heldman AW, McNiece I, Hare JM. Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. *Circ Res* 2010; **107**: 913-922 [PMID: 20671238 DOI: 10.1161/circresaha.110.222703]
  - 132 **Williams AR**, Hatzistergos KE, Addicott B, McCall F, Carvalho D, Suncion V, Morales AR, Da Silva J, Sussman MA, Heldman AW, Hare JM. Enhanced effect of combining human cardiac stem cells and bone marrow mesenchymal stem cells to reduce infarct size and to restore cardiac function after myocardial infarction. *Circulation* 2013; **127**: 213-223 [PMID: 23224061 DOI: 10.1161/circulationaha.112.131110]
  - 133 **Le Blanc K**, Tammik C, Rosendahl K, Zetterberg E, Ringdén O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003; **31**: 890-896 [PMID: 14550804 DOI: 10.1016/S0301-472X(03)00110-3]
  - 134 **Batten P**, Sarathchandra P, Antoniow JW, Tay SS, Lowdell MW, Taylor PM, Yacoub MH. Human mesenchymal stem cells induce T cell anergy and downregulate T cell allo-responses via the TH2 pathway: relevance to tissue engineering human heart valves. *Tissue Eng* 2006; **12**: 2263-2273 [PMID: 16968166 DOI: 10.1089/ten.2006.12.2263]
  - 135 **Chiesa S**, Morbelli S, Morando S, Massollo M, Marini C, Bertoni A, Frasson F, Bartolomé ST, Sambucetti G, Traggiai E, Uccelli A. Mesenchymal stem cells impair in vivo T-cell priming by dendritic cells. *Proc Natl Acad Sci USA* 2011; **108**: 17384-17389 [PMID: 21960443 DOI: 10.1073/pnas.1103650108]
  - 136 **Li W**, Ren G, Huang Y, Su J, Han Y, Li J, Chen X, Cao K, Chen Q, Shou P, Zhang L, Yuan ZR, Roberts AI, Shi S, Le AD, Shi Y. Mesenchymal stem cells: a double-edged sword in regulating immune responses. *Cell Death Differ* 2012; **19**: 1505-1513 [PMID: 22421969 DOI: 10.1038/cdd.2012.26]
  - 137 **Huang XP**, Sun Z, Miyagi Y, McDonald Kinkaid H, Zhang L, Weisel RD, Li RK. Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. *Circulation* 2010; **122**: 2419-2429 [PMID: 21098445 DOI: 10.1161/circulationaha.110.955971]
  - 138 **Mirotsov M**, Jayawardena TM, Schmeckpeper J, Gnecci M, Dzau VJ. Paracrine mechanisms of stem cell reparative and regenerative actions in the heart. *J Mol Cell Cardiol* 2011; **50**: 280-289 [PMID: 20727900 DOI: 10.1016/j.yjmcc.2010.08.005]
  - 139 **Amado LC**, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S, Durand DJ, Fitton T, Kuang JQ, Stewart G, Lehrke S, Baumgartner WW, Martin BJ, Heldman AW, Hare JM. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc Natl Acad Sci USA* 2005; **102**: 11474-11479 [PMID: 16061805 DOI: 10.1073/pnas.0504388102]
  - 140 **Dai W**, Hale SL, Martin BJ, Kuang JQ, Dow JS, Wold LE, Kloner RA. Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short- and long-term effects. *Circulation* 2005; **112**: 214-223 [PMID: 15998673 DOI: 10.1161/circulationaha.104.527937]
  - 141 **Williams AR**, Suncion VY, McCall F, Guerra D, Mather J, Zambrano JP, Heldman AW, Hare JM. Durable scar size reduction due to allogeneic mesenchymal stem cell therapy regulates whole-chamber remodeling. *J Am Heart Assoc* 2013; **2**: e000140 [PMID: 23686370 DOI: 10.1161/jaha.113.000140]
  - 142 **Zhao Y**, Li T, Wei X, Bianchi G, Hu J, Sanchez PG, Xu K, Zhang P, Pittenger MF, Wu ZJ, Griffith BP. Mesenchymal stem cell transplantation improves regional cardiac remodeling following ovine infarction. *Stem Cells Transl Med* 2012; **1**: 685-695 [PMID: 23197875 DOI: 10.5966/sctm.2012-0027]
  - 143 **Hare JM**, Traverse JH, Henry TD, Dib N, Strumpf RK, Schulman



- SP, Gerstenblith G, DeMaria AN, Denktas AE, Gammon RS, Hermiller JB, Reisman MA, Schaer GL, Sherman W. A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. *J Am Coll Cardiol* 2009; **54**: 2277-2286 [PMID: 19958962 DOI: 10.1016/j.jacc.2009.06.055]
- 144 **Hare JM**, Fishman JE, Gerstenblith G, DiFede Velazquez DL, Zambrano JP, Suncion VY, Tracy M, Ghersin E, Johnston PV, Brinker JA, Breton E, Davis-Sproul J, Schulman IH, Byrnes J, Mendizabal AM, Lowery MH, Rouy D, Altman P, Wong Po Foo C, Ruiz P, Amador A, Da Silva J, McNiece IK, Heldman AW, George R, Lardo A. Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. *JAMA* 2012; **308**: 2369-2379 [PMID: 23117550 DOI: 10.1001/jama.2012.25321]
- 145 **Hamamoto H**, Gorman JH, Ryan LP, Hinmon R, Martens TP, Schuster MD, Plappert T, Kiupel M, St John-Sutton MG, Itescu S, Gorman RC. Allogeneic mesenchymal precursor cell therapy to limit remodeling after myocardial infarction: the effect of cell dosage. *Ann Thorac Surg* 2009; **87**: 794-801 [PMID: 19231391 DOI: 10.1016/j.athoracsur.2008.11.057]
- 146 **Gronthos S**, Fitter S, Diamond P, Simmons PJ, Itescu S, Zannettino AC. A novel monoclonal antibody (STRO-3) identifies an isoform of tissue nonspecific alkaline phosphatase expressed by multipotent bone marrow stromal stem cells. *Stem Cells Dev* 2007; **16**: 953-963 [PMID: 18158854 DOI: 10.1089/scd.2007.0069]
- 147 **Psaltis PJ**, Paton S, See F, Arthur A, Martin S, Itescu S, Worthley SG, Gronthos S, Zannettino AC. Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. *J Cell Physiol* 2010; **223**: 530-540 [PMID: 20162565 DOI: 10.1002/jcp.22081]
- 148 **See F**, Seki T, Psaltis PJ, Sondermeijer HP, Gronthos S, Zannettino AC, Govaert KM, Schuster MD, Kurlansky PA, Kelly DJ, Krum H, Itescu S. Therapeutic effects of human STRO-3-selected mesenchymal precursor cells and their soluble factors in experimental myocardial ischemia. *J Cell Mol Med* 2011; **15**: 2117-2129 [PMID: 21155976 DOI: 10.1111/j.1582-4934.2010.01241.x]
- 149 **Dixon JA**, Gorman RC, Stroud RE, Bouges S, Hirotsugu H, Gorman JH, Martens TP, Itescu S, Schuster MD, Plappert T, St John-Sutton MG, Spinal FG. Mesenchymal cell transplantation and myocardial remodeling after myocardial infarction. *Circulation* 2009; **120**: S220-S229 [PMID: 19752372 DOI: 10.1161/circulationaha.108.842302]
- 150 **Houtgraaf JH**, de Jong R, Kazemi K, de Groot D, van der Spoel TI, Arslan F, Hoefler I, Pasterkamp G, Itescu S, Zijlstra F, Geleijnse ML, Serruys PW, Duckers HJ. Intracoronary infusion of allogeneic mesenchymal precursor cells directly after experimental acute myocardial infarction reduces infarct size, abrogates adverse remodeling, and improves cardiac function. *Circ Res* 2013; **113**: 153-166 [PMID: 23658436 DOI: 10.1161/circresaha.112.300730]
- 151 **Penn MS**, Ellis S, Gandhi S, Greenbaum A, Hodes Z, Mendelsohn FO, Strasser D, Ting AE, Sherman W. Adventitial delivery of an allogeneic bone marrow-derived adherent stem cell in acute myocardial infarction: phase I clinical study. *Circ Res* 2012; **110**: 304-311 [PMID: 22052917 DOI: 10.1161/circresaha.111.253427]
- 152 **Ménard C**, Hagège AA, Agbulut O, Barro M, Morichetti MC, Brasselet C, Bel A, Messas E, Bissery A, Bruneval P, Desnos M, Pucéat M, Menasché P. Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. *Lancet* 2005; **366**: 1005-1012 [PMID: 16168783 DOI: 10.1016/s0140-6736(05)67380-1]
- 153 **Caspi O**, Huber I, Kehat I, Habib M, Arbel G, Gepstein A, Yankelson L, Aronson D, Beyar R, Gepstein L. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. *J Am Coll Cardiol* 2007; **50**: 1884-1893 [PMID: 17980256 DOI: 10.1016/j.jacc.2007.07.054]
- 154 **Takahashi K**, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]
- 155 **Riggs JW**, Barrilleaux BL, Varlakhanova N, Bush KM, Chan V, Knoepfler PS. Induced pluripotency and oncogenic transformation are related processes. *Stem Cells Dev* 2013; **22**: 37-50 [PMID: 22998387 DOI: 10.1089/scd.2012.0375]
- 156 **Sanz-Ruiz R**, Santos ME, Muñoz MD, Martín IL, Parma R, Fernández PL, Fernández-Avilés F. Adipose tissue-derived stem cells: the friendly side of a classic cardiovascular foe. *J Cardiovasc Transl Res* 2008; **1**: 55-63 [PMID: 20559958 DOI: 10.1007/s12265-007-9006-9]
- 157 **Sánchez PL**, Sanz-Ruiz R, Fernández-Santos ME, Fernández-Avilés F. Cultured and freshly isolated adipose tissue-derived cells: fat years for cardiac stem cell therapy. *Eur Heart J* 2010; **31**: 394-397 [PMID: 20037147 DOI: 10.1093/eurheartj/ehp403]
- 158 **Wang L**, Deng J, Tian W, Xiang B, Yang T, Li G, Wang J, Gruwel M, Kashour T, Rendell J, Glogowski M, Tomanek B, Freed D, Deslauriers R, Arora RC, Tian G. Adipose-derived stem cells are an effective cell candidate for treatment of heart failure: an MR imaging study of rat hearts. *Am J Physiol Heart Circ Physiol* 2009; **297**: H1020-H1031 [PMID: 19574490 DOI: 10.1152/ajpheart.01082.2008]
- 159 **Valina C**, Pinkernell K, Song YH, Bai X, Sadat S, Campeau RJ, Le Jemtel TH, Alt E. Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodeling after acute myocardial infarction. *Eur Heart J* 2007; **28**: 2667-2677 [PMID: 17933755 DOI: 10.1093/eurheartj/ehm426]
- 160 **Houtgraaf JH**, den Dekker WK, van Dalen BM, Springeling T, de Jong R, van Geuns RJ, Geleijnse ML, Fernandez-Aviles F, Zijlstra F, Serruys PW, Duckers HJ. First experience in humans using adipose tissue-derived regenerative cells in the treatment of patients with ST-segment elevation myocardial infarction. *J Am Coll Cardiol* 2012; **59**: 539-540 [PMID: 22281257 DOI: 10.1016/j.jacc.2011.09.065]
- 161 **Perin EC**, Sanz-Ruiz R, Sánchez PL, Lasso J, Pérez-Cano R, Alonso-Farto JC, Pérez-David E, Fernández-Santos ME, Serruys PW, Duckers HJ, Kastrup J, Chamuleau S, Zheng Y, Silva GV, Willerson JT, Fernández-Avilés F. Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: The PRECISE Trial. *Am Heart J* 2014; **168**: 88-95.e2 [PMID: 24952864 DOI: 10.1016/j.ahj.2014.03.022]
- 162 **Ellison GM**, Torella D, Dellegrottaglie S, Perez-Martinez C, Perez de Prado A, Vicinanza C, Purushothaman S, Galuppo V, Iaconetti C, Waring CD, Smith A, Torella M, Cuellas Ramon C, Gonzalo-Orden JM, Agosti V, Indolfi C, Galiñanes M, Fernandez-Vazquez F, Nadal-Ginard B. Endogenous cardiac stem cell activation by insulin-like growth factor-1/hepatocyte growth factor intracoronary injection fosters survival and regeneration of the infarcted pig heart. *J Am Coll Cardiol* 2011; **58**: 977-986 [PMID: 21723061 DOI: 10.1016/j.jacc.2011.05.013]
- 163 **Koudstaal S**, Bastings MM, Feyen DA, Waring CD, van Slochteren FJ, Dankers PY, Torella D, Sluijter JP, Nadal-Ginard B, Doevendans PA, Ellison GM, Chamuleau SA. Sustained delivery of insulin-like growth factor-1/hepatocyte growth factor stimulates endogenous cardiac repair in the chronic infarcted pig heart. *J Cardiovasc Transl Res* 2014; **7**: 232-241 [PMID: 24395494 DOI: 10.1007/s12265-013-9518-4]
- 164 **Penn MS**, Mendelsohn FO, Schaer GL, Sherman W, Farr M, Pastore J, Rouy D, Clemens R, Aras R, Losordo DW. An open-label dose escalation study to evaluate the safety of administration of nonviral stromal cell-derived factor-1 plasmid to treat symptomatic ischemic heart failure. *Circ Res* 2013; **112**: 816-825 [PMID: 23429605 DOI: 10.1161/circresaha.111.300440]
- 165 **Jufo K**, Ii M, Sekiguchi H, Klyachko E, Misener S, Tanaka T, Tongers J, Roncalli J, Renault MA, Thorne T, Ito A, Clarke T, Kamide C, Tsurumi Y, Hagiwara N, Qin G, Asahi M, Losordo DW.



CXC-chemokine receptor 4 antagonist AMD3100 promotes cardiac functional recovery after ischemia/reperfusion injury via endothelial

nitric oxide synthase-dependent mechanism. *Circulation* 2013; **127**: 63-73 [PMID: 23204107 DOI: 10.1161/circulationaha.112.099242]

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## Mesenchymal stem cells as a therapeutic tool to treat sepsis

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targeting components of the derailed host response have failed. Therefore, there is a dramatic need for new and mechanistically alternative therapies to treat this syndrome. Based on their immunomodulatory properties, adult mesenchymal stem or stromal cells (MSCs) can be a novel therapeutic tool to treat sepsis. Indeed, MSCs reduce mortality in experimental models of sepsis by modulating the deregulated inflammatory response against bacteria through the regulation of multiple inflammatory networks, the reprogramming of macrophages and neutrophils towards a more anti-inflammatory phenotype and the release of anti-microbial peptides. This report will review the current knowledge on the effects of MSC treatment in preclinical experimental small animal models of sepsis.

**Key words:** Adult mesenchymal stem cells; Therapy; Sepsis

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**Core tip:** Sepsis remains as the most frequent cause of death in hospitalized patients and, therefore, new therapeutic alternatives are needed. Adult mesenchymal stem cells reduce mortality in experimental models of sepsis by modulating the deregulated inflammatory response against bacteria through the regulation of multiple inflammatory networks, the reprogramming of macrophages and neutrophils towards a more anti-inflammatory phenotype and the release of anti-microbial peptides. In this report we aim to provide a comprehensive snapshot of the potential clinical use of cell therapy with mesenchymal stem cells for sepsis.

### Abstract

Sepsis is a clinical syndrome caused by a deregulated host response to an infection. Sepsis is the most frequent cause of death in hospitalized patients. Although knowledge of the pathogenesis of sepsis has increased substantially during the last decades, attempts to design effective and specific therapies

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## INTRODUCTION

Sepsis is a clinical syndrome caused by a deregulated host response to an infection. Sepsis is the most frequent cause of death in hospitalized patients. Although knowledge of the pathogenesis of sepsis has increased substantially during the last decades, attempts to design effective and specific therapies targeting components of the derailed host response have failed. Sepsis will remain an important clinical problem in the future, especially in light of the ageing population and emerging antibiotic resistance. Therefore, there is a dramatic need for new and mechanistically alternative therapies to treat this syndrome. Based on their immunomodulatory properties, adult mesenchymal stem or stromal cells (MSCs) can be a novel therapeutic tool to treat sepsis. This report will review the current knowledge on the effects of MSC treatment in preclinical experimental small animal models of sepsis.

## SEPSIS

### Epidemiology

The incidence of sepsis varies between different reports, largely due to the use of different case definitions and diagnosis codes<sup>[1,2]</sup>. Nevertheless, sepsis clearly is a leading cause of death, and the most frequent cause of death in non-coronary intensive care units (ICUs) in the developed world<sup>[2]</sup>. In the United States the yearly incidence of severe sepsis is estimated at 300 cases per 100000 person-years population, which accounts for 10% of all ICU admissions<sup>[3]</sup>. The incidence of severe sepsis was recently reported to increase<sup>[4]</sup>, although it is uncertain whether this signifies a true increase or altered coding and registration practices<sup>[2,5]</sup>. Mayr *et al.*<sup>[2]</sup> have recently reported that the mortality of severe sepsis and septic shock lies between 25%-50%, with the extent and number of organ failures as the strongest predictors of an adverse outcome. Notably, the case fatality rate for sepsis has declined in the past decade, most likely due to improved general care in the ICU<sup>[6]</sup>.

The most common sources of sepsis are in descending order pneumonia, intra-abdominal-, urinary tract- and soft tissue infections<sup>[5]</sup>. Blood cultures are positive in only one third of cases, and up to a third of cases are culture negative from all body sites. The most commonly isolated Gram-positive bacterial pathogens are *Staphylococcus aureus* and *Streptococcus pneumoniae*, and the most common Gram-negative pathogens are *Escherichia coli*, *Klebsiella spp.*, and *Pseudomonas aeruginosa*<sup>[7]</sup>. While Gram-positive infections had been reported as surpassing Gram-negative infections in recent years<sup>[8]</sup>, a recent study encompassing 14000 ICU patients in 75 countries found that 62% of positive isolates were Gram-negative bacteria, vs 47% Gram-positive and 19% fungal<sup>[9]</sup>.

### Pathophysiology and host response

Sepsis occurs when the body's response to infection

injures the host's tissues and organs. The deregulated host response during sepsis entails both excessive proinflammatory and immune suppressive anti-inflammatory components<sup>[7,10]</sup>.

Immune cells can sense pathogens *via* so-called pattern-recognition receptors (PRRs), which recognize conserved motifs expressed by microorganisms called pathogen-associated molecular patterns or PAMPs<sup>[7,11]</sup>. Four classes of PRRs have been identified: Toll-like receptors (TLRs), C-type lectin receptors, RIG-I-like receptors and NOD-like receptors<sup>[11]</sup>. Activation of PRRs by PAMPs causes upregulation of inflammatory gene transcription and initiation of innate immunity, a response aimed at eliminating the invading pathogen. However, when bacteria overcome the ability of the innate immune system to clear the infection, resulting in progression to sepsis, the interactions between pathogens and PRRs advances into a deregulated response that no longer benefits the host. During such injurious host response inflammation can be perpetuated by stimulation of PRRs by so-called danger-associated molecular patterns (DAMPs or alarmins), which are endogenous molecules released by injured or dying cells<sup>[12]</sup>. Alarmins are also released during sterile injury such as after trauma or severe pancreatitis, which contributes to the concept that the pathogenesis of multiple organ failure in sepsis and non-infectious critical illness is not fundamentally different<sup>[5,13]</sup>.

Cytokines are an important component of the "hyperinflammatory" response to severe infection. Experimental sepsis induced by systemic challenge with high bacterial doses is associated with enhanced release of multiple cytokines, and elimination or inhibition of several of these proinflammatory mediators [including tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-12, IL-17, IL-18, interferon- $\gamma$ , and macrophage migration inhibitory factor] improves survival in these models<sup>[14]</sup>. However and importantly, these systemic challenge models do not adequately mimic the clinical syndrome of sepsis. Many trials evaluating the efficacy of proinflammatory cytokine inhibition, especially targeting TNF- $\alpha$  and IL-1, or other anti-inflammatory strategies have failed<sup>[15]</sup>. Other proinflammatory mediators implicated in sepsis pathogenesis include high mobility group box 1 (HMGB1) and S100 proteins.

Activation of the complement systems forms a fundamental part of the innate immune response to infection<sup>[16]</sup>. Sepsis is associated with systemic activation of the complement system, which can be harmful in the setting of fulminant sepsis. Indeed, neutralization or genetic absence of complement factor C5a and its receptors results in increased survival during abdominal sepsis or endotoxemia in mice. Other hallmark features of the sepsis host response include activation of the coagulation system and vascular dysfunction. The most severe manifestation of coagulopathy is the syndrome of disseminated intravascular coagulation, with an



estimated incidence between 30%-50% in severe sepsis, caused by tissue factor-driven activation of coagulation with concurrent impairment of anticoagulant and fibrinolytic mechanisms<sup>[17]</sup>. Organ dysfunction in sepsis is at least in part caused by tissue hypoperfusion, secondary to hypotension, microvascular thrombosis and/or dysfunction of the vascular endothelium with loss of barrier function<sup>[5]</sup>. Mitochondrial dysfunction and altered cellular bioenergetics have been implicated in sepsis-induced organ dysfunction, although further research is warranted to establish a causal relationship<sup>[18]</sup>.

While proinflammatory responses definitely contribute to sepsis pathogenesis, immune suppression is also a common feature in patients with sepsis<sup>[7,10]</sup>. Autopsy studies have revealed strong deficiencies of splenocytes harvested from patients who had died of sepsis to produce cytokines upon stimulation<sup>[19]</sup>. The mechanisms that underlie this phenomenon have not been fully elucidated, although likely anti-inflammatory cytokines, particularly IL-10 and transforming growth factor (TGF)- $\beta$ , and inhibition of signalling by PRRs, partially due to epigenetic modifications of essential promoter regions, are involved. Moreover, apoptosis of immune cells has been implicated in immune dysfunction and mortality in sepsis<sup>[10]</sup>. Most cells that undergo enhanced apoptosis in sepsis are of lymphoid origin (B cells, CD4 T cells), but also dendritic cells are affected. Preclinical studies have suggested that enhanced apoptosis of lymphocytes contributes to sepsis lethality<sup>[10]</sup>.

## MSC

MSCs have emerged in recent years as therapeutic tools based on four important features: (1) differentiation potential; (2) capacity to modulate immune responses; (3) pro-angiogenic and repair promoting capacities; and (4) low immunogenicity; the latter feature may allow allogeneic treatments. MSCs have been found in a variety of adult tissues of mesodermal origin, such as bone marrow, adipose tissue, placenta, umbilical cord, dental pulp or synovium<sup>[20-25]</sup>. Although sharing the main characteristics, differences between MSCs from different sources can be found, for instance at the RNA and protein expression profiles levels<sup>[26-28]</sup>. MSCs are considered a promising tool for cell therapy, in particular for inflammatory diseases, based on their immunomodulatory properties and paracrine effects through trophic factors with anti-fibrotic, anti-apoptotic or pro-angiogenic properties<sup>[29,30]</sup>. MSCs regulate the function of a broad range of immune cells<sup>[30-37]</sup>, and are activated by inflammatory mediators released from activated immune cells (*i.e.*, IFN $\gamma$ , IL1 $\beta$  and TNF $\alpha$ )<sup>[38,39]</sup>. The mechanisms involved in the immunoregulatory activity of MSCs are still under investigation but rely on both cell contact-dependent mechanisms (*i.e.*, Jagged1-Notch1 interaction, Fas-Fas-L interaction)<sup>[40,41]</sup> and

paracrine effects through the release of soluble factors including hepatocyte growth factor, prostaglandin-E2 (PGE2), TGF- $\beta$ 1, nitric oxide (NO), IL-10, IL-6, heme oxygenase-1 (HO-1), HLA-G5 or the enzymatic activity of indoleamine 2,3-dioxygenase<sup>[42]</sup>. In addition to the direct effect of these soluble factors, MSCs may also modulate immune responses through the generation of immune cells with regulatory phenotype, including regulatory T cells or anti-inflammatory macrophages<sup>[43-45]</sup>.

MSCs have also been reported to show antimicrobial activities against different pathogens upon activation with inflammatory cytokines<sup>[46]</sup>. Noteworthy in the context of sepsis, the functionality of MSCs can also be modulated by activators of TLRs<sup>[47]</sup>. It has been described that MSCs can be polarized *in vitro* towards either anti-inflammatory or pro-inflammatory phenotypes, depending on the TLR ligand time/concentration used for activation<sup>[48]</sup>. Furthermore, it has been recently described that interaction of gastrointestinal bacteria (*Salmonella typhimurium* or *Lactobacillus acidophilus*) with MSCs increased their capacity to inhibit T lymphocyte proliferation *in vitro* through a PGE2-dependent mechanism, indicating that bacteria may also enhance the immunomodulating properties of MSCs<sup>[49]</sup>.

MSCs can sense inflammatory signals through the expression of cytokine/chemokine receptors and integrins, and subsequently migrate to sites of inflammation<sup>[50]</sup>. Moreover, homing of systemically administered MSCs to lymphoid organs (draining lymph nodes and spleen) and the subsequent generation of functional Tregs have also been reported<sup>[51-53]</sup>. MSCs do not long-term engraft at the inflammation site and cells seem to be cleared shortly after administration. This suggests that transient effects through soluble factors and cell-to-cell contacts play a main role in MSC-mediated initial controlling and balancing of local inflammation.

Allogeneic MSCs are regarded as a preferred source for treatment as they would allow treatment with a ready to use, off-the-shelf product, available for a large number of patients, specially, in acute life threatening indications like sepsis in which isolation and expansion of autologous MSCs is not an option. In that context, MSCs are considered immune privileged as they express constitutively only low levels of cell-surface HLA class I molecules and lack expression of HLA class II, CD40, CD80 and CD86 which would lead to reduced activation of the innate and adaptive immune responses<sup>[54]</sup>. This immune privilege of MSCs therefore supports the feasibility of allogeneic treatments without the requirement of suppression of host immunity<sup>[55,56]</sup>. However the immunogenic features of MSCs are currently under review as there is some evidence of immunogenicity in experimental animal models that coincides with immunomodulatory effects by MSCs<sup>[57]</sup>.



## MSC IN EXPERIMENTAL MODELS OF SEPSIS

Sepsis being a disease that results as a consequence of deregulated inflammatory and immune responses against an infection can lead to tissue damage, multiorgan failure and death. Interest in investigating the therapeutic effect of MSCs on experimental models of sepsis emerged recently, and is based on their immunomodulatory properties<sup>[58]</sup>. A description of the therapeutic effects of MSCs in experimental small animal models of sepsis and the mechanisms involved are described in the following sections. A summary of the data is provided in Table 1.

### Experimental models of sepsis

In order to study sepsis pathophysiology, animal models of sepsis have been established. These models are normally used to preliminarily test potential therapeutic treatments prior to human clinical trials. On the basis of the initiating agent, sepsis models can be divided into three categories: toxemia models (exogenous administration of a bacterial toxin, such as lipopolysaccharide), bacterial infection models (exogenous administration of a bacteria) and host barrier disruption models (alteration of the animal's endogenous colonic protective barrier allowing bacterial leakage)<sup>[59]</sup>. These experimental models have in common high inflammatory responses against endotoxins or bacteria, subsequent organ injury and failure and, as a consequence, high mortality rates within few hours or days. All models have contributed significantly to our understanding of sepsis pathophysiology, although no single one fully mimics the course of human disease. Two limitations of sepsis models compared to human disease are the timing of disease progression (the progression to multiorgan failure and death occurs in hours to days in most animal models, whereas in human sepsis this occurs in days to weeks) and lack of supportive therapeutic intervention (*i.e.*, intubation and mechanical ventilation, fluid therapy), in particular in small animal models. Therefore, extrapolation of efficacy results obtained in small sepsis animal models to the human disease has to be made with caution<sup>[58,59]</sup>.

The toxemia models involve the administration by intraperitoneal or intravenous injection of a bacterial toxin. Thus, a single injection of high dose LPS (normally 10-20 mg/kg) is the most commonly used toxemia model (LPS model). LPS administration induces a very rapid and transient increase in systemic cytokine levels, hypodynamic cardiovascular activity and a shock-like state. The injection of LPS may result within hours in high mortality rates that may vary with the dose, type of LPS, age and strain of animal. The bacterial infection models consist on an exogenous bacterial infection and the severity of the model may vary depending on the bacterial strain (*i.e.*, *Escherichia coli*, *Pseudomonas aeruginosa*) and route of infection

(intravenous, intraperitoneal, intratracheal) used. The clinical progression of the disease is rapid with hypodynamic cardiovascular state, high cytokine levels and progression towards death within hours. The host-barrier disruption models require the surgical disruption of the shielding barrier that protects sterile compartments from pathogens, allowing bacteria to spread. These models have become the most relevant sepsis models because they create a focus of infection that can disseminate throughout the body, mimicking the human situation. The caecal ligation and puncture model (CLP) is considered to be one of the most clinically relevant models for sepsis research. The model involves surgical ligation of the distal cecum with suture followed by one or two small punctures distal to the ligation. This allows the leakage of intestinal content into the peritoneal cavity, which results in polymicrobial sepsis (several bacterial species can be found in the blood and other organs of CLP animals). Technical variations (needle size and number of punctures) can influence the severity of the CLP model (mortality within hours or several days)<sup>[59,60]</sup>.

### Effect of MSC treatment on mortality and organ injury induced by sepsis

The therapeutic effect of MSC treatment has been tested using different sepsis animal models, MSC types, dosing, timing and routes of administration. These studies have consistently reported improvement on survival rates of animals treated with MSCs (Table 1). In mice, one single dose of between  $3 \times 10^5$  and  $10^6$  MSCs administered by intraperitoneal, intravenous or intratracheal route was able to significantly reduce sepsis-related mortality in LPS, CLP, *P. aeruginosa* peritonitis and *E.coli* pneumonia mouse models<sup>[52,61-68]</sup>. Similar therapeutic effects have been observed using autologous, allogeneic or xenogeneic MSCs<sup>[52,61]</sup>. Noteworthy, treatment with fibroblasts has not been reported to increase survival of septic mice, despite the shared immunomodulating properties of fibroblasts with MSCs<sup>[61,63,64]</sup>.

The effects on survival might depend on the dose (low/high, one/multiple) and the timing of administration (early/late after insult). Gonzalez-Rey *et al.*<sup>[52]</sup> reported that one dose of  $10^6$  human ASCs administered intraperitoneally 30 min after LPS injection in mice had a higher protective effect on mortality than one dose of  $3 \times 10^5$  cells. Mei *et al.*<sup>[62]</sup> found that intravenous administration of one dose of  $2.5 \times 10^5$  mouse BM-MSCs after 6 h of CLP did not significantly protect mice, unless MSCs were administered concomitantly with antibiotics. These results might be related to different experimental settings because, compared to other studies carried out in CLP mouse models, Mei *et al.*<sup>[62]</sup> administered a lower dose of MSCs ( $2.5 \times 10^5$  vs  $10^6$  cells) and at a later time point (6 h after CLP vs a range between 24 h before and 4 h after CLP)<sup>[52,61,66,68]</sup>. On the other hand, Hall *et al.* reported that three intravenous



Table 1 Efficacy preclinical studies on experimental models of sepsis using mesenchymal stem cells, mesenchymal stem cell-conditioned medium or mesenchymal stem cell-derived macrophages

Animal model	MSC type	Route/time	Dose	Number of doses	Therapeutic effects					MoA	Ref.
					Survival	Cytokines	Inflammatory infiltration	Organ injury	Bacterial load		
LPS, mouse	hASCs (xeno)	I.P./after 0.5 h or I.P./after 0.5 h	$3 \times 10^5$ $10^6$	1 1	Improved Improved	Reduced pro-inflammatory cytokines in serum, liver, lung and intestine Increased anti-inflammatory cytokine (IL10) in liver, lung and intestine	Reduced lymphocyte, neutrophil and macrophage infiltration in peritoneum, liver, lung and intestine	ND	ND	ND	Gonzalez-Rey <i>et al</i> <sup>[23]</sup>
LPS, mouse	hBM-MSCs (xeno) normal/ senescent	I.P./after 0.5 h	$10^6$	1	Improved only by normal cells	Reduced pro-inflammatory cytokines in serum and lungs (normal cells) Reduced anti-inflammatory cytokine (IL10) in serum (normal cells)	ND	ND	ND	ND	Sepúlveda <i>et al</i> <sup>[61]</sup>
LPS, mouse	hASC/BM-MSC-CM	I.P./at 0 h	1 mL CM (from $2 \times 10^6$ cells per milliliter)	1	Improved only by hBM-MSC CM	ND	Reduced neutrophil infiltration in kidney (hBM-MSC CM)	Improved kidney, liver and lung damage (hBM-MSC CM)	ND	ND	Elman <i>et al</i> <sup>[63]</sup>
LPS, rat	hASCs (xeno)	I.V./after 0.5 h	$2 \times 10^6$	1	ND	Reduced pro-inflammatory cytokines in lung No effect on anti-inflammatory cytokine (IL10)	ND	Improved kidney, liver and lung damage	ND	ND	Shin <i>et al</i> <sup>[73]</sup>
LPS, rat	hBM-MSCs (xeno)	I.M./at 0 h	$2 \times 10^6$	1	ND	ND	ND	Improved kidney, liver and lung damage	ND	ND	Yagi <i>et al</i> <sup>[73]</sup>
LPS, rat	hBM-MSCs (xeno)	I.M./at 0 h	$2 \times 10^6$	1	ND	Reduced pro-inflammatory cytokines in serum	Reduced neutrophil and macrophage infiltration in kidney, liver and lung	ND	ND	MSC release of sTNFR1	Yagi <i>et al</i> <sup>[76]</sup>
LPS, rat	mBM-MSCs (xeno)	I.P./after 1 h	$2 \times 10^6$	1	ND	Reduced pro-inflammatory cytokines in serum and myocardium Increased anti-inflammatory cytokine (IL10) in serum but not in myocardium	ND	Improved myocardial damage Cells from female donors showed higher effect	ND	Higher expression of anti-apoptotic proteins in myocardium	Manukyan <i>et al</i> <sup>[71]</sup>
LPS, rat	mBM-MSCs (xeno)	I.P./after 1 h	$2 \times 10^6$	1	ND	Reduced pro-inflammatory cytokines in serum and myocardium Increased anti-inflammatory cytokine (IL10) in serum but not in myocardium	ND	Improved myocardial damage	ND	ND	Weil <i>et al</i> <sup>[72]</sup>
LPS, rat	rBM-MSCs (auto)	I.V./after 1 h	$2.5 \times 10^6$	1	ND	Reduced pro-inflammatory cytokines in serum and myocardium Increased anti-inflammatory cytokine (IL10) in serum but not in myocardium	ND	Improved myocardial damage	ND	ND	Weil <i>et al</i> <sup>[74]</sup>



CLP, mouse	hASCs (xeno) mASCs (auto/allo)	I.P./after 4 h	10 <sup>6</sup>	1	Improved	Reduced pro-inflammatory cytokines in serum, liver, lung and intestine Increased anti-inflammatory cytokine (IL10) in liver, lung and intestine	Reduced lymphocyte, neutrophil and macrophage infiltration in peritoneum, liver, lung and intestine	ND	ND	ND	Gonzalez-Rey <i>et al.</i> <sup>[52]</sup>
CLP, mouse	mBM-MSCs (auto/allo)	I.V./24 h prior or I.V./after 1 h	10 <sup>6</sup> 10 <sup>6</sup>	1 1	Improved Improved	Reduced pro-inflammatory cytokines in serum Increased anti-inflammatory cytokine (IL10) in serum	Reduced neutrophil infiltration in peritoneum, liver and kidney	Improved kidney, liver, pancreatic and spleen damage and vascular permeability	Reduced bacterial counts in blood	Anti-inflammatory Mph (IL10) induced by MSCs through PGE2	Németh <i>et al.</i> <sup>[61]</sup>
CLP, mouse	mBM-MSCs (auto)	I.V./after 6 h	2.5 × 10 <sup>5</sup>	1	Improved	Reduced pro-inflammatory cytokines in serum and BAL No effect on anti-inflammatory cytokine (IL10) in serum and BAL	Reduced neutrophil infiltration in peritoneum, liver and kidney	Improved kidney and lung damage No effect on liver and pancreatic damage	Reduced bacterial counts in spleen	Increased phagocytic activity of macrophages and neutrophils	Mei <i>et al.</i> <sup>[62]</sup>
CLP, mouse	mBM-MSCs (auto)	I.V./after 2 h and I.V./after 24 h and I.V./after 48 h	5 × 10 <sup>5</sup> 2.5 × 10 <sup>5</sup> 2.5 × 10 <sup>5</sup> total: 10 <sup>6</sup>	3	Improved	ND	Reduced neutrophil infiltration in bowel	Improved bowel, kidney, liver and spleen damage	Reduced bacterial counts in peritoneum and blood	Increased phagocytic activity of neutrophils	Hall <i>et al.</i> <sup>[64]</sup>
CLP, mouse	mBM-MSCs (auto)	I.V./after 3 h	10 <sup>6</sup>	1	Improved	Reduced pro-inflammatory cytokines in serum Increased anti-inflammatory cytokine (IL10) in serum	Reduced neutrophil infiltration in kidney	Improved kidney damage	Reduced bacterial counts in blood	ND	Luo <i>et al.</i> <sup>[66]</sup>
CLP, mouse	ASC-derived mouse Mph	I.P./after 4 h or I.P./after 6 h or I.P./after 12 h or I.P./after 24 h	10 <sup>6</sup> 10 <sup>6</sup> 10 <sup>6</sup> 10 <sup>6</sup>	1 1 1 1	Improved Improved Improved No effect	Reduced pro-inflammatory cytokines in serum (only treatment at 4 h tested)	Reduced lymphocyte, neutrophil and macrophage infiltration in peritoneum, lung, liver and intestine (only treatment at 4 h tested)	ND	ND	IL10 secreted by Mph	Anderson <i>et al.</i> <sup>[77]</sup>
CLP, mouse	hUC-MSCs (xeno) wt/Poly I:C preactivated	I.V./after 1 h	10 <sup>6</sup>	1	Improved Better preactivated	Reduced pro-inflammatory cytokines in plasma Better preactivated	Reduced inflammatory infiltration in kidney, liver and lung	Improved kidney, liver damage and pancreatic damage Better preactivated	Reduced bacterial counts in peritoneum and blood Better preactivated	Poly I:C inhibition of MMR-143 expression by MSCs	Zhao <i>et al.</i> <sup>[68]</sup>
CLP, rat	rASCs (auto) living/apoptotic	I.P./after 0.5 h and I.P./after 6 h and I.P./after 18 h	1.2 × 10 <sup>6</sup> 1.2 × 10 <sup>6</sup> 1.2 × 10 <sup>6</sup> total: 3.6 × 10 <sup>6</sup>	3	Higher mortality by living cells Improved by apoptotic cells	Reduced TNFα (apoptotic rASC treated rats)	ND	Improved kidney, liver, lung and myocardial damage (apoptotic rASCs)	ND	ND	Chang <i>et al.</i> <sup>[69]</sup>



<i>E.coli</i> pneumonia, mouse	hBM-MSCs (xeno)	I.T/after 4 h	10 <sup>6</sup>	1	ND	Reduced pro-inflammatory cytokines in bronchoalveolar liquid (BAL)	Reduced neutrophil infiltration in BAL	Improved lung epithelial and endothelial permeability	Reduced bacterial counts in BAL	MSC release of the antimicrobial peptide LL-37	Krasnodems-kaya <i>et al</i> <sup>[70]</sup>
<i>P.aeruginosa</i> peritonitis, mouse	hBM-MSCs (xeno)	I.V/after 1 h	10 <sup>6</sup>	1	Improved	No changes in serum or peritoneal fluid levels of pro or anti-inflammatory mediators	ND	ND	Reduced bacterial counts in peripheral blood, peritoneal fluid, lung, and spleen	Increased phagocytic activity of macrophages Generation of anti-inflammatory macrophages in spleen	Krasnodems-kaya <i>et al</i> <sup>[65]</sup>

LPS: Lipopolysaccharide; CLP: Caecal ligation and puncture; h: Human; r: Rat; m: Mouse; ASCs: Adipose mesenchymal stem cells; BM-MSCs: Bone marrow mesenchymal stem cells; UC-MSCs: Umbilical cord mesenchymal stem cells; Mphi: Macrophages; I.P: Intraperitoneal; I.V: Intravenous; I.T: Intratracheal; I.M: Intramuscular; ND: Not determined; CM: Conditioned medium.

administrations of  $5 \times 10^5$ ,  $2.5 \times 10^5$  and  $2.5 \times 10^5$  mouse BM-MSCs at 2, 24 and 48 h after CLP, respectively, also reduced mortality rates on mice, although they did not compare with the effects of one single dose<sup>[64]</sup>. Unfortunately, so far, different timing of administration have only been compared in the same study by Németh *et al*<sup>[61]</sup> who reported that a prophylactic intravenous treatment with mouse BM-MSCs 24 h prior to CLP had similar therapeutic effects than a therapeutic treatment after 1 h of CLP. However, due to the urgent and acute condition of sepsis, a prophylactic treatment is clinically not feasible and comparing in the same study single vs multiple dosing and different time regimens (early or late after the induction of sepsis) might be very important in order to understand if there is a time window in which MSC

treatment is most efficacious and, therefore, maximize the therapeutic benefit of MSC therapy.

In addition to the dose and time, the “fitness” of the cells at the time of administration might also affect the therapeutic effect of MSCs. Thus, Sepúlveda *et al*<sup>[67]</sup> found that while intraperitoneal administration of normal human BM-MSCs 30 min after LPS injection reduced mortality in an LPS sepsis model in mice, senescent human BM-MSCs failed to protect them, despite the fact they conserved the capacity to modulate the function of lymphocytes and macrophages *in vitro*. However, in contrast to these positive results, Chang *et al*<sup>[69]</sup> reported that three intraperitoneal administrations of  $1.2 \times 10^6$  living rat ASCs (at 0.5, 6 and 18 h) did not reduce, but moderately increased, mortality in a rat model of CLP, whereas the administration of apoptotic rat ASCs protected rats from death. Further studies will be needed to better understand these results.

The effects of MSC treatment on survival are a consequence of the reduction of inflammation-associated organ injury and the improvement in organ function. MSC treatment has been reported to reduce damage in kidney (*i.e.*, reduced levels of apoptotic cells, serum creatinine and tubular injury score), liver (*i.e.*, reduced levels of apoptotic cells, serum liver enzymes and blood urea nitrogen), pancreatic (*i.e.*, reduced levels of serum amylase), spleen (*i.e.*, reduced levels of apoptotic cells), lung (*i.e.*, reduced levels of apoptotic cells and vascular leakage) and heart function (*i.e.*, improved cardiac depression) in a variety of sepsis models and experimental settings<sup>[61,62,64-66,68-75]</sup>. Improvement in organ damage correlates with reduction on neutrophil infiltration and myeloperoxidase (MPO) activity in target organs<sup>[52,61]</sup>. Notably, these effects can also be obtained by intraperitoneal administration of conditioned medium from BM-MSCs or ASCs in a LPS mouse model, suggesting that the therapeutic effects of MSCs might be mediated, at least in part, by soluble factors<sup>[65]</sup>. Of note, Manukyan *et al*<sup>[71]</sup> observed that female mouse BM-MSCs injected intraperitoneally had a better improvement of cardiac function of LPS septic rats than male mouse BM-MSCs. This effect correlated with a higher expression of the anti-apoptotic protein Bcl-XL in the myocardium of female MSC treated rats.

No specific side effects of MSC treatment have been reported in sepsis models (only Chang *et al*<sup>[69]</sup> reported increased mortality when using living rat ASCs compared to the untreated group in a rat model of CLP). The fate of MSCs in sepsis models have been also investigated in some studies. When MSCs were administered intravenously, cells were always detected in the lungs and eventually, to a lesser extent, in spleen, liver, kidney or lymph nodes<sup>[61,66,74]</sup>. When MSCs were administered



intramuscularly, cells were only detected in the muscle up to 24 h after administration<sup>[76]</sup>.

### Effects of MSCs on inflammation induced by sepsis

The pathogenesis of sepsis is characterized by massive infiltration of immune cells in target organs and high pro-inflammatory cytokine levels systemically and locally, that can lead to tissue damage, multiple organ failure and death. Treatment with MSCs reduces the infiltration of neutrophils and monocyte/macrophages to target organs, including liver, lung, intestine and kidney<sup>[52,61,62,64-66,68,70,76]</sup>. Furthermore, MSC treatment has also been reported to reduce the levels of proinflammatory cytokines (*i.e.*, IFN $\gamma$ , TNF $\alpha$ , IL1 $\beta$  or IL6) in several organs including serum, liver, lung, intestine and myocardium<sup>[52,61,66-68,70-72,74,76]</sup>. These anti-inflammatory effects can be enhanced by preactivation of UC-MSCs with Poly I:C which results in the inhibition of miR-143 expression by MSCs<sup>[68]</sup>. The reduction on the levels of anti-inflammatory cytokines was accompanied by the increase on the levels of the anti-inflammatory cytokine IL10<sup>[52,61,66-68,70-72,74,76]</sup>, although other authors have reported either no effect on IL10 levels or even a reduction<sup>[62,63,67,75]</sup>. These differences might be related to differences in the experimental settings, such as the use of different animal models, MSCs, dosing and time of sample collection. Nevertheless, there is evidence that IL10 plays an important role in the therapeutic effects of MSCs in sepsis. Thus, injection of a neutralizing antibody against IL10 or IL10 receptor prior to CLP abrogated the therapeutic effects of mouse BM-MSCs<sup>[61]</sup>. *In vitro* studies showed that IL10 was not directly produced by MSC, but by macrophages through a mechanism that required MSC-secretion of PGE2<sup>[61,77]</sup>. Moreover, a role of IL10 in inhibiting the migration of neutrophils into the infected tissues has also been suggested<sup>[61]</sup>. In addition to IL10, other mediators of the therapeutic effect of MSCs have been identified. Thus, Yagi *et al.*<sup>[73]</sup> observed that blockade of sTNFR1, which is released by MSCs in response to inflammation, partially impaired the anti-inflammatory effects of MSC treatment.

The MSC-mediated reprogramming of macrophages towards a regulatory and anti-inflammatory M2 phenotype has also been reported in sepsis models by other authors. Krasnodemskaya *et al.*<sup>[63]</sup> observed a larger population of monocytes expressing CD206 (a marker of alternative activated M2 macrophages) in the spleen of MSC-treated mice and a higher phagocytic capacity of blood monocytes. Furthermore, Anderson *et al.*<sup>[77]</sup> provided strong evidence of the important role that MSC-induced regulatory macrophages play in the therapeutic effects of ASCs in sepsis. The authors generated "ASC-mediated regulatory macrophages" (ASC-Mph) by *in vitro* culture of mouse bone marrow macrophages and ASCs (either mouse or human) and injected 10<sup>6</sup> ASC-Mph intraperitoneally in septic mice

at different time points after CLP. These treatments resulted in reduced mortality rates when ASC-Mph were administered between 4 h and 12 h (but not at 24 h) after CLP by a mechanism that required the production of IL10 by ASC-Mph<sup>[77]</sup>. Moreover, these regulatory ASC-Mph also reduced levels of pro-inflammatory cytokines in serum and infiltration of inflammatory cells in the peritoneum, lung, liver and intestine. Finally, the relevance of monocytes/macrophages, but also neutrophils, in mediating the therapeutic effects of MSCs is highlighted by the fact that depletion of monocyte/macrophages (by using clodronate-filled liposomes) or neutrophils (by using anti-Ly6G antibody) completely abrogated the protective effects of MSCs *in vivo*<sup>[61,64]</sup>.

The effects of MSC treatment on transcriptional inflammatory pathways in target organs of CLP septic mice treated with MSCs have been investigated by microarray analysis of total RNA expression. The results show that MSC treatment affects an ample range of transcriptional networks (it was estimated that up to a 13% of total murine genome was transcriptionally reprogrammed after MSC treatment compared to control septic mice including: (1) downregulation of TLR, NF- $\kappa$ B or IL6 signaling pathways; (2) upregulation of NF-AT-related genes; (3) upregulation of genes involved in phagocytosis, antigen presentation, bacterial killing, coagulation, complement regulation and platelet activation; and (4) upregulation of genes involved in cell-to-cell interaction and endothelial/vascular integrity<sup>[62,78]</sup>.

### Effect of MSCs on bacterial burden in sepsis

The mechanism by which MSCs protect from sepsis is not only limited to reducing the production of inflammatory cytokines and migration of inflammatory cells to infected organs, but also includes direct anti-microbial properties, as well as the improvement of the phagocytic properties of monocyte/macrophages and neutrophils. Gonzalez-Rey *et al.*<sup>[52]</sup> and Németh *et al.*<sup>[61]</sup> first reported a reduction on bacterial load in target organs (*i.e.*, peritoneal cavity, blood, spleen or liver) in MSC-treated septic mice, despite the MSC-mediated reduction of the inflammatory response. Krasnodemskaya *et al.*<sup>[70]</sup> determined that MSC have intrinsic anti-microbial activity because they secrete the anti-microbial peptide LL-37 in response to the stimulation with *Escherichia coli* or *Pseudomonas aeruginosa*. Intratracheal administration of human BM-MSCs in a mouse pneumonia model highly reduced bacterial counts in bronchoalveolar lavage (BAL). However, when a LL-37 neutralizing antibody was also administered to mice, the anti-microbial effects of MSCs were only partially lost, suggesting that additional anti-microbial mechanisms might be involved. This potential direct killing of bacteria by MSCs needs to be further confirmed as Gonzalez-Rey *et al.*<sup>[52]</sup> did not observe direct killing of *Escherichia coli* by MSCs *in vitro* in the absence of other cells.



In addition, the enhancement of the phagocytic properties of monocyte/macrophages and neutrophils have also been reported to improve bacterial clearance by MSCs. Noteworthy, MSCs seem not to have the capacity to phagocyte bacteria *in vitro*<sup>[62,64]</sup>. Mei *et al.*<sup>[62]</sup> found that MSC treatment in a mouse CLP model increased the phagocytic capacity of peritoneal and spleen CD11b positive cells (mainly monocyte/macrophages and neutrophils) in MSC treated mice. Krasnodembskaya *et al.*<sup>[63]</sup> observed a reduction on bacterial counts in several organs, but more significantly in peripheral blood of MSC treated mice infected with *Pseudomonas aeruginosa*, which was also associated to an increased capacity of peripheral blood monocytes to phagocyte bacteria. Hall *et al.*<sup>[64]</sup> determined that MSCs, but not fibroblasts, also enhanced the phagocytic properties of neutrophils *in vitro* and in a CLP mouse model. In fact, depletion of neutrophils *in vivo* abrogated the ability of MSCs to promote bacterial clearance<sup>[64]</sup>. Notably, Németh *et al.*<sup>[61]</sup> noticed that while infiltration of neutrophils to target organs was inhibited in MSC treated mice, their presence in circulation was concomitantly increased and suggested that this mechanism might help to clear bacteria from circulation and minimize organ injury due to leukocyte infiltration. Interestingly, preactivation with Poly I:C increased the *in vivo* anti-microbial effects of UC-MSCs in a CLP mouse model through a mechanism that requires the inhibition of the expression of miR-143<sup>[68]</sup>.

## CONCLUSION

Sepsis is a leading cause of death and the most frequent cause of death in non-coronary ICUs in the developed world and, despite improvement in treatments, the mortality of severe sepsis and septic shock remains very high, showing that current treatments are not sufficient to combat this syndrome. The use of MSCs in experimental animal models of sepsis has reported strong evidence of the therapeutic potential of MSC therapy in this indication. These studies have been mainly focused on the effects of MSCs on the pro-inflammatory phase of sepsis, while the effects of MSCs on the subsequent anti-inflammatory/immune exhaustion phase of the disease has not been elucidated so far and will need further investigation. The mechanisms by which MSCs improve survival in sepsis models rely on the collective effects of their immunomodulatory and anti-microbial properties: MSC treatment modulates inflammation in septic mice by a mechanism that requires the reprogramming of macrophages towards a more anti-inflammatory phenotype (release of anti-inflammatory IL10), resulting in reduced levels of pro-inflammatory cytokines in blood and organs and attenuated infiltration of immune cells in infected tissues (monocytes and neutrophils). Moreover, MSCs show direct (release of LL-37 peptide) and indirect (increase of phagocytic properties of

monocyte/macrophages and neutrophils) anti-microbial effects. The combined effect of reducing both the inflammatory response and the bacterial burden results in an improvement of organ function and higher survival rates. The promising results obtained in these, small animal, preclinical efficacy studies are encouraging and suggest that MSCs might be a therapeutic option to treat sepsis in patients. Importantly, efficacy of MSCs in large animal models that better replicate the inflammatory response, organ failure and disease in humans (e.g., sheep models) will be additionally relevant to support further testing of the therapeutic potential of allogeneic MSC treatment in humans. Such clinical trials should be prospective, controlled, and randomized so to guarantee a clear outcome of the MSC treatment effect. Moreover, taking into consideration the complexity and heterogeneity of sepsis and the poor results up to now in sepsis clinical trials, we believe that such trials should first be done in well defined and homogeneous sepsis patient populations.

## REFERENCES

- 1 **Lagu T**, Rothberg MB, Shieh MS, Pekow PS, Steingrub JS, Lindenauer PK. What is the best method for estimating the burden of severe sepsis in the United States? *J Crit Care* 2012; **27**: 414.e1-414.e9 [PMID: 22516143 DOI: 10.1016/j.jcrc.2012.02.004]
- 2 **Mayr FB**, Yende S, Angus DC. Epidemiology of severe sepsis. *Virulence* 2014; **5**: 4-11 [PMID: 24335434 DOI: 10.4161/viru.27372]
- 3 **Angus DC**, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med* 2001; **29**: 1303-1310 [PMID: 11445675]
- 4 **Lagu T**, Rothberg MB, Shieh MS, Pekow PS, Steingrub JS, Lindenauer PK. Hospitalizations, costs, and outcomes of severe sepsis in the United States 2003 to 2007. *Crit Care Med* 2012; **40**: 754-761 [PMID: 21963582 DOI: 10.1097/CCM.0b013e318232db65]
- 5 **Angus DC**, van der Poll T. Severe sepsis and septic shock. *N Engl J Med* 2013; **369**: 840-851 [PMID: 23984731 DOI: 10.1056/NEJMr1208623]
- 6 **Kaukonen KM**, Bailey M, Suzuki S, Pilcher D, Bellomo R. Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000-2012. *JAMA* 2014; **311**: 1308-1316 [PMID: 24638143 DOI: 10.1001/jama.2014.2637]
- 7 **van der Poll T**, Opal SM. Host-pathogen interactions in sepsis. *Lancet Infect Dis* 2008; **8**: 32-43 [PMID: 18063412]
- 8 **Martin GS**, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 2003; **348**: 1546-1554 [PMID: 12700374]
- 9 **Vincent JL**, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, Moreno R, Lipman J, Gomersall C, Sakr Y, Reinhart K. International study of the prevalence and outcomes of infection in intensive care units. *JAMA* 2009; **302**: 2323-2329 [PMID: 19952319 DOI: 10.1001/jama.2009.1754]
- 10 **Hotchkiss RS**, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 2013; **13**: 862-874 [PMID: 24232462 DOI: 10.1038/nri3552]
- 11 **Takeuchi O**, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010; **140**: 805-820 [PMID: 20303872 DOI: 10.1016/j.cell.2010.01.022]
- 12 **Chan JK**, Roth J, Oppenheim JJ, Tracey KJ, Vogl T, Feldmann M, Horwood N, Nanchahal J. Alarmins: awaiting a clinical response. *J Clin Invest* 2012; **122**: 2711-2719 [PMID: 22850880 DOI: 10.1172/JCI62423]
- 13 **Deutschman CS**, Tracey KJ. Sepsis: current dogma and new



- perspectives. *Immunity* 2014; **40**: 463-475 [PMID: 24745331 DOI: 10.1016/j.immuni.2014.04.001]
- 14 **Wiersinga WJ**, Leopold SJ, Cranendonk DR, van der Poll T. Host innate immune responses to sepsis. *Virulence* 2014; **5**: 36-44 [PMID: 23774844 DOI: 10.4161/viru.25436]
  - 15 **Marshall JC**. Why have clinical trials in sepsis failed? *Trends Mol Med* 2014; **20**: 195-203 [PMID: 24581450 DOI: 10.1016/j.molmed.2014.01.007]
  - 16 **Bosmann M**, Ward PA. Role of C3, C5 and anaphylatoxin receptors in acute lung injury and in sepsis. *Adv Exp Med Biol* 2012; **946**: 147-159 [PMID: 21948367 DOI: 10.1007/978-1-4614-0106-3\_9]
  - 17 **Levi M**, van der Poll T. Inflammation and coagulation. *Crit Care Med* 2010; **38**: S26-S34 [PMID: 20083910 DOI: 10.1097/CCM.0b013e3181c98d21]
  - 18 **Singer M**. The role of mitochondrial dysfunction in sepsis-induced multi-organ failure. *Virulence* 2014; **5**: 66-72 [PMID: 24185508 DOI: 10.4161/viru.26907]
  - 19 **Boomer JS**, To K, Chang KC, Takasu O, Osborne DF, Walton AH, Bricker TL, Jarman SD, Kreisel D, Krupnick AS, Srivastava A, Swanson PE, Green JM, Hotchkiss RS. Immunosuppression in patients who die of sepsis and multiple organ failure. *JAMA* 2011; **306**: 2594-2605 [PMID: 22187279 DOI: 10.1001/jama.2011.1829]
  - 20 **Friedenstein AJ**, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976; **4**: 267-274 [PMID: 976387]
  - 21 **Zuk PA**, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; **13**: 4279-4295 [PMID: 12475952]
  - 22 **Fukuchi Y**, Nakajima H, Sugiyama D, Hirose I, Kitamura T, Tsuji K. Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells* 2004; **22**: 649-658 [PMID: 15342929]
  - 23 **Romanov YA**, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. *Stem Cells* 2003; **21**: 105-110 [PMID: 12529557]
  - 24 **Gronthos S**, Mankani M, Brahimi J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci USA* 2000; **97**: 13625-13630 [PMID: 11087820]
  - 25 **De Bari C**, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* 2001; **44**: 1928-1942 [PMID: 11508446]
  - 26 **Noël D**, Caton D, Roche S, Bony C, Lehmann S, Casteilla L, Jorgensen C, Cousin B. Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. *Exp Cell Res* 2008; **314**: 1575-1584 [PMID: 18325494 DOI: 10.1016/j.yexcr.2007.12.022]
  - 27 **Skalnikova H**, Motlik J, Gadher SJ, Kovarova H. Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines. *Proteomics* 2011; **11**: 691-708 [PMID: 21241017 DOI: 10.1002/pmic.201000402]
  - 28 **De Ugarte DA**, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J, Hedrick MH. Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* 2003; **174**: 101-109 [PMID: 12835573]
  - 29 **Singer NG**, Caplan AI. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol* 2011; **6**: 457-478 [PMID: 21073342 DOI: 10.1146/annurev-pathol-011110-130230]
  - 30 **Bernardo ME**, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* 2013; **13**: 392-402 [PMID: 24094322 DOI: 10.1016/j.stem.2013.09.006]
  - 31 **Di Nicola M**, Carlo-Stella C, Magni M, Milanese M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; **99**: 3838-3843 [PMID: 11986244]
  - 32 **Krampera M**, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, Dazzi F. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003; **101**: 3722-3729 [PMID: 12506037]
  - 33 **Ghannam S**, Pène J, Moquet-Torcy G, Jorgensen C, Yssel H. Mesenchymal stem cells inhibit human Th17 cell differentiation and function and induce a T regulatory cell phenotype. *J Immunol* 2010; **185**: 302-312 [PMID: 20511548 DOI: 10.4049/jimmunol.0902007]
  - 34 **Prigione I**, Benvenuto F, Bocca P, Battistini L, Uccelli A, Pistoia V. Reciprocal interactions between human mesenchymal stem cells and gammadelta T cells or invariant natural killer T cells. *Stem Cells* 2009; **27**: 693-702 [PMID: 19096038 DOI: 10.1634/stemcells.2008-0687]
  - 35 **Corcione A**, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Rizzo M, Gualandi F, Mancardi GL, Pistoia V, Uccelli A. Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006; **107**: 367-372 [PMID: 16141348]
  - 36 **Raffaghelli L**, Bianchi G, Bertolotto M, Montecucco F, Busca A, Dallegri F, Ottonello L, Pistoia V. Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells* 2008; **26**: 151-162 [PMID: 17932421]
  - 37 **DeLaRosa O**, Sánchez-Correa B, Morgado S, Ramírez C, del Río B, Menta R, Lombardo E, Tarazona R, Casado JG. Human adipose-derived stem cells impair natural killer cell function and exhibit low susceptibility to natural killer-mediated lysis. *Stem Cells Dev* 2012; **21**: 1333-1343 [PMID: 21867426 DOI: 10.1089/scd.2011.0139]
  - 38 **Krampera M**, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, Santarlasci V, Mazzinghi B, Pizzolo G, Vinante F, Romagnani P, Maggi E, Romagnani S, Annunziato F. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006; **24**: 386-398 [PMID: 16123384]
  - 39 **Prasanna SJ**, Gopalakrishnan D, Shankar SR, Vasandan AB. Pro-inflammatory cytokines, IFN-gamma and TNF-alpha, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. *PLoS One* 2010; **5**: e9016 [PMID: 20126406 DOI: 10.1371/journal.pone.0009016]
  - 40 **Liotta F**, Angeli R, Cosmi L, Fili L, Manuelli C, Frosali F, Mazzinghi B, Maggi L, Pasini A, Lisi V, Santarlasci V, Consoloni L, Angelotti ML, Romagnani P, Parronchi P, Krampera M, Maggi E, Romagnani S, Annunziato F. Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. *Stem Cells* 2008; **26**: 279-289 [PMID: 17962701]
  - 41 **Akiyama K**, Chen C, Wang D, Xu X, Qu C, Yamaza T, Cai T, Chen W, Sun L, Shi S. Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand/FAS-mediated T cell apoptosis. *Cell Stem Cell* 2012; **10**: 544-555 [PMID: 22542159 DOI: 10.1016/j.stem.2012.03.007]
  - 42 **Doorn J**, Moll G, Le Blanc K, van Blitterswijk C, de Boer J. Therapeutic applications of mesenchymal stromal cells: paracrine effects and potential improvements. *Tissue Eng Part B Rev* 2012; **18**: 101-115 [PMID: 21995703 DOI: 10.1089/ten.TEB.2011.0488]
  - 43 **Maccario R**, Podestà M, Moretta A, Cometa A, Comoli P, Montagna D, Daudt L, Ibatici A, Piaggio G, Pozzi S, Frassoni F, Locatelli F. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. *Haematologica* 2005; **90**: 516-525 [PMID: 15820948]
  - 44 **Gonzalez-Rey E**, Gonzalez MA, Varela N, O'Valle F, Hernandez-Cortes P, Rico L, Büscher D, Delgado M. Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis. *Ann Rheum Dis* 2010; **69**: 241-248 [PMID: 19124525 DOI: 10.1136/ard.2008.101881]
  - 45 **Eggenhofer E**, Hoogduijn MJ. Mesenchymal stem cell-educated macrophages. *Transplant Res* 2012; **1**: 12 [PMID: 23369493 DOI: 10.1186/2047-1440-1-12]
  - 46 **Meisel R**, Brockers S, Heseler K, Digestirici O, Bülle H, Woite C, Stuhlsatz S, Schwippert W, Jäger M, Sorg R, Henschler R, Seissler J, Dillloo D, Däubener W. Human but not murine multipotent



- mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. *Leukemia* 2011; **25**: 648-654 [PMID: 21242993 DOI: 10.1038/leu.2010.310]
- 47 **Delarosa O**, Dalemans W, Lombardo E. Toll-like receptors as modulators of mesenchymal stem cells. *Front Immunol* 2012; **3**: 182 [PMID: 22783256 DOI: 10.3389/fimmu.2012.00182]
  - 48 **Waterman RS**, Tomchuck SL, Henkle SL, Betancourt AM. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. *PLoS One* 2010; **5**: e10088 [PMID: 20436665 DOI: 10.1371/journal.pone.0010088]
  - 49 **Kol A**, Foutouhi S, Walker NJ, Kong NT, Weimer BC, Borjesson DL. Gastrointestinal microbes interact with canine adipose-derived mesenchymal stem cells in vitro and enhance immunomodulatory functions. *Stem Cells Dev* 2014; **23**: 1831-1843 [PMID: 24803072 DOI: 10.1089/scd.2014.0128]
  - 50 **Yagi H**, Soto-Gutierrez A, Parekkadan B, Kitagawa Y, Tompkins RG, Kobayashi N, Yarmush ML. Mesenchymal stem cells: Mechanisms of immunomodulation and homing. *Cell Transplant* 2010; **19**: 667-679 [PMID: 20525442 DOI: 10.3727/096368910X508762]
  - 51 **González MA**, Gonzalez-Rey E, Rico L, Büscher D, Delgado M. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. *Arthritis Rheum* 2009; **60**: 1006-1019 [PMID: 19333946 DOI: 10.1002/art.24405]
  - 52 **Gonzalez-Rey E**, Anderson P, González MA, Rico L, Büscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009; **58**: 929-939 [PMID: 19136511 DOI: 10.1136/gut.2008.168534]
  - 53 **González MA**, Gonzalez-Rey E, Rico L, Büscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009; **136**: 978-989 [PMID: 19135996 DOI: 10.1053/j.gastro.2008.11.041]
  - 54 **Kahan BD**. Individuality: the barrier to optimal immunosuppression. *Nat Rev Immunol* 2003; **3**: 831-838 [PMID: 14523389]
  - 55 **Le Blanc K**, Tammik C, Rosendahl K, Zetterberg E, Ringdén O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003; **31**: 890-896 [PMID: 14550804]
  - 56 **Mitchell JB**, McIntosh K, Zvonick S, Garrett S, Floyd ZE, Kloster A, Di Halvorsen Y, Storms RW, Goh B, Kilroy G, Wu X, Gimble JM. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 2006; **24**: 376-385 [PMID: 16322640]
  - 57 **Griffin MD**, Ritter T, Mahon BP. Immunological aspects of allogeneic mesenchymal stem cell therapies. *Hum Gene Ther* 2010; **21**: 1641-1655 [PMID: 20718666 DOI: 10.1089/hum.2010.156]
  - 58 **Wannemuehler TJ**, Manukyan MC, Brewster BD, Rouch J, Poynter JA, Wang Y, Meldrum DR. Advances in mesenchymal stem cell research in sepsis. *J Surg Res* 2012; **173**: 113-126 [PMID: 2225756 DOI: 10.1016/j.jss.2011.09.053]
  - 59 **Buras JA**, Holzmann B, Sitkovsky M. Animal models of sepsis: setting the stage. *Nat Rev Drug Discov* 2005; **4**: 854-865 [PMID: 16224456]
  - 60 **Nemzek JA**, Hugunin KM, Opp MR. Modeling sepsis in the laboratory: merging sound science with animal well-being. *Comp Med* 2008; **58**: 120-128 [PMID: 18524169]
  - 61 **Németh K**, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009; **15**: 42-49 [PMID: 19098906 DOI: 10.1038/nm.1905]
  - 62 **Mei SH**, Haitsma JJ, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, Liles WC, Stewart DJ. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 2010; **182**: 1047-1057 [PMID: 20558630 DOI: 10.1164/rccm.201001-0010OC]
  - 63 **Krasnodembkaya A**, Samarani G, Song Y, Zhuo H, Su X, Lee JW, Gupta N, Petrini M, Matthay MA. Human mesenchymal stem cells reduce mortality and bacteremia in gram-negative sepsis in mice in part by enhancing the phagocytic activity of blood monocytes. *Am J Physiol Lung Cell Mol Physiol* 2012; **302**: L1003-L1013 [PMID: 22427530 DOI: 10.1152/ajplung.00180.2011]
  - 64 **Hall SR**, Tsouyi K, Ith B, Padera RF, Lederer JA, Wang Z, Liu X, Perrella MA. Mesenchymal stromal cells improve survival during sepsis in the absence of heme oxygenase-1: the importance of neutrophils. *Stem Cells* 2013; **31**: 397-407 [PMID: 23132816 DOI: 10.1002/stem.1270]
  - 65 **Elman JS**, Li M, Wang F, Gimble JM, Parekkadan B. A comparison of adipose and bone marrow-derived mesenchymal stromal cell secreted factors in the treatment of systemic inflammation. *J Inflamm (Lond)* 2014; **11**: 1 [PMID: 24397734 DOI: 10.1186/1476-9255-11-1]
  - 66 **Luo CJ**, Zhang FJ, Zhang L, Geng YQ, Li QG, Hong Q, Fu B, Zhu F, Cui SY, Feng Z, Sun XF, Chen XM. Mesenchymal stem cells ameliorate sepsis-associated acute kidney injury in mice. *Shock* 2014; **41**: 123-129 [PMID: 24169208 DOI: 10.1097/SHK.0000000000000080]
  - 67 **Sepúlveda JC**, Tomé M, Fernández ME, Delgado M, Campisi J, Bernad A, González MA. Cell senescence abrogates the therapeutic potential of human mesenchymal stem cells in the lethal endotoxemia model. *Stem Cells* 2014; **32**: 1865-1877 [PMID: 24496748 DOI: 10.1002/stem.1654]
  - 68 **Zhao X**, Liu D, Gong W, Zhao G, Liu L, Yang L, Hou Y. The toll-like receptor 3 ligand, poly(I: C), improves immunosuppressive function and therapeutic effect of mesenchymal stem cells on sepsis via inhibiting MiR-143. *Stem Cells* 2014; **32**: 521-533 [PMID: 24105952 DOI: 10.1002/stem.1543]
  - 69 **Chang CL**, Leu S, Sung HC, Zhen YY, Cho CL, Chen A, Tsai TH, Chung SY, Chai HT, Sun CK, Yen CH, Yip HK. Impact of apoptotic adipose-derived mesenchymal stem cells on attenuating organ damage and reducing mortality in rat sepsis syndrome induced by cecal puncture and ligation. *J Transl Med* 2012; **10**: 244 [PMID: 23217183 DOI: 10.1186/1479-5876-10-244]
  - 70 **Krasnodembkaya A**, Song Y, Fang X, Gupta N, Serikov V, Lee JW, Matthay MA. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 2010; **28**: 2229-2238 [PMID: 20945332 DOI: 10.1002/stem.544]
  - 71 **Manukyan MC**, Weil BR, Wang Y, Abarbanell AM, Herrmann JL, Poynter JA, Brewster BD, Meldrum DR. Female stem cells are superior to males in preserving myocardial function following endotoxemia. *Am J Physiol Regul Integr Comp Physiol* 2011; **300**: R1506-R1514 [PMID: 21451141 DOI: 10.1152/ajpregu.00518.2010]
  - 72 **Weil BR**, Manukyan MC, Herrmann JL, Wang Y, Abarbanell AM, Poynter JA, Meldrum DR. Mesenchymal stem cells attenuate myocardial functional depression and reduce systemic and myocardial inflammation during endotoxemia. *Surgery* 2010; **148**: 444-452 [PMID: 20434747 DOI: 10.1016/j.surg.2010.03.010]
  - 73 **Yagi H**, Soto-Gutierrez A, Kitagawa Y, Tilles AW, Tompkins RG, Yarmush ML. Bone marrow mesenchymal stromal cells attenuate organ injury induced by LPS and burn. *Cell Transplant* 2010; **19**: 823-830 [PMID: 20573305 DOI: 10.3727/096368910X508942]
  - 74 **Weil BR**, Manukyan MC, Herrmann JL, Abarbanell AM, Poynter JA, Wang Y, Meldrum DR. The immunomodulatory properties of mesenchymal stem cells: implications for surgical disease. *J Surg Res* 2011; **167**: 78-86 [PMID: 20869073 DOI: 10.1016/j.jss.2010.07.019]
  - 75 **Shin S**, Kim Y, Jeong S, Hong S, Kim I, Lee W, Choi S. The therapeutic effect of human adult stem cells derived from adipose tissue in endotoxemic rat model. *Int J Med Sci* 2013; **10**: 8-18 [PMID: 23289000 DOI: 10.7150/ijms.5385]
  - 76 **Yagi H**, Soto-Gutierrez A, Navarro-Alvarez N, Nahmias Y, Goldwasser Y, Kitagawa Y, Tilles AW, Tompkins RG, Parekkadan B, Yarmush ML. Reactive bone marrow stromal cells attenuate



systemic inflammation via sTNFR1. *Mol Ther* 2010; **18**: 1857-1864 [PMID: 20664529 DOI: 10.1038/mt.2010.155]

- 77 **Anderson P**, Souza-Moreira L, Morell M, Caro M, O'Valle F, Gonzalez-Rey E, Delgado M. Adipose-derived mesenchymal stromal cells induce immunomodulatory macrophages which protect from experimental colitis and sepsis. *Gut* 2013; **62**: 1131-1141

[PMID: 22637701 DOI: 10.1136/gutjnl-2012-302152]

- 78 **dos Santos CC**, Murthy S, Hu P, Shan Y, Haitsma JJ, Mei SH, Stewart DJ, Liles WC. Network analysis of transcriptional responses induced by mesenchymal stem cell treatment of experimental sepsis. *Am J Pathol* 2012; **181**: 1681-1692 [PMID: 23083833 DOI: 10.1016/j.ajpath.2012.08.009]

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## Transplantation of stem cell-derived astrocytes for the treatment of amyotrophic lateral sclerosis and spinal cord injury

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### Abstract

Neglected for years, astrocytes are now recognized to fulfill and support many, if not all, homeostatic functions

of the healthy central nervous system (CNS). During neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and spinal cord injury (SCI), astrocytes in the vicinity of degenerating areas undergo both morphological and functional changes that might compromise their intrinsic properties. Evidence from human and animal studies show that deficient astrocyte functions or loss-of-astrocytes largely contribute to increased susceptibility to cell death for neurons, oligodendrocytes and axons during ALS and SCI disease progression. Despite exciting advances in experimental CNS repair, most of current approaches that are translated into clinical trials focus on the replacement or support of spinal neurons through stem cell transplantation, while none focus on the specific replacement of astroglial populations. Knowing the important functions carried out by astrocytes in the CNS, astrocyte replacement-based therapies might be a promising approach to alleviate overall astrocyte dysfunction, deliver neurotrophic support to degenerating spinal tissue and stimulate endogenous CNS repair abilities. Enclosed in this review, we gathered experimental evidence that argue in favor of astrocyte transplantation during ALS and SCI. Based on their intrinsic properties and according to the cell type transplanted, astrocyte precursors or stem cell-derived astrocytes promote axonal growth, support mechanisms and cells involved in myelination, are able to modulate the host immune response, deliver neurotrophic factors and provide protective molecules against oxidative or excitotoxic insults, amongst many possible benefits. Embryonic or adult stem cells can even be genetically engineered in order to deliver missing gene products and therefore maximize the chance of neuroprotection and functional recovery. However, before broad clinical translation, further preclinical data on safety, reliability and therapeutic efficiency should be collected. Although several technical challenges need to be overcome, we discuss the major hurdles that have already been met or solved by targeting the astrocyte population



in experimental ALS and SCI models and we discuss avenues for future directions based on latest molecular findings regarding astrocyte biology.

**Key words:** Neuroprotection; Stem cell; Cell therapy; Astrocyte; Transplantation; Amyotrophic lateral sclerosis; Spinal cord injury

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**Core tip:** Amyotrophic lateral sclerosis (ALS) and spinal cord injury (SCI) result in incurable neurological dysfunction due to loss of spinal motor neurons and axonal degeneration, amongst other mechanisms. Astrocytes are increasingly recognized as being necessary for neuroprotection and regeneration in the central nervous system as they promote axonal growth and deliver essential neurotrophic factors under both physiological and pathophysiological conditions. Given the central role played by astrocytes, we gathered convincing results from ALS and SCI literature that argue in favor of stem cell-based astrocyte replacement therapies and stress the scientific community to investigate more deeply the molecular understanding of astrocyte biology.

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## MULTIPLE FACETS OF ASTROCYTES IN THE CENTRAL NERVOUS SYSTEM

### Astrocyte functions

Astrocytes are the most abundant cells in the central nervous system (CNS), outnumbering neuronal cells by several fold in some CNS regions. They have long been relegated to a secondary position, behind neurons or oligodendrocytes, as many thought that astrocytes were barely by-standers of the CNS. Accounting for a large fraction of the brain volume, their particular shape and tissue distribution are known to elaborate an extensive network of fine interconnected processes. Their star-shaped morphology projecting long branched processes provides a large coverage of CNS structures and the ensheathment of the brain or spinal cord in the pia matter. They draw the whole brain microanatomy by secreting astrocyte-derived extracellular matrix proteins (*e.g.*, chondroitin sulfate proteoglycans, hyaluronan, tenascin proteins family, thrombospondin), thereby providing structural support for nervous system cells. Beside their structural role, astrocytes are recognized to fulfill and support many, if

not all, functions of the healthy CNS<sup>[1]</sup>. Astrocyte end-feet cover more than 90% of the CNS vasculature and come in contact with endothelial cells. Expressing key glucose transporter-1, astrocytes convey glucose from blood vessels to nervous system cells, most of them being devoid of direct access to this high-end source of energy. Hence, astrocytes synthesize *via* specific metabolic pathways glycogen and lactate, main energy fuels for neurons or distant synapses. Through humoral factors released at the perivascular space, astrocytes control local cerebral blood flow and blood-brain barrier (BBB) integrity. Transforming growth factor-beta, glial-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2) and angiopoietin 1 (binding the endothelium-specific receptor TIE2), all secreted at the vascular end-feet, act on endothelial cells in order to induce or maintain an operational BBB<sup>[2,3]</sup>. Astrocytes-released growth factors [*e.g.*, brain-derived neurotrophic factor, BDNF; ciliary neurotrophic factor (CNTF)] exert beneficial effects far beyond the perivascular space and act as neurotrophic factors on all CNS cells<sup>[4,5]</sup>. The perivascular end-feet of astrocytes show other specialized features characteristic of this location, including a high density of orthogonal arrays containing the water channel aquaporin 4 (AQP4) and the Kir4.1 K<sup>+</sup> channel<sup>[6,7]</sup>. Those proteins closely interact with endothelial basal lamina and regulate ions/water fluxes through the BBB<sup>[7-10]</sup>. Astrocytes finely tune the dynamics of cerebral blood flow in order to increase the availability of oxygen and glucose locally to highly active brain areas; this process is mediated via secreted vasoactive compounds (*i.e.*, endothelin-1, arachidonic acid, prostaglandins, nitric oxide) and intercellular Ca<sup>2+</sup> signaling<sup>[11-13]</sup>. As a member of the tripartite synapse, astrocytes are responsible for proper neurotransmission. Astrocyte end-feet express Kir4.1 channel, which is responsible for the rapid uptake of K<sup>+</sup> released in the interstitial CNS fluid and thus for spatial extracellular potassium siphoning *needed* during high-frequency firing of neurons<sup>[14-16]</sup>. Astrocytes also express membrane-bound transporters and receptors involved in the re-uptake and the response to neurotransmitters (*i.e.*, glutamate, GABA, glycine), whose synaptic concentrations must be tightly controlled in the synaptic cleft. In the case of glutamate homeostasis, two major CNS glutamate transporters, GLT1/EAAT2 and GLAST/EAAT1, are expressed almost exclusively by astrocytes in the adult mammals. GLT1 accounts for upward of 90% of glutamate uptake in most CNS regions<sup>[17]</sup>. By regulating extracellular glutamate homeostasis, GLT1 assures proper synaptic function and prevents excitotoxic insult to susceptible neurons, axons and oligodendrocytes. In addition, astrocytes form a gap junction-coupled network of very narrow overlapping domains throughout the CNS<sup>[18,19]</sup>. Each domain covers a CNS area encompassing from 20000 neuronal synapses in rodents up to 2000000 in humans and connects neighboring astrocytes by



junctional complexes made of connexin (Cx) family proteins. On a functional point-of-view, the astrocyte syncytium supports ATP-mediated long-distance propagation of  $\text{Ca}^{2+}$  waves<sup>[20,21]</sup>, which is involved in the control of local cerebral blood flow and the fine tuning of electrical neuronal activity<sup>[22]</sup>. Cx channels allow the intercellular passage of monovalent ions ( $\text{Na}^+$ ,  $\text{K}^+$ ) or small molecules up to 1.5 kDa (e.g., ATP, glutamate, d-serine), contributing to signaling, metabolic cooperation and ionic spatial buffering<sup>[23,24]</sup>. Astrocytes express Cx26, Cx30 and Cx43 that interact with homologous connexins in astrocyte-to-astrocyte (A/A) gap junctions and that can also bind in a heterologous fashion Cx29, Cx32 and Cx47 on oligodendrocytes partners<sup>[24-26]</sup>. In mammals at least, the presence of these 6 connexins suggests a high level of complexity in coupling partners within the glial cell populations and reveals close cellular interactions between astrocytes and oligodendrocytes. Astrocyte-oligodendrocyte (A/O) coupling via connexins is necessary to sustain myelin formation and maintenance throughout life. In humans, mutations in Cx29, Cx32 and Cx47 genes lead to myelin formation abnormalities and are linked to Charcot-Marie-Tooth disease and several forms of leukodystrophies<sup>[27]</sup>. Beyond mutations targeting oligodendroglial connexins, the effect of loss-of-function of astrocyte connexins has been experimentally investigated using knockout mice. Double Cx30<sup>-/-</sup> and Cx43<sup>-/-</sup> knockout mice showed white matter pathology comprising vacuolated oligodendrocytes and intramyelinic edema<sup>[28]</sup>. Histopathological changes were accompanied by significant sensorimotor and cognitive deficits. Similar findings were shown when double-deleting Cx43 and Cx32 in mice<sup>[29]</sup>. All of these findings suggest an essential role of A/A and A/O coupling in maintaining overall CNS functions<sup>[30]</sup> and pave the way for developing integrated therapies targeting the astrocyte syncytium and its dysfunction(s) during neurodegenerative conditions.

### Wealth through diversity

A rapid look at the morphology of white matter astrocytes compared to gray matter astrocytes reveals the complexity and heterogeneity of this class of cells. Their different morphologies are most likely to be related with their wide range of functions, their neuroanatomical sites and the stem cells from which they derive (reviewed by<sup>[31]</sup>). During development, astrocytes mainly arise from radial glial cells located in the brain and spinal cord. During adulthood, astrocytes are still generated from differentiating progenitors in stem cell niches<sup>[32]</sup> or from dividing mature astrocytes in specific brain regions<sup>[33]</sup>. Historically, two classes of astrocytes were described: "type I" fibrous astrocytes mostly found in white matter tracts and "type II" protoplasmic astrocytes found in the grey matter<sup>[34,35]</sup>. Nowadays, the scientific community agrees that astrocyte complexity, in particular within

the protoplasmic subfamily, has increased along with phylogenetic evolution. As far as we know, this complexity culminates in the human CNS. Compared to rodents<sup>[19]</sup>, human astrocytes have a greater size, a more complex morphology, a large pleiomorphism<sup>[36]</sup> and are able to propagate calcium waves five times more rapidly<sup>[31,37,38]</sup>. Although mice represent a useful tool to study astrocytes and their *in vivo* functions through genetic manipulation, one limitation of rodent-to-human extrapolations is the wider diversity of human counterparts. For instance, primate brain contains two types of astrocytes not found in rodent brain: interlaminar and varicose astrocytes, whose functions are as of yet undetermined but seem to be related to the higher complexity of neuronal layers inside the human cortex<sup>[37]</sup>. Despite this heterogeneity, astrocytes share similarities such as the expression of several common proteins. Intermediate filament proteins are very abundant in the cytoplasmic compartment among all astrocytes types: glial fibrillary acidic protein (GFAP), vimentin, desmin and synemin. Recently, the cell surface marker CD44, the receptor of extracellular matrix hyaluronan, has been described to distinguish, more accurately than GFAP, protoplasmic astrocytes from fibrous-like astrocytes<sup>[36]</sup>. Resting astrocytes also display specific immunoreactivity for aldehyde dehydrogenase 1 family member L1, AQP4, S100, GLT1, GLAST and glutamine synthetase. Under pathological conditions affecting CNS [e.g., Alzheimer's disease, multiple sclerosis, stroke, amyotrophic lateral sclerosis (ALS), spinal cord injury (SCI)], astrocytes can switch to an activated phenotype. Activated astrocytes, which can in some case include a population of proliferating astrocytes, are commonly observed around focal CNS lesions, in areas of neuronal loss/damage, in demyelinating areas, during neuroinflammation or during CNS repair. Astrocyte hypertrophy and upregulation of GFAP, vimentin, nestin or S100 are hallmarks of activated astrocytes. For instance, new protein markers may also be expressed. Expression of S100A6, a calcium binding protein, is drastically increased in astrocytes from ALS spinal cord<sup>[39,40]</sup>, after traumatic CNS injury<sup>[41]</sup> or in Alzheimer brain tissue<sup>[42]</sup>. Given that ALS and SCI affect either diffuse or discrete areas, respectively within the CNS (*i.e.*, in ALS from motor cortex to bulbar respiratory nuclei to the lumbar spinal motor neurons; in SCI, both gray and white matter astrocytes are lost over a distance of several millimeters), therapeutically-targeting astrocytes would undoubtedly take into account their diversity of shape and function, with respect to their location. Our knowledge about astrocyte heterogeneity now allows for generating *in vitro* subpopulations of stem cell-derived astrocytes endowed with specific fates or defined functional abilities. These cells, isolated and expanded *in vitro*, can be further used in transplantation paradigms aiming at providing neuroprotection or replacing astrocyte-deficient



functions observed during neurodegenerative and traumatic diseases of the spinal cord such as ALS or SCI.

## SOURCES OF STEM CELL-DERIVED ASTROCYTES

### *Glial-restricted precursor cells*

Use of lineage-restricted progenitors as a source for deriving astrocytes has gained wide popularity in recent years due to their ability to generate specific glial types following transplantation. Glial-restricted progenitors are present in the embryonic spinal cord and through their ability to self-renew and differentiate give rise to oligodendrocytes and astrocyte populations. The first type of glial restricted progenitor that was originally characterized was the A2B5<sup>+</sup> O-2A progenitor cell. This was isolated from optic nerve of embryonic and post-natal rats and through *in vitro* differentiation was shown to be able to differentiate into oligodendrocytes and a particular type of astrocyte called the type-2 astrocyte<sup>[43-45]</sup>. More recently, a different type of progenitor cell was characterized that is able to differentiate into oligodendrocytes and two different populations of astrocytes. This cell type was isolated from E13.5 rat spinal cord and is also positive for the A2B5 antigen. *In vitro* conditions that supported their above mentioned differentiation included culture medium supplemented with fetal calf serum or platelet-derived growth factor (PDGF) and T3 thyroid hormone. Clonal analysis confirmed that these individual GRPs are tripotent. Furthermore, these cells are capable of extensive self-renewal in the presence of PDGF and FGF2. These GRPs differed from the original A2B5<sup>+</sup> O-2A progenitor cell in several aspects such as absence of mitogen requirements, responsiveness to PDGF and morphologies<sup>[46]</sup>.

### *Neural stem cells or radial glia*

Using cell genesis and fate specifying processes, neural tube-derived neural stem cells give rise to 10<sup>11</sup> neurons and at least 10<sup>12</sup> glial cells of many different phenotypes in the adult brain<sup>[47-49]</sup>. Neural stem cells (NSCs) are an attractive source for generating astroglial cells through their multipotency and high potential of self-renewal. NSCs represent an endogenous population existing during brain development and to a lesser extent in adult brain niches. In adult CNS, they are found around the sub-ventricular zone, the sub-granular layer of the hippocampus and spinal ependymal canal. Their phenotype is characterized by an astrocyte-like morphology and GFAP expression<sup>[50,51]</sup> together with other markers of an undifferentiated state (e.g., nestin, SOX2). The most common sources for NSC isolation include embryonic brain cortex and adult sub-ventricular zones. One of the basic features of NSCs is that they can easily divide *in vitro* either as

spherical aggregates called “neurospheres” or adherent layers of cells when supplemented with epidermal growth factor and fibroblast growth factor 2. Culture conditions involving growth factor withdrawal and serum exposure elicit a differentiation program into either neurons, oligodendrocytes or GFAP<sup>+</sup> astrocytes, with various yields<sup>[52-54]</sup>. Transplantation of NSCs into the CNS results in the generation of multiple cell types, including astrocytes<sup>[53]</sup> and oligodendrocytes<sup>[55]</sup>. Another source of astrocytes in the adult CNS is radial glial cells<sup>[56,57]</sup>. Several lines of evidence support the existence of a small pool of such cells expressing classic radial glial markers (*i.e.*, NG2, RC1, 3CB2, brain lipid-binding protein)<sup>[58]</sup> and located in limited regions of ongoing neurogenesis in the adult CNS<sup>[59-61]</sup>.

### *Embryonic or Induced-pluripotent stem cells*

Embryonic stem cells (ESCs) and induced-pluripotent stem cells (iPSCs) represent an ideal pluripotent source for deriving human astrocytes. Even though early reports demonstrated differentiation of astrocytes from human iPSCs or ESCs, there was a great diversity in terms of reported methods and the total yields of neural cells were not high. Furthermore, astrocytes generated by these protocols were not defined in terms of their subtype identity. On the other hand, specific protocols to generate astrocytes with regional or subtype identity were not reported<sup>[62-68]</sup>. This gap was fulfilled by a recently reported protocol, which allows directed differentiation of astrocytes *via* a prior differentiation of human stem cell derived neuro-epithelial cells into glial progenitor cells by repression of neurogenesis. Astrocytes generated by this protocol were characterized by specific gene expression profiles, neurotransmitter and ionic uptake, support of neuronal maturation, and importantly expression of specific sets of homeodomain transcription factors that specify functional identity<sup>[69]</sup>. On the basis of this, another protocol was recently reported for an efficient generation of immature astrocytes from human pluripotent stem cells (hPSCs)<sup>[70]</sup>. The protocol consists of three steps. In the first stage, hPSCs were differentiated into neuroepithelial cells either by co-culturing with mouse embryonic fibroblasts or by differentiation in feeder free conditions. These appeared in the form of columnar epithelia that organized into neural tube-like rosettes at about day 10-15 of differentiation. Addition of morphogens at days 8 to 15 allows differentiation of specific astrocyte subtypes. In the second stage, floating neural progenitor aggregates were expanded with epidermal growth factor, FGF2 and mitogenic factors. Neuronal differentiation was repressed and potential for gliogenic progenitor was enhanced by weekly mechanical trituration. By around 90 d, most of the progenitors expressed markers for astrocyte progenitors or astrocytes such as CD44 and S100. Thus at this stage, they are referred to as “astrospheres”. In the third and



final stage, astroglial progenitors were differentiated into functional astrocytes by removal of mitogens. Addition of CNTF for 6 d stimulated gliogenic gene expression. If neuroepithelial cells were patterned in stage 1, terminally differentiated astrocytes expressed specific markers such as Hoxb4 for posterior identity or Nkx2.1 for ventral identity.

#### ***In vivo or in vitro transdifferentiation***

Some cases of *in vivo* transdifferentiation into astrocytes have been described in the literature following transplantation of bone marrow-derived stem cells or mesenchymal stem cells into rodent CNS. It is not yet clear whether transdifferentiation normally occurs in healthy CNS and, if so, whether it requires a de-differentiation step. Several criteria must be filled to truly establish transdifferentiation into astrocytes: original cells must lose their committed-lineage differentiation markers; they have to adopt a astrocyte-like morphology and express astrocyte markers (*e.g.*, GFAP, S100). Several reports showed that GFP reporter-expressing bone marrow stem cells transplanted into adult rodent CNS gave rise to GFP<sup>+</sup> GFAP<sup>+</sup> astrocytes<sup>[71-73]</sup>. Similarly, mesenchymal stem cells (MSCs) isolated from bone marrow, when grafted into neonatal mouse brain, have been shown to migrate extensively and differentiate into olfactory bulb granule cells and astrocytes<sup>[74]</sup>. In an original experimental approach, Boucherie and colleagues intrathecally grafted MSCs in ALS-affected rodent spinal cord and observed that MSCs were able to extensively migrate while differentiating mostly into astrocytes at the sites of neurodegeneration<sup>[75]</sup>. In recent years, *in vitro* transdifferentiation, starting from single skin fibroblasts, has also made possible the modeling of CNS diseases in a dish, bypassing invasive neurosurgical procedures to get human diseased tissue. Recent cell reprogramming protocols describe the direct conversion of human fibroblasts to "induced" neural stem cells, able to further differentiate into the three CNS lineages (neurons, astrocytes and oligodendrocytes) following appropriate culture conditions. Artificial and temporal expression of specific transcription factors such as Oct-3/4, Sox2, Klf4, Brn4 and c-myc seems to govern the cell conversion towards a neural fate<sup>[76-78]</sup>. Molecular pathways and key transcription factors making transdifferentiation possible are currently under investigation for different cell types<sup>[79]</sup>.

## **RATIONALE FOR ASTROCYTE REPLACEMENT IN ALS AND SCI**

#### ***What is amyotrophic lateral sclerosis?***

Amyotrophic lateral sclerosis (ALS) is a common adult-onset neurodegenerative disease of the motor system, with a prevalence of 2-5/100000 people. It is characterized by a rapidly progressing neurode-

generation selectively affecting cortical, brainstem and spinal motor neurons. ALS remains an incurable disease, leading to fatal respiratory failure usually within 5 years following diagnosis<sup>[80]</sup>. Pathophysiology of ALS is poorly understood and likely multifactorial. Proposed starting points for this complex disease targeting the motor neuron population include mitochondrial dysfunction, intracellular protein aggregation, disturbances of RNA metabolism, extracellular toxic environment, impairment at the level of axonal transport and at the neuromuscular synapse (reviewed by<sup>[81]</sup>), together with extrinsic events: blood-brain barrier breakdown, glial cell reaction/dysfunction and neuroinflammation<sup>[82-88]</sup>. It is known that dying motor neurons influence surrounding cells, of which astrocytes are most commonly investigated. Astrocytes shift from an anti-inflammatory and neuroprotective role to one that is pro-inflammatory and neurotoxic, thus adding to complexity of this fatal cascade<sup>[80]</sup>. In 5%-10% of patients, ALS is inherited (familial ALS). In those cases, every fifth patient carries a detected mutation in superoxide dismutase 1 (SOD1), which is at present the most reliable and most widely used genetic animal model for ALS<sup>[89]</sup>. Among others, the most commonly described mutations include genes encoding the DNA/RNA-binding proteins FUS, TDP43<sup>[90]</sup>, the ubiquitin-like protein Ubiquilin 2<sup>[91]</sup> and optineurin<sup>[92]</sup>. On the other hand, an intronic hexanucleotide repeat in the C9ORF72 gene was discovered in both familial and sporadic cases of ALS<sup>[93]</sup>.

#### ***What are spinal cord injuries?***

SCI are devastating and diverse set of conditions that result from damage to spinal cord grey matter and white matter, as well as corresponding spinal nerves. The National Statistics for SCI estimates approximately 12000 new cases each year in the United States alone, most of which result from preventable causes (*e.g.*, motor vehicle accidents, falls, sports or violence). Some common outcomes of SCI include dysfunctions in the musculoskeletal, respiratory, uro-genital and gastrointestinal systems. The diversity of this condition results from differences in the location, type and severity of trauma, as well as on the consequent types and degree of functional impairment. Despite this diversity, all forms of SCI are linked to specific phenotypic changes in populations of spinal cord astrocytes. Some examples of these changes are: (1) acquisition of specific protective functions such as glial scar formation to constrain the secondary expansion of the lesion; (2) loss of certain crucial homeostatic functions such as the astrocyte glutamate transporter system that are key to normal CNS physiology; and (3) gain of toxic functions such as the generation of pro-inflammatory signaling molecules that contribute to degeneration, neuronal hyperexcitability and other detrimental effects. The response of astrocytes is a graded response such that there is diversity in these changes that vary with the type and severity of trauma and with proximity



to the lesion. Furthermore, even within a population of astrocytes within the spinal cord, the response of individual astrocytes to the same injury can vary, likely reflecting the normal heterogeneity amongst astrocytes that is now becoming increasingly appreciated.

### ***Defective astrocyte glutamate handling: one rationale among others***

ALS and SCI are both spinal cord disorders involving all CNS cells: neurons, oligodendrocytes, microglial cells, endothelial cells and astrocytes. In both cases, there is significant motor, as well as sensory, dysfunction, although sensory involvement is still a matter of debate in ALS. To some extent, they share histopathological features in the spinal cord tissue: death of spinal motor neurons, axonal damage in white matter tracts, anterograde axonal degeneration, BBB dysfunction, impaired astrocyte function and neuroinflammation. In the case where neurodegeneration reaches the cervical level of the spinal cord, the loss of phrenic motor neurons that control contraction of the diaphragm leads to severe respiratory deficits and represents a life-threatening condition. ALS, historically characterized by the loss of upper and lower motor neurons, is more and more recognized as endowed with a non-cell-autonomous component, in which microglia and astrocytes act as significant contributors to overall spinal cord dysfunction. It has been shown that microglia carrying ALS-linked mutant SOD1 drive ALS disease progression by fostering neuroinflammation and a toxic environment for spinal motor neurons<sup>[81,94]</sup>. With respect to astrocytes in ALS, independent groups demonstrated that astrocytes bearing mutant SOD1 are key determinants of disease progression<sup>[95-97]</sup>. In the absence of any pathology, normal astrocytes uptake glutamate, released at the synaptic cleft, through their glutamate transporters (GLT1/EAAT2 and GLAST/EAAT1). Furthermore, normal astrocytes protect motor neurons from glutamate excitotoxicity by stimulating the neuronal expression of AMPA-GluR2, which is less permeable to calcium making motor neurons less sensitive to excitotoxicity<sup>[98]</sup>. During ALS disease course, it is known from human and animal model samples that astrocyte-based glutamate uptake is compromised due to an early loss of astrocyte GLT1. Drastic GLT1 downregulation is most likely to be linked with deleterious extrasynaptic glutamate accumulation, which gave rise to the theory of glutamate-induced excitotoxic motor neuron death and to the unique FDA-approved drug that slows ALS: Riluzole, based on its anti-glutamatergic action.

Following SCI, the entire architecture of the spinal cord is disrupted at the lesion site; the trauma produces both immediate and delayed cell death affecting all nervous system populations: neurons, astrocytes and oligodendrocytes. Shortly after injury, it has been demonstrated that glutamate starts to accumulate in

the extracellular space<sup>[99]</sup>, and can persist for over a week, depending on injury severity<sup>[100]</sup>. Overload of CNS extracellular glutamate may cause excitotoxic damage to neurons, axons and oligodendrocytes *via* overactivation of both AMPA and NMDA receptors<sup>[101,102]</sup>. Failure of long-term extrasynaptic glutamate clearance is suspected to be one major cause of secondary cell loss following SCI. Noteworthy, we and others demonstrated that astrocyte GLT1 was chronically lost at the injury epicenter following SCI but also downregulated in spinal cord regions distant from the lesion core<sup>[103-106]</sup>. Furthermore, experimental data showed that the newly-generated astrocytes arising during the SCI repair phase lacked GLT1 expression, possibly compromising long-term astrocyte glutamate homeostasis<sup>[107]</sup>. Other consequences of astrocyte dysfunction or loss should be considered in ALS and SCI: shortage of neurotrophic factors important for neuronal survival, overwhelming of anti-oxidative defenses, lack of support to maintain endothelial BBB integrity, impaired water, ionic and metabolic transport, release of harmful proinflammatory cytokines and synthesis of glial scar-related ECM proteins that block axonal regrowth<sup>[1]</sup>. Regardless of whether astrocyte dysfunction is a cause of disease or a consequence of neuronal loss, altered physiology of pathologic astrocytes likely results in further susceptibility to CNS tissue loss, justifying the rationale for transplantation-based astrocyte replacement<sup>[108]</sup>. Numerous studies indicate that transplantation of stem cell-derived astrocytes has exhibited beneficial effects on histological/functional outcomes in ALS and SCI animal models. In this review, we summarize the data from recent literature regarding transplantation of stem cell-derived astrocytes targeting the replacement of deficient or lost astrocytes during ALS and SCI.

## **METHODOLOGICAL CONSIDERATIONS**

### ***Feasibility of astrocyte transplantation in healthy spinal cord***

Pilot studies were done on spinal GRPs isolated and characterized from the human aborted embryos<sup>[46,109,110]</sup>. Upon transplantation into neonatal and adult rodent brain, human GRPs survived, migrated and differentiated into (im)mature oligodendrocytes and into GFAP-expressing astrocytes<sup>[111]</sup>. These results were later confirmed by another group who analyzed the fate of GRPs harvested from alkaline phosphatase-expressing transgenic rats injected into intact and injured spinal cord. In the first set of experiments, GRPs survived at least 6 wk<sup>[112]</sup> and in the second experiment up to 15 mo post-transplantation<sup>[113]</sup>, demonstrating their long-term integration. Cells showed morphological maturation and differentiated along astrocyte and oligodendrocyte lineages and not into neuronal lineages. Interestingly, cells grafted into the intact spinal cord showed a particular tropism for white matter tracts and robust migration capacities



since they were found more than 15 mm away from the injection sites<sup>[112,113]</sup>. Transplantation of GRP-derived astrocytes seems to be a safe procedure, at least experimentally, since no tumor formation or pronounced immune response were observed at the graft sites. Isolated human GRPs express *in vitro* functional glutamate transporters EAAT1, EAAT3 and EAAT4 but not GLT1/EAAT2<sup>[114]</sup>. According to the nature of *in vitro* pre-differentiation signals, GRPs do not give rise to homogenous astrocyte populations upon transplantation<sup>[115,116]</sup>. Following bone morphogenic protein 4 (BMP4) driven differentiation, GRPs start to express EAAT2/GLT1 together with AQP4, AKAP12, Cx43 as markers of mature astrocytes<sup>[116]</sup>. On the contrary, *in vitro* GRP exposure to CNTF give rise to mature astrocytes expressing FGF receptor-3 and several axon growth inhibitory proteoglycans, such as neurocan, brevican and phosphacan, suggestive of a phenotype of reactive astrocytes.

### Routes of administration

Considering the route of administration for a stem cell therapy product is a significant factor when targeting the CNS and the spinal cord in particular. Stem cell transplantation into the spinal cord was successfully reported, in terms of homing and long-term engraftment, using different cell populations and different route of administration. Intraparenchymal injections of MSCs and NSCs into the thoracolumbar region<sup>[117-123]</sup> and of GRPs into the cervical region<sup>[104,124]</sup> have been successfully reported in animal models of ALS. Although direct targeting of the spinal cord is technically challenging and an invasive application, this route promises to be the most versatile and accurate method of targeted CNS therapeutic delivery, and therefore has been chosen for clinical translation of stem cell-based therapies in ALS patients. As both ALS and SCI affect long segments of spinal cord (several millimeters long and sometimes independent regions within the cord), a need for multiple injections adds to the complexity of the procedure. Aware of this issue, neurosurgeons are developing new surgical techniques or tools (e.g., radially-branched cannulae) able to deliver therapeutic agents to large CNS areas in a single shot<sup>[125]</sup>. Other attempts to find the optimal delivery route used intranasal<sup>[126]</sup>, intrathecal<sup>[127-129]</sup>, intraperitoneal<sup>[130]</sup>, intramuscular<sup>[131]</sup>, intravenous<sup>[132-135]</sup> and intra-bone marrow cell<sup>[136]</sup> transplantation. Major shortcomings are poor CNS homing of the therapeutic product compared to the initial dose given (dilution of cells in unwanted organs/sites) and adverse effects such as blockade of intravenously-injected stem cells in lung capillaries or spleen (reviewed by<sup>[137]</sup>). In many cases, researchers reported beneficial effects of peripherally-delivered stem cells associated with improved histological and functional outcomes in ALS animal models. While various routes of stem cell

delivery have been investigated in ALS therapy (Table 1), experimental SCI pathology was mainly targeted by focal stem cell injections in and around the lesion core, regardless of the spinal level affected (Table 2). Although the most efficient delivery method is still a matter of debate, the dose of transplanted cells, the timing, the location, the type of cells for particular needs, and their migratory abilities are additional parameters to take into account in designing CNS stem cell-based therapies. Numerous preclinical studies using animal transplantation paradigms will still be required to assess biodistribution, viability, integration into host tissue, differentiation into functional cells, lack of tumorigenicity and safety of delivery before broad clinical application<sup>[138-140]</sup>.

## LESSONS FROM TRANSPLANTATION PARADIGMS IN ALS MODELS

Taking into account that neurons represent only a portion of the various nervous system cell types, it is not surprising that during onset and especially during progression of disease non-neuronal cells contribute significantly to neuronal dysfunction and death. For example, it has been shown that wild-type motor neurons in close proximity to mutant SOD1-containing non-neuronal cells became affected by pathological chain reactions specific for ALS and eventually die with many features of this disease<sup>[141]</sup>. In the same way, wild type non-neuronal cells extend survival of SOD1 mutant motor neurons<sup>[142]</sup>. It has been clearly shown that reduction of mutant SOD1 selectively in astrocytes results in a prolongation of disease duration, but has no effects on disease onset. At least a part of this process is modulated by dysfunction and decrease in levels of the primary astrocyte glutamate transporter, GLT1, in areas of motor neuron loss<sup>[143]</sup>. Taken together, these results suggest a particular role for astrocytes in later progression of disease<sup>[81,97]</sup>. Main studies that have assessed replacement of astrocytes based on transplantation in ALS animal models have been gathered in Table 1.

Transplantation of wild-type astrocytes or their precursors into CNS tissue affected by ALS represents a promising experimental approach. With the aim to rescue motor neurons responsible for breathing, which is the primary cause of death in human ALS, GRPs were transplanted into the cervical spinal cord of the SOD1<sup>G93A</sup> rat model. GRPs survived in diseased tissue (32.2% ± 4.6% of transplanted cells survived at least 80 d post-transplantation), differentiated efficiently into astrocytes, and reduced microgliosis in the cervical spinal cord. Most notably, GRPs extended survival and disease duration, attenuated motor neuron loss, and slowed declines in forelimb motor and respiratory function. Since GRPs that did not express the glutamate transporter GLT1 did not have similar effects on behavioral measures or



**Table 1** Transplantation-based astrocyte replacement in amyotrophic lateral sclerosis animal models

Animal	Type of cells	Delivery	Effect on disease	Main outcomes	Ref.
SOD1 <sup>G93A</sup> ALS rats (80 d-old)	GRPs from rat E13.5 spinal cord, wild-type and overexpressing GLT1	Injections into C4-C6 cervical spinal cord, 6 sites, bilateral $1.5 \times 10^5$ cells/site	Delayed decline in motor function and survival extension	Differentiation into functional astrocytes. Prevented motor neurons loss independently from growth factors secretion, sustained GLT1 levels, alleviated microgliosis	[104]
SOD1 <sup>G93A</sup> ALS mice (75 d-old)	Human neural precursors (hNPs) overexpressing BDNF, IGF-1, VEGF, NT-3, or GDNF	Injection in cisterna magna and cerebral ventricles	No effect on motor function or animal survival	Differentiation in GFAP <sup>+</sup> GLT1 <sup>+</sup> expressing and growth factors-secreting astrocytes. Prevented motor neurons loss	[147]
SOD1 <sup>G93A</sup> ALS rats (90 d-old)	Rat adult MSCs	Intrathecal delivery in lumbar cisterna magna, $1.95 \times 10^6$ cells	Preserved motor function and survival extension	Differentiation into astroglial cells. Decreased neuroinflammation	[75]
SOD1 <sup>G93A</sup> ALS mice (24-26 wk-old)	Human umbilical cord blood cells overexpressing VEGF and FGF2	Intravenous delivery, $1 \times 10^6$ cells	Not investigated	Differentiation in S100 <sup>+</sup> astrocytes	[146]
SOD1 <sup>G93A</sup> ALS rats (14-26 wk-old)	NSCs from rat E16 brain cortex	Intravenous delivery, $1 \times 10^7$ cells	Not investigated	Preferential homing to late symptomatic ALS brain and spinal cord. Differentiation into neurons and astrocytes	[135]
SOD1 <sup>G93A</sup> ALS mice (50-60 d-old)	hGRPs from fetal cadaver brain tissue (week 17-24 of gestational)	Injections into C4-C5 cervical spinal cord, 4 sites, bilateral $1.2 \times 10^5$ cells/site	No effect on histological or functional outcomes	Poor cell survival	[124]

ALS: Amyotrophic lateral sclerosis; BDNF: Brain-derived neurotrophic factor; C: Cervical; E: Embryonic; FGF2: Fibroblast growth factor 2; GDNF: Glial cell-derived neurotrophic factor; GFAP: Glial fibrillary acidic protein; GLT1: Glutamate transporter 1; GRP: Glial-restricted precursor; IGF-1: Insulin-like growth factor 1; MSC: Mesenchymal stem cell; NP: Neural precursor; NSC: Neural stem cell; NT-3: Neutrophin-3; SOD1: Superoxide dismutase 1; VEGF: Vascular endothelial growth factor.

animal survival, this highly suggested that glutamate-relevant pathways contribute to the cascade of events leading to cell death in this model and that the focal beneficial effects of GRP transplantation could be explained, at least in part, by increases in glutamate transporter expression<sup>[104]</sup>.

With the aim to translate astrocyte-based transplantation towards treatment of patients, human GRPs have been transplanted into SOD1<sup>G93A</sup> mice. At disease end-stage, 10% of initial hGRP transplants survived and distributed throughout of the cervical spinal cord and up to 80% of all human derived cells co-expressed the astrocyte marker GFAP. Unlike the results obtained with rodent-derived GRPs, this xenograft transplantation paradigm using human GRPs did not provide a phenotypic preservation of motor function<sup>[124]</sup>.

In another study, Boucherie and colleagues tested a different approach: they injected MSCs into the cerebrospinal fluid of symptomatic SOD1<sup>G93A</sup> rats. They successfully penetrated the CNS parenchyma and accumulated at sites of motor neuron degeneration. They differentiated into astrocytes and, most notably, decreased motor neuron loss in the lumbar spinal cord, preserving hindlimb motor function and extending the survival of SOD1<sup>G93A</sup> rats. This neuroprotection correlated with decreased inflammation, as shown by a decrease in the proliferation of microglial cells and reduced expression of inflammatory-related genes, COX-2 and NOX-2<sup>[75]</sup>. Recent publications confirmed that transplantation of stem cells indeed reduces neuroinflammation and suggests that alleviating

astrocytosis or microgliosis is an important parameter leading to both histological and functional improvement in ALS animal models<sup>[144,145]</sup>.

Another approach was tested by Mitrećić *et al.*<sup>[135]</sup>. They transplanted NSCs into the rat model of ALS using the intravascular route. It was clearly shown that in animals affected by disease cells did cross the blood-brain barrier and accumulated in the regions affected by disease (motor cortex, ventral horns of the spinal cord) more than in healthy non-diseased animals. In the diseased CNS tissue, although only 6% of the initial stem cell dose was found at 7 d post-infusion, the transplanted cells differentiated into neurons and astrocytes<sup>[135]</sup>. Transplantation of genetically-engineered cells is another strategy to provide additional support or growth factors to ALS-affected cells and could also unexpectedly influence (stem) cell differentiation. This is what happened in a study from Rizvanov *et al.*<sup>[146]</sup> who showed that wild-type human umbilical cord blood cells differentiated into endothelial and microglial lineages after transplantation into SOD1<sup>G93A</sup> mice, while the same cells genetically modified to overexpress vascular endothelial growth factor (VEGF) and FGF2 exhibited preferentially an astrocytic differentiation<sup>[146]</sup>. In the same vein, human neural precursors were also genetically engineered to express insulin-like growth factor-1, neurotrophin-3, BDNF, VEGF or GDNF. These cells were then transplanted into the spinal cord or brain of SOD1<sup>G93A</sup> mice where they migrated and differentiated into neurons, oligodendrocytes or GLT1-expressing astrocytes. Unfortunately, although cells of interest



Table 2 Transplantation-based astrocyte replacement in spinal cord injury animal models

Animal and type of SCI	Type of cells	Delivery	Effects on disease	Main outcomes	Ref.
Rat, aspiration of C3 fasciculus gracilis	E14 rat spinal cord astrocytes	Intraparenchymal graft at lesion site	Worsened hindlimb function compared to controls	Migration of grafted GFAP <sup>+</sup> astrocytes toward the nucleus gracilis of the host medulla	[184]
Rat, L3 hemisection	Neonatal rat cortical astrocytes	Intraparenchymal injection at lesion site, 2.5 × 10 <sup>5</sup> cells, in suspension or in gelfoam	Not investigated	Migration more than 4 mm away from the injection site, reduced glial scarring	[185]
Rat, photochemically-induced infarction of dorsal funiculus	Neonatal rat mixed glial cells (close to type-1 astrocytes) to type-2 astrocytes) CG4-mixed glial cells (differentiated into type-2 astrocytes)	Intraparenchymal injection at lesion site	Not investigated	Produced dense clusters of astrocytes surrounded by meningeal cells within the cyst Produced cells that filled the cyst with a loose network devoid of meningeal cell infiltration at the lesion Filled the cyst with a loose network and increased the density of blood vessels in the lesion core	[186]
Rat, T9/T10 contusion	GRPs from rat E13.5 spinal cord	Intraparenchymal injection at lesion site, 5 × 10 <sup>5</sup> cells	Not investigated	Differentiated into oligodendrocytes and astrocytes. Reducing glial scar and proteoglycans synthesis. Supported axonal regrowth in the lesion but not on long-distance	[155]
Rat, T8 dorsal hemisection	P3 rat neonatal cortical astrocytes (mainly type 1 astrocytes)	Intraparenchymal injection at lesion site, 2.5 × 10 <sup>5</sup> astrocytes in a collagen I scaffold	Modest temporary improvements of locomotor function	No migration of astroglial cells out of the implant. Significant increase in the number of ingrowing axonal fibres	[152]
Rat, T8/T9 contusion	Mixed NRPs and GRPs (ratio 1:3) from rat E13.5 spinal cord	Intraparenchymal injections at and around lesion site, 3 sites, 1 × 10 <sup>6</sup> cells	Improvement of bladder, sensory and motor functions	Differentiation into neurons and glia. Volume of spinal cord spared was increased and local lumbosacral circuitry was modified	[187]
Rat, T8 complete transection	Adult rat cortical astrocytes	Intraparenchymal injection below lesion site (T11), 1.5 × 10 <sup>5</sup> cells	Not investigated	Massive rostral migration (8 mm)	[188]
Rat, C1/2 or C3/4 dorsal hemisection	GDAs <sup>BM/IN</sup> from rat E13.5 spinal cord	Intraparenchymal injections at and around lesion site, 6 sites, 2-3 × 10 <sup>4</sup> cells/site	Functional locomotor recovery	Significant axonal regrowth, decreased synthesis of inhibitory proteoglycans, suppression of axotomized neurons atrophy	[156]
Rat, C1/2 or C3/4 dorsal hemisection	GDAs <sup>BM/IN</sup> from rat E13.5 spinal cord	Intraparenchymal injections at and around lesion site, 6 sites, 3 × 10 <sup>4</sup> cells/site	Same for GDAs <sup>BM/IN</sup> as in [156], GRPs and GDAs <sup>CNTF</sup> caused mechanical allodynia and thermal hyperalgesia	Same results for GDAs <sup>BM/IN</sup> as in [156], GRPs and GDAs <sup>CNTF</sup> failed to support axonal regrowth	[115]
Rat, C3/4 dorsal hemisection	GDAs <sup>BM/IN</sup> and GDAs <sup>CNTF</sup> from human embryonic spinal cord tissue (week 9 of gestation)	Intraparenchymal injections at and around lesion site, 6 sites, 3 × 10 <sup>4</sup> cells/site	No improvement of locomotor or sensory functions	GDAs <sup>BM/IN</sup> supported axonal regrowth, neuronal survival more efficiently than GDAs <sup>CNTF</sup>	[116]
Mouse, T9/10 contusion	Mouse iPSC-derived astrocytes	Intraparenchymal injection at lesion site, 1 × 10 <sup>5</sup> cells	No improvement of locomotor or sensory functions	No tumor formation. Long GFAP <sup>+</sup> processes from transplanted cells. No interaction with host cells.	[189]
Athymic rats, T10 contusion	hGRPs from fetal cadaver brain tissue (week 18-24 of gestational)	Intraparenchymal injections at and around lesion site, 3 sites, 1 × 10 <sup>6</sup> cells	No significant improvements in motor function recovery. hGRP grafts attenuated hyperactive bladder reflexes	Differentiation for 80% of grafted cells into GFAP <sup>+</sup> astrocytes	[159]
Athymic rat, C4/5 dorsal hemisection	GRPs, GDAs <sup>BM/IN</sup> and GDAs <sup>CNTF</sup> from human and rat embryonic tissue	Intraparenchymal injection at lesion site, 1 site, 6 × 10 <sup>5</sup> cells	Not investigated	In all 3 groups differentiation into astrocytes generating a permissive environment for axonal regrowth, but not out of the lesion	[157]
Rat, T9 contusion	GDAs <sup>BM/IN</sup> from rat E14 spinal cord and overexpressing D15A	Intraparenchymal injections around lesion site, 4 sites, 4 × 10 <sup>5</sup> cells	Improved locomotor function. No changes in neuropathic pain	Differentiation into GFAP <sup>+</sup> astrocytes not secreting CSFG and allowing robust axonal regeneration. Increased spared white matter and decreased injury size compared to controls	[181]
Athymic rat, C4/5 dorsal hemisection	GRPs, GDAs <sup>BM/IN</sup> and GDAs <sup>CNTF</sup> from fetal cadaver brain tissue (week 20-21 of gestation)	Intraparenchymal injection at lesion site, 1 site, 6 × 10 <sup>5</sup> cells	Not investigated	Differentiated astrocytes from all 3 groups generated a permissive environment for axonal regrowth	[160]
Rat, T8 contusion	GDAs <sup>BM/IN</sup> from rat E13.5 spinal cord	Intraparenchymal injection at and around lesion site, 12 sites, 1.5 × 10 <sup>4</sup> cells/site	Improved hindlimb motor function	Promoted axonal regrowth, reduced glial scarring, inhibited neuroinflammation	[190]

BM/IN: Bone morphogenic protein 4; CNTF: Ciliary neurotrophic factor; E: Embryonic; CSFG: Chondroitin sulfate proteoglycans; GDA: GRP-derived astrocyte; GFAP: Glial fibrillary acidic protein; GRP: Glial-restricted precursor; h: Human; iPSC: Induced-pluripotent stem cells; L: Lumbar; NRP: Neural-restricted precursor; P: Post-natal; SCI: Spinal cord injury; T: Thoracic.



were generated and produced substantial amount of growth factors, they were unable to modify ALS disease onset or progression<sup>[147]</sup>.

In the last few years, we have been witnessing translation of the basic research into clinical trials for ALS. Maybe too prematurely, clinical trials have been launched around the world, without consensus on the cell types used, the delivery methods, or targeted outcome measurements. The majority of the these studies have focused on safety measurements, and all of them have reported transplantation of stem cells as a safe procedure (reviewed by<sup>[148]</sup>). As we are approaching the stage when some of these trials are moving from phase 1/2 to phase 2/3, the field is eagerly awaiting detailed reports of the clinical benefits of these cellular interventions. Currently, there are approximately 15 ongoing clinical trials in the EU and United States. Most of them are focused on intraspinal delivery of fetal/mesenchymal/neural stem cells (United States, Italy, Spain) and some used intrathecal delivery of autologous bone marrow-derived cells; so far, none of them is specifically addressing the replacement of ALS-affected astrocytes.

## LESSONS FROM TRANSPLANTATION PARADIGMS IN SCI MODELS

Some of the major experimental studies addressing the replacement of astrocytes based on transplantation paradigm in SCI animal models are summarized in Table 2. Historical studies conducted in the laboratories of George Smith and Jerry Silver tested the therapeutic potential of rodent neonatal astrocytes by transplantation following CNS insults. In this paradigm, cerebral midline was lesioned causing severing of callosal axons. A nitrocellulose bridge was introduced at the site of injury. When conducted at postnatal day 8 or younger ages, no necrosis was observed within 24 h. Furthermore, glial cells migrated and integrated into the graft, which provided a substrate for axon growth. However, when conducted later, there was extensive tissue degeneration and the implant was covered with a scar like mixture of fibroblasts and astrocytes. This glial scar failed to promote axon regeneration. Interestingly, when this paradigm was modified such that glial cells from younger pups were grafted into lesions of the older pups, they retained their ability to promote axon outgrowth. These pioneering studies demonstrated an age dependent decrease in the ability of endogenous and transplanted astrocytes to promote axon regeneration<sup>[149]</sup>.

Successive *in vitro* studies conducted by these researchers confirmed age dependent changes in intrinsic properties and molecular basis for supporting axon growth in astrocytes. The rate and extent of chick, as well as rodent, axon outgrowth was consistently greater over the surface of immature compared to mature astrocytes. In terms of molecular properties,

NCAM and G4/L1 antibodies reduced the rate of outgrowth over immature but not mature astrocytes, whereas integrin B1 receptor antibody reduced outgrowth on immature, and to a lesser extent, over mature astrocytes<sup>[150]</sup>.

Furthermore, there are differences in the motile properties of the two cell populations such that following transplantation into adult brain immature astrocytes demonstrate extensive migration and interaction with host blood vessels, unlike mature astrocytes. This ability may be linked to their potential to limit glial scar formation and support axon outgrowth<sup>[151]</sup>. Transplantation of neonatal astrocytes at the site of thoracic SCI resulted in outgrowth of axons along the transplants, as well as some motor recovery<sup>[152]</sup>.

Even though these early studies produced promising results, because of the difficulty in harvesting these beneficial immature astrocytes from human tissues, further studies explored more practically relevant cell types such as multipotent NSCs as a viable source to generate transplantable astrocyte populations. In studies conducted by the Whittemore lab, NSCs isolated from E14 rat cortex were transplanted into intact and injured spinal cord, without prior differentiation. In the intact spinal cord, over a 2-mo period, a 50% of multipotent stem cells differentiated into GFAP positive astrocytes and a small percentage differentiated into oligodendrocytes and neurons. Following injury to the spinal cord, astrocyte differentiation dropped to 35% and neuronal differentiation was not observed at all<sup>[153]</sup>, demonstrating an effect of the micro-environment on the differentiation pattern of the transplanted stem cells. To overcome this, in a separate study, NSCs were transfected with Ngn-2, followed by transplantation into injured spinal cord. This resulted in a significant reduction in astrocyte differentiation and an increase in differentiation into oligodendrocytes and neurons. Furthermore, Ngn-2-NSC treated rats showed improved motor function. These studies also showed that transplantation of control NSCs enhanced neuropathic pain associated with SCI, whereas this unexpected side effect was not observed in Ngn-2-NSC transplanted rats<sup>[154]</sup>.

While NSCs provide the benefit of being able to give rise to neurons as well as glia, the use of GRPs harvested from fetal CNS has gained wide popularity in recent years for targeting astrocytes. Early studies documented extensive migration of transplanted GRPs in injured and intact spinal cord and differentiation into cells of the glial lineage, predominantly into astrocytes<sup>[112]</sup>. Transplantation of these cells into thoracic SCI reduced scar formation and expression of growth-inhibiting proteoglycans; however, no axon outgrowth was observed<sup>[155]</sup>. This obstacle was overcome by pre-differentiating GRPs into astrocytes (GDAs) by treatment with BMP-4. These GDAs were able to promote axon outgrowth when transplanted into lesioned spinal cord without causing neuropathic



pain<sup>[156]</sup>. These investigators also differentiated GRPs into astrocytes by treatment with a different estrogenic signaling molecule, CNTF. This type of GDA however failed to promote axon regeneration and promoted thermal hyperalgesia and mechanical allodynia following transplantation into lesioned spinal cord<sup>[115]</sup>. These studies highlighted that heterogeneous astrocyte populations may have distinct therapeutic potentials. Contrasting results from the Fischer lab indicated that, although heterogeneous, both BMP-4 and CNTF GDAs promoted axon outgrowth to a similar extent<sup>[157]</sup>. Although it is not possible to compare in detail the precursor populations used by the two labs, the properties of specific glial precursor populations, differences in the culture conditions, and different transplantation and tracing protocols may have led to these contrasting results. Advantages derived from the graft are manifold. For example, a recent report from the Proschel lab illustrated an underlying mechanism through which BMP4-GDAs promote axon regeneration involving secretion of periostin<sup>[158]</sup>.

While the above-mentioned studies involving rodent GRPs provided an understanding of their properties and therapeutic potential, further studies involving more clinically relevant human GRPs were designed based on these previous studies. hGRPs harvested from fetal human brain were pre-differentiated into astrocytes (hGDAs) by treatment with BMP-4 and transplanted into injured spinal cords of athymic rats. Graft expansion was seen over an 8 wk time period, and in both hGRP and hGDA transplant groups, cyst and glial scar formation was reduced. Although there was no significant motor recovery, lack of neuropathic pain and permissive properties of these cells make them promising therapeutic candidates after SCI<sup>[159]</sup>. Based on their rodent GRP work, the Fischer group generated GDAs by treatment with either CNTF or BMP-4 and showed that these cells have similar therapeutic potential in terms of promotion of axon generation<sup>[160]</sup>, whereas the Davies and Proschel groups again reported contrasting results<sup>[116]</sup>. These differences may be related to the age, region and technique of isolation of hGRPs, as well as differences in the culture conditions used by the two labs.

In recent years, the discovery of induced pluripotent stem cells (iPSC) cells has generated great excitement as large quantities of clinically relevant mature cell types can be generated from these pluripotent cells, without facing the ethical concerns presented by pluripotent embryonic stem cells (ESCs). However, this field is in its infancy, especially in the context of astrocytes. Recently, the Zhang laboratory developed a protocol for producing large quantities of regionally specific astrocytes from ES and iPSC cells<sup>[69]</sup>. A shortcoming of this study however is that it required 6 mo of culturing. Shorter duration protocols were subsequently reported, but these produced mainly populations of immature astrocytes<sup>[161,162]</sup>. Astrocyte maturation takes place in

two distinct phases and marker expression for these phases has been characterized, which led to the development of protocols that generate astrocytes with mature phenotypes. While the treatment of immature astrocytes with FGF1 or FGF2 promoted up-regulation of maturation markers such as glutamate transporters and downregulation of GFAP, treatment with tumor necrosis factor- pushed cells toward a reactive astrocyte phenotype. These cells can be used to model human pathological processes *in vitro* and *in vivo* as well as for therapeutic transplantation<sup>[163]</sup>. More recently the Zhang laboratory reported another shorter duration protocol for generating astrocytes from iPSCs by differentiation of these cells into neuroepithelial cells, followed by removal of mitogen and treatment with CNTF to induce differentiation into astrocytes<sup>[70]</sup>. Recently, the Maragakis lab conducted transplantation of iPSC- and ESC-derived immature astrocytes into rodent spinal cord. Although limited, the grafts showed long-term survival, with less than 5% of the transplanted cells surviving at 12 wk post-transplantation. Furthermore, graft derived astrocytes expressed markers of mature, quiescent astrocytes such as AQP4, EAAT1, EAAT2, as assessed by immunostaining analysis and gene expression profiling, suggesting that these cells are a promising resource for transplantation therapies<sup>[164]</sup>. Additional reports suggest that it is important to evaluate the *in vivo* safety of these cells, especially in terms of their tumor formation tendency, prior to their use for clinical transplantation<sup>[165,166]</sup>. Recent work from the Lepore lab, conducted as an extension of their work related to rodent GRPs, generated glial progenitors from human iPSCs, followed by further differentiation into astrocytes. Following transplantation into injured spinal cord, these cells showed long-term survival, no tumor formation, and efficient differentiation into GFAP expressing cells. These cells could even be engineered to express GLT1, suggesting that hiPSCs could be used as a safe source for transplantation therapies for targeted replacement of astrocytes in SCI and other CNS diseases (unpublished data).

Until recently, the SCI field primarily focused on the role of astrocytes with regard to scar formation and their inhibitory effects on axon re-growth. Recent studies however have elucidated their heterogeneity and the changes exhibited by these cells following SCI, thereby underscoring the need to preserve their normal physiological functions. Studies mentioned above, as well as other studies that are not detailed here, are indicative of the growing appreciation of cell transplantation therapies for therapeutically targeting astrocytes in SCI.

With a special focus on chronic SCI conditions, many clinical trials based on the transplantation of unmodified mesenchymal, neural or bone-marrow stem cells have been launched around the world<sup>[167-171]</sup>. Among phase 1 studies, most of them have focused on safety measurements and graft survival according to different routes of administration (mostly intrathecal



or focal intraparenchymal delivery). All of these studies have reported transplantation of stem cells as a risk-free procedure. To our best knowledge, none of the current human trials are specifically addressing the replacement of lost astrocytes following SCI.

## CONCLUDING REMARKS AND FUTURE DIRECTIONS

We must admit that, despite numerous preclinical animal studies demonstrating improvement in a variety of functional outcomes following astrocyte transplantation, the variability and the poor reproducibility of the results still hinder full clinical translation. The variability might be partly explained by the limited survival of transplanted cells in diseased spinal cord tissue. In published preclinical ALS and SCI studies, cell graft survival was found to be usually between 5% and 32% of the initial dose and was often assessed at early time points post-transplantation. Inter-laboratory discrepancies might also be due to the source or type of astrocytes (or precursors) used in the transplantation paradigms. Indeed, astrocytes, in rodents and even more in humans, are a broad class of CNS cells endowed with specific functions according to their location, their morphology, their degree of maturity and their activation state. Finally, lack of standardization, different type of cells, different animal species and single targeting strategies of multifactorial diseases may also be part of the failures. Therefore, future protocols should aim at standardizing the culture conditions, verifying the astrocyte population homogeneity, the cell purity and, above all, at enhancing cell survival after *in vivo* transplantation. Researchers are also encouraged to provide preclinical data about long-term follow-up of engrafted cells, including survival time, differentiation pattern and CNS integration several months post-transplantation.

During this last decade, detailed molecular dissection of astroglial populations has shown that astrocytes are active players in CNS homeostasis and far more than basic fibroblast-like cells of the CNS. Understanding the molecular mechanisms underlying the normal biology of astrocytes as well as their behavior during pathological conditions will provide us with undoubtedly novel astrocyte-specific therapeutic targets. Especially regarding ALS and SCI that share several common pathophysiology mechanisms, targeting astrocytes, whose dysfunction is now well established from animal and human studies, might be a promising therapeutic strategy. As far as we know, oxidative stress, neuroinflammation and glutamate excitotoxicity are all pathological events in which astrocytes are actively involved. Therapeutics that address each separately or all-in-one these specific pathological events are of great relevance to ALS and SCI.

Stem cell based therapy for ALS and SCI includes three main aims: (1) replacement of lost nervous cells for restoring homeostatic functions of the CNS;

(2) neuroprotection of unaffected surrounding tissue *i.e.*, by secretion of neurotrophic factors or anti-inflammatory molecules; and (3) enhancement of the endogenous repair process. Based on the biology and the known functions of astrocytes, here are a few examples of neuroprotection-based mechanisms that are under investigation and might deserve particular attention in future transplantation paradigms targeting astrocytes.

Increased oxidative stress has been implicated in the pathogenesis of ALS and SCI, leading to the testing of different antioxidants in affected patients. Several studies have demonstrated that astrocyte-secreted glutathione greatly contributes to neuroprotection against oxidative stress. One of the main regulators of glutathione production and release is the transcription factor Nrf2. ALS mice with astrocyte driven Nrf2 overexpression showed increased lifespan, better functional outcomes and lower glial reactivity in spinal cord tissue<sup>[172]</sup>. Accordingly, it would be interesting to investigate the effect of transplantation of Nrf2-overexpressing astrocytes. Stat3 is another transcription factor regulating GSH synthesis<sup>[173]</sup>, as well as a "master-switch" that mediates transition from quiescent to reactive astrocyte. Following SCI, transgenic mice ablated for Stat3 in the astroglial population showed less reactive astrocytes in the spinal cord, resulting in more widespread neuroinflammation, motor neuron loss and axonal damage and thus worsened motor deficits compared to wild-type counterparts. This study and others demonstrates that Stat3 is a key transcription factor controlling astrocyte reactivity during neurodegenerative processes, providing a potential target for intervention<sup>[174,175]</sup>.

Far from being science fiction, researchers can genetically modify (stem) cells to make them produce more growth factors or proteins of interest, as Trojan horses to deliver molecules to specific CNS locations. For instance, NSCs engineered to overexpress CNTF exert neuroprotection by *in situ* differentiation into supportive astrocytes in a mouse model of photoreceptor degeneration<sup>[176]</sup>. Similarly, NSCs overexpressing nerve growth factor showed beneficial effects on cognitive functions after CNS transplantation and astrocyte differentiation in a learning deficit mouse model<sup>[177]</sup>. Another example is illustrated in a recent report from Gowing<sup>[178]</sup>. This group engineered GDNF-secreting human neural progenitors that were able to survive up to 7.5 mo after intraspinal transplantation, differentiate mainly into astrocytes and still maintain locally high levels of GDNF. More specifically, intranigral transplantation of astrocytes transduced with a lentiviral vector expressing GDNF resulted in sustained local production of growth factor and provided neuroprotection in a rat model of Parkinson<sup>[179]</sup>. Lastly, genetically-modified astrocytes designed to secrete BDNF promoted retinal ganglion cell survival<sup>[180]</sup>, and transplantation of neurotrophin-releasing GRPs improved functional and histological outcomes after



SCI<sup>[181]</sup>. Growth factors released from transplanted cells act in a paracrine manner on surrounding host tissue but are likely to also act in an autocrine manner on grafted cells to promote survival and/or a specific differentiation pattern. All aforementioned examples should encourage us to investigate more deeply the therapeutic potential of grafted exogenous astrocytes or stem cell-derived astrocytes in neurodegenerative and traumatic CNS diseases, as well as to think about novel genetically-modified cells that specifically target pathogenic events occurring during ALS or SCI.

Inspired from encouraging results obtained following transplantation of GLT1-overexpressing GRPs in the rat model of ALS<sup>[104]</sup>, the Lepore lab is studying their applicability and evaluating their efficacy in clinically-relevant SCI models<sup>[182,183]</sup>. The rationale behind such an approach is based on the findings that, following SCI, astrocyte GLT1 expression and function are compromised, resulting in excitotoxicity-induced cell death during the delayed secondary injury phase<sup>[105]</sup>. Unpublished data from our group show that transplantation of astrocytes engineered to overexpress GLT1 can prevent excitotoxicity and protect respiratory phrenic motor neurons following cervical SCI. While unmodified transplants showed robust survival, they expressed relatively low levels of GLT1 in injured spinal cord. Excitingly, GLT1 overexpressing transplant-derived astrocytes continued to express high levels of GLT1 protein following transplantation into the injured spinal cord and promoted survival of these important phrenic motor neurons and preservation of diaphragm function (unpublished work).

Finally, although ALS and SCI share many similarities including the death of spinal motor neurons, it must be emphasized that important differences between two diseases exist. ALS is a progressive neurodegenerative condition starting with discrete and asymptomatic loss of motor neurons in remote specific regions of the CNS (upper and lower motor neurons), whose symptomatology becomes apparent at approximately 50% of motor neuron loss and fatally progresses when disease affects bulbar and/or spinal respiratory centers. Therefore, therapeutic strategies in ALS, once diagnosed, should focus on astrocyte-mediated neuroprotection that prevent further motor neuron loss, worsening of functional outcomes and delayed fatal respiratory failure. Unlike ALS, SCI is characterized by different temporal sequence of events. First, acute and sub-acute phases following the initial trauma in the first hours-by-days post-injury result in a hostile environment in the spinal cord around the lesion epicenter, including inflammation, excitotoxicity and oxidative stress that lead to more cell loss and thus extension of initial lesion. This narrow time window would be appropriate for interventional strategies targeting astrocytes that favor neuroprotection or compensate for lost astrocyte-specific functions. At later time points, during the chronic stage of SCI, characterized by the resolution of subacute events, glial scarring and reorganization of spinal tissue architecture, one can imagine designing astrocyte transplants to make

scar tissue permeable to axonal regrowth, to promote invasion by remyelinating oligodendroglial precursors, or to facilitate overall rewiring of ascending/descending tracts in the spinal cord.

## REFERENCES

- 1 **Sofroniew MV**, Vinters HV. Astrocytes: biology and pathology. *Acta Neuropathol* 2010; **119**: 7-35 [PMID: 20012068 DOI: 10.1007/s00401-009-0619-8]
- 2 **Abbott NJ**. Astrocyte-endothelial interactions and blood-brain barrier permeability. *J Anat* 2002; **200**: 629-638 [PMID: 12162730 DOI: 10.1046/j.1469-7580.2002.00064.x]
- 3 **Abbott NJ**, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. *Neurobiol Dis* 2010; **37**: 13-25 [PMID: 19664713 DOI: 10.1016/j.nbd.2009.07.030]
- 4 **Svendsen CN**. The amazing astrocyte. *Nature* 2002; **417**: 29-32 [PMID: 11986650 DOI: 10.1038/417029a]
- 5 **Chu T**, Zhou H, Li F, Wang T, Lu L, Feng S. Astrocyte transplantation for spinal cord injury: current status and perspective. *Brain Res Bull* 2014; **107**: 18-30 [PMID: 24878447 DOI: 10.1016/j.brainresbull.2014.05.003]
- 6 **Nagelhus EA**, Mathiisen TM, Ottersen OP. Aquaporin-4 in the central nervous system: cellular and subcellular distribution and coexpression with KIR4.1. *Neuroscience* 2004; **129**: 905-913 [PMID: 15561407 DOI: 10.1016/j.neuroscience.2004.08.053]
- 7 **Nielsen S**, Nagelhus EA, Amiry-Moghaddam M, Bourque C, Agre P, Ottersen OP. Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. *J Neurosci* 1997; **17**: 171-180 [PMID: 8987746]
- 8 **Haj-Yasein NN**, Vindedal GF, Eilert-Olsen M, Gundersen GA, Skare Ø, Laake P, Klungland A, Thorén AE, Burkhardt JM, Ottersen OP, Nagelhus EA. Glial-conditional deletion of aquaporin-4 (Aqp4) reduces blood-brain water uptake and confers barrier function on perivascular astrocyte endfeet. *Proc Natl Acad Sci USA* 2011; **108**: 17815-17820 [PMID: 21990350 DOI: 10.1073/pnas.1110655108]
- 9 **Nagelhus EA**, Ottersen OP. Physiological roles of aquaporin-4 in brain. *Physiol Rev* 2013; **93**: 1543-1562 [PMID: 24137016 DOI: 10.1152/physrev.00011.2013]
- 10 **Amiry-Moghaddam M**, Ottersen OP. The molecular basis of water transport in the brain. *Nat Rev Neurosci* 2003; **4**: 991-1001 [PMID: 14682361 DOI: 10.1038/nrn1252]
- 11 **Gordon GR**, Mulligan SJ, MacVicar BA. Astrocyte control of the cerebrovasculature. *Glia* 2007; **55**: 1214-1221 [PMID: 17659528 DOI: 10.1002/glia.20543]
- 12 **Iadecola C**, Nedergaard M. Glial regulation of the cerebral microvasculature. *Nat Neurosci* 2007; **10**: 1369-1376 [PMID: 17965657 DOI: 10.1038/nn2003]
- 13 **Koehler RC**, Roman RJ, Harder DR. Astrocytes and the regulation of cerebral blood flow. *Trends Neurosci* 2009; **32**: 160-169 [PMID: 19162338 DOI: 10.1016/j.tins.2008.11.005]
- 14 **Heuser K**, Eid T, Lauritzen F, Thoren AE, Vindedal GF, Taubøll E, Gjerstad L, Spencer DD, Ottersen OP, Nagelhus EA, de Lanerolle NC. Loss of perivascular Kir4.1 potassium channels in the sclerotic hippocampus of patients with mesial temporal lobe epilepsy. *J Neuropathol Exp Neurol* 2012; **71**: 814-825 [PMID: 22878665 DOI: 10.1097/NEN.0b013e318267b5af]
- 15 **Nagelhus EA**, Horio Y, Inanobe A, Fujita A, Haug FM, Nielsen S, Kurachi Y, Ottersen OP. Immunogold evidence suggests that coupling of K<sup>+</sup> siphoning and water transport in rat retinal Müller cells is mediated by a coenrichment of Kir4.1 and AQP4 in specific membrane domains. *Glia* 1999; **26**: 47-54 [PMID: 10088671 DOI: 10.1002(SICI)1098-1136(199903)26]
- 16 **Haj-Yasein NN**, Jensen V, Vindedal GF, Gundersen GA, Klungland A, Ottersen OP, Hvalby O, Nagelhus EA. Evidence that compromised K<sup>+</sup> spatial buffering contributes to the epileptogenic effect of mutations in the human Kir4.1 gene (KCNJ10). *Glia* 2011; **59**: 1635-1642 [PMID: 21748805 DOI: 10.1002/glia.21205]
- 17 **Maragakis NJ**, Rothstein JD. Glutamate transporters: animal



- models to neurologic disease. *Neurobiol Dis* 2004; **15**: 461-473 [PMID: 15056453 DOI: 10.1016/j.nbd.2003.12.007]
- 18 **Nedergaard M**, Ransom B, Goldman SA. New roles for astrocytes: redefining the functional architecture of the brain. *Trends Neurosci* 2003; **26**: 523-530 [PMID: 14522144 DOI: 10.1016/j.tins.2003.08.008]
  - 19 **Ogata K**, Kosaka T. Structural and quantitative analysis of astrocytes in the mouse hippocampus. *Neuroscience* 2002; **113**: 221-233 [PMID: 12123700 DOI: 10.1016/S0306-4522(02)00041-6]
  - 20 **Haas B**, Schipke CG, Peters O, Söhl G, Willecke K, Kettenmann H. Activity-dependent ATP-waves in the mouse neocortex are independent from astrocytic calcium waves. *Cereb Cortex* 2006; **16**: 237-246 [PMID: 15930372 DOI: 10.1093/cercor/bhi101]
  - 21 **Orthmann-Murphy JL**, Abrams CK, Scherer SS. Gap junctions couple astrocytes and oligodendrocytes. *J Mol Neurosci* 2008; **35**: 101-116 [PMID: 18236012 DOI: 10.1007/s12031-007-9027-5]
  - 22 **Anderson CM**, Nedergaard M. Astrocyte-mediated control of cerebral microcirculation. *Trends Neurosci* 2003; **26**: 340-344; author reply 340-344 [PMID: 12850427 DOI: 10.1016/S0166-2236(03)00141-3]
  - 23 **Simard M**, Arcuino G, Takano T, Liu QS, Nedergaard M. Signaling at the gliovascular interface. *J Neurosci* 2003; **23**: 9254-9262 [PMID: 14534260]
  - 24 **Nagy JI**, Ionescu AV, Lynn BD, Rash JE. Coupling of astrocyte connexins Cx26, Cx30, Cx43 to oligodendrocyte Cx29, Cx32, Cx47: Implications from normal and connexin32 knockout mice. *Glia* 2003; **44**: 205-218 [PMID: 14603462 DOI: 10.1002/glia.10278]
  - 25 **Magnotti LM**, Goodenough DA, Paul DL. Functional heterotypic interactions between astrocyte and oligodendrocyte connexins. *Glia* 2011; **59**: 26-34 [PMID: 21046554 DOI: 10.1002/glia.21073]
  - 26 **Orthmann-Murphy JL**, Freidin M, Fischer E, Scherer SS, Abrams CK. Two distinct heterotypic channels mediate gap junction coupling between astrocyte and oligodendrocyte connexins. *J Neurosci* 2007; **27**: 13949-13957 [PMID: 18094232 DOI: 10.1523/JNEUROSCI.3395-07.2007]
  - 27 **Nagy JI**, Ionescu AV, Lynn BD, Rash JE. Connexin29 and connexin32 at oligodendrocyte and astrocyte gap junctions and in myelin of the mouse central nervous system. *J Comp Neurol* 2003; **464**: 356-370 [PMID: 12900929 DOI: 10.1002/cne.10797]
  - 28 **Lutz SE**, Zhao Y, Gulino M, Lee SC, Raine CS, Brosnan CF. Deletion of astrocyte connexins 43 and 30 leads to a dysmyelinating phenotype and hippocampal CA1 vacuolation. *J Neurosci* 2009; **29**: 7743-7752 [PMID: 19535586 DOI: 10.1523/JNEUROSCI.0341-09.2009]
  - 29 **Magnotti LM**, Goodenough DA, Paul DL. Deletion of oligodendrocyte Cx32 and astrocyte Cx43 causes white matter vacuolation, astrocyte loss and early mortality. *Glia* 2011; **59**: 1064-1074 [PMID: 21538560 DOI: 10.1002/glia.21179]
  - 30 **Cotrina ML**, Nedergaard M. Brain connexins in demyelinating diseases: therapeutic potential of glial targets. *Brain Res* 2012; **1487**: 61-68 [PMID: 22789906 DOI: 10.1016/j.brainres.2012.07.003]
  - 31 **Oberheim NA**, Goldman SA, Nedergaard M. Heterogeneity of astrocytic form and function. *Methods Mol Biol* 2012; **814**: 23-45 [PMID: 22144298 DOI: 10.1007/978-1-61779-452-0\_3]
  - 32 **Molofsky AV**, Krenick R, Ullian EM, Tsai HH, Deneen B, Richardson WD, Barres BA, Rowitch DH. Astrocytes and disease: a neurodevelopmental perspective. *Genes Dev* 2012; **26**: 891-907 [PMID: 22549954 DOI: 10.1101/gad.188326.112]
  - 33 **Emsley JG**, Macklis JD. Astroglial heterogeneity closely reflects the neuronal-defined anatomy of the adult murine CNS. *Neuron Glia Biol* 2006; **2**: 175-186 [PMID: 17356684 DOI: 10.1017/S1740925X06000202]
  - 34 **Raff MC**, Abney ER, Cohen J, Lindsay R, Noble M. Two types of astrocytes in cultures of developing rat white matter: differences in morphology, surface gangliosides, and growth characteristics. *J Neurosci* 1983; **3**: 1289-1300 [PMID: 6343560]
  - 35 **Raff MC**, Miller RH, Noble M. A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* 1983; **303**: 390-396 [PMID: 6304520 DOI: 10.1038/303390a0]
  - 36 **Sosunov AA**, Wu X, Tsankova NM, Guilfoyle E, McKhann GM, Goldman JE. Phenotypic heterogeneity and plasticity of isocortical and hippocampal astrocytes in the human brain. *J Neurosci* 2014; **34**: 2285-2298 [PMID: 24501367 DOI: 10.1523/JNEUROSCI.4037-13.2014]
  - 37 **Oberheim NA**, Takano T, Han X, He W, Lin JH, Wang F, Xu Q, Wyatt JD, Pilcher W, Ojemann JG, Ransom BR, Goldman SA, Nedergaard M. Uniquely hominid features of adult human astrocytes. *J Neurosci* 2009; **29**: 3276-3287 [PMID: 19279265 DOI: 10.1523/JNEUROSCI.4707-08.2009]
  - 38 **Oberheim NA**, Wang X, Goldman S, Nedergaard M. Astrocytic complexity distinguishes the human brain. *Trends Neurosci* 2006; **29**: 547-553 [PMID: 16938356 DOI: 10.1016/j.tins.2006.08.004]
  - 39 **Hoyaux D**, Alao J, Fuchs J, Kiss R, Keller B, Heizmann CW, Pochet R, Frermann D. S100A6, a calcium- and zinc-binding protein, is overexpressed in SOD1 mutant mice, a model for amyotrophic lateral sclerosis. *Biochim Biophys Acta* 2000; **1498**: 264-272 [PMID: 11108968 DOI: 10.1016/S0167-4889(00)00101-4]
  - 40 **Hoyaux D**, Boom A, Van den Bosch L, Belot N, Martin JJ, Heizmann CW, Kiss R, Pochet R. S100A6 overexpression within astrocytes associated with impaired axons from both ALS mouse model and human patients. *J Neuropathol Exp Neurol* 2002; **61**: 736-744 [PMID: 12152788]
  - 41 **Fang B**, Liang M, Yang G, Ye Y, Xu H, He X, Huang JH. Expression of S100A6 in rat hippocampus after traumatic brain injury due to lateral head acceleration. *Int J Mol Sci* 2014; **15**: 6378-6390 [PMID: 24739809 DOI: 10.3390/ijms15046378]
  - 42 **Boom A**, Pochet R, Authélet M, Pradier L, Borghgraef P, Van Leuven F, Heizmann CW, Brion JP. Astrocytic calcium/zinc binding protein S100A6 over expression in Alzheimer's disease and in PS1/APP transgenic mice models. *Biochim Biophys Acta* 2004; **1742**: 161-168 [PMID: 15590066 DOI: 10.1016/j.bbamer.2004.09.011]
  - 43 **Miller RH**. Oligodendrocyte origins. *Trends Neurosci* 1996; **19**: 92-96 [PMID: 9054062 DOI: 10.1016/S0166-2236(96)80036-1]
  - 44 **Noble M**, Gutowski N, Bevan K, Engel U, Linskey M, Urenjak J, Bhakoo K, Williams S. From rodent glial precursor cell to human glial neoplasia in the oligodendrocyte-type-2 astrocyte lineage. *Glia* 1995; **15**: 222-230 [PMID: 8586459 DOI: 10.1002/glia.440150304]
  - 45 **Raff MC**. Glial cell diversification in the rat optic nerve. *Science* 1989; **243**: 1450-1455 [PMID: 2648568 DOI: 10.1126/science.2648568]
  - 46 **Rao MS**, Noble M, Mayer-Pröschel M. A tripotential glial precursor cell is present in the developing spinal cord. *Proc Natl Acad Sci USA* 1998; **95**: 3996-4001 [PMID: 9520481]
  - 47 **Freeman MR**. Specification and morphogenesis of astrocytes. *Science* 2010; **330**: 774-778 [PMID: 21051628 DOI: 10.1126/science.1190928]
  - 48 **Zhou Q**, Anderson DJ. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* 2002; **109**: 61-73 [PMID: 11955447 DOI: 10.1016/S0092-8674(02)00677-3]
  - 49 **Levison SW**, Druckman SK, Young GM, Basu A. Neural stem cells in the subventricular zone are a source of astrocytes and oligodendrocytes, but not microglia. *Dev Neurosci* 2003; **25**: 184-196 [PMID: 12966216 DOI: 10.1159/000072267]
  - 50 **Doetsch F**, Caillé I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 1999; **97**: 703-716 [PMID: 10380923 DOI: 10.1016/S0092-8674(00)80783-7]
  - 51 **Doetsch F**, Garcia-Verdugo JM, Alvarez-Buylla A. Regeneration of a germinal layer in the adult mammalian brain. *Proc Natl Acad Sci USA* 1999; **96**: 11619-11624 [PMID: 10500226 DOI: 10.1073/pnas.96.20.11619]
  - 52 **Chiasson BJ**, Tropepe V, Morshead CM, van der Kooy D. Adult mammalian forebrain ependymal and subependymal cells demonstrate proliferative potential, but only subependymal cells have neural stem cell characteristics. *J Neurosci* 1999; **19**: 4462-4471 [PMID: 10341247]
  - 53 **Gage FH**. Mammalian neural stem cells. *Science* 2000; **287**:



- 1433-1438 [PMID: 10688783 DOI: 10.1126/science.287.5457.1433]
- 54 **Temple S**, Alvarez-Buylla A. Stem cells in the adult mammalian central nervous system. *Curr Opin Neurobiol* 1999; **9**: 135-141 [PMID: 10072370 DOI: 10.1016/S0959-4388(99)80017-8]
- 55 **Seidenfaden R**, Desoeuvre A, Bosio A, Virard I, Cremer H. Glial conversion of SVZ-derived committed neuronal precursors after ectopic grafting into the adult brain. *Mol Cell Neurosci* 2006; **32**: 187-198 [PMID: 16730456 DOI: 10.1016/j.mcn.2006.04.003]
- 56 **Chanas-Sacre G**, Rogister B, Moonen G, Leprince P. Radial glia phenotype: origin, regulation, and transdifferentiation. *J Neurosci Res* 2000; **61**: 357-363 [PMID: 10931521 DOI: 10.1002/1097-4547(20000815)61:4<357::AID-JNRI>3.0.CO;2-7]
- 57 **Richardson WD**, Young KM, Tripathi RB, McKenzie I. NG2-glia as multipotent neural stem cells: fact or fantasy? *Neuron* 2011; **70**: 661-673 [PMID: 21609823 DOI: 10.1016/j.neuron.2011.05.013]
- 58 **Kulbatski I**, Mothe AJ, Parr AM, Kim H, Kang CE, Bozkurt G, Tator CH. Glial precursor cell transplantation therapy for neurotrauma and multiple sclerosis. *Prog Histochem Cytochem* 2008; **43**: 123-176 [PMID: 18706353 DOI: 10.1016/j.proghi.2008.04.001]
- 59 **Goldman S**. Glia as neural progenitor cells. *Trends Neurosci* 2003; **26**: 590-596 [PMID: 14585598 DOI: 10.1016/j.tins.2003.09.011]
- 60 **McDermott KW**, Barry DS, McMahon SS. Role of radial glia in cytotogenesis, patterning and boundary formation in the developing spinal cord. *J Anat* 2005; **207**: 241-250 [PMID: 16185248 DOI: 10.1111/j.1469-7580.2005.00462.x]
- 61 **Mori T**, Buffo A, Götz M. The novel roles of glial cells revisited: the contribution of radial glia and astrocytes to neurogenesis. *Curr Top Dev Biol* 2005; **69**: 67-99 [PMID: 16243597 DOI: 10.1016/S0070-2153(05)69004-7]
- 62 **Hu BY**, Weick JP, Yu J, Ma LX, Zhang XQ, Thomson JA, Zhang SC. Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. *Proc Natl Acad Sci USA* 2010; **107**: 4335-4340 [PMID: 20160098 DOI: 10.1073/pnas.0910012107]
- 63 **Itsykson P**, Ilouz N, Turetsky T, Goldstein RS, Pera MF, Fishbein I, Segal M, Reubinoff BE. Derivation of neural precursors from human embryonic stem cells in the presence of noggin. *Mol Cell Neurosci* 2005; **30**: 24-36 [PMID: 16081300 DOI: 10.1016/j.mcn.2005.05.004]
- 64 **Johnson MA**, Weick JP, Pearce RA, Zhang SC. Functional neural development from human embryonic stem cells: accelerated synaptic activity via astrocyte coculture. *J Neurosci* 2007; **27**: 3069-3077 [PMID: 17376968 DOI: 10.1523/JNEUROSCI.4562-06.2007]
- 65 **Reubinoff BE**, Itsykson P, Turetsky T, Pera MF, Reinhartz E, Itzik A, Ben-Hur T. Neural progenitors from human embryonic stem cells. *Nat Biotechnol* 2001; **19**: 1134-1140 [PMID: 11731782 DOI: 10.1038/nbt1201-1134]
- 66 **Ruiz S**, Brennan K, Panopoulos AD, Herreras A, Gage FH, Izpisua-Belmonte JC. High-efficient generation of induced pluripotent stem cells from human astrocytes. *PLoS One* 2010; **5**: e15526 [PMID: 21170306 DOI: 10.1371/journal.pone.0015526]
- 67 **Tabar V**, Panagiotakos G, Greenberg ED, Chan BK, Sadelain M, Gutin PH, Studer L. Migration and differentiation of neural precursors derived from human embryonic stem cells in the rat brain. *Nat Biotechnol* 2005; **23**: 601-606 [PMID: 15852001 DOI: 10.1038/nbt1088]
- 68 **Zhang SC**, Wernig M, Duncan ID, Brüstle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* 2001; **19**: 1129-1133 [PMID: 11731781 DOI: 10.1038/nbt1201-1129]
- 69 **Krencik R**, Weick JP, Liu Y, Zhang ZJ, Zhang SC. Specification of transplantable astroglial subtypes from human pluripotent stem cells. *Nat Biotechnol* 2011; **29**: 528-534 [PMID: 21602806 DOI: 10.1038/nbt.1877]
- 70 **Krencik R**, Zhang SC. Directed differentiation of functional astroglial subtypes from human pluripotent stem cells. *Nat Protoc* 2011; **6**: 1710-1717 [PMID: 22011653 DOI: 10.1038/nprot.2011.405]
- 71 **Corti S**, Locatelli F, Donadoni C, Strazzer S, Salani S, Del Bo R, Caccialanza M, Bresolin N, Scarlato G, Comi GP. Neuroectodermal and microglial differentiation of bone marrow cells in the mouse spinal cord and sensory ganglia. *J Neurosci Res* 2002; **70**: 721-733 [PMID: 12444594 DOI: 10.1002/jnr.10455]
- 72 **Kopen GC**, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci USA* 1999; **96**: 10711-10716 [PMID: 10485891 DOI: 10.1073/pnas.96.19.10711]
- 73 **Nakano K**, Migita M, Mochizuki H, Shimada T. Differentiation of transplanted bone marrow cells in the adult mouse brain. *Transplantation* 2001; **71**: 1735-1740 [PMID: 11455251]
- 74 **Deng J**, Petersen BE, Steindler DA, Jorgensen ML, Laywell ED. Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. *Stem Cells* 2006; **24**: 1054-1064 [PMID: 16322639 DOI: 10.1634/stemcells.2005-0370]
- 75 **Boucherie C**, Schäfer S, Lavand'homme P, Maloteaux JM, Hermans E. Chimerization of astroglial population in the lumbar spinal cord after mesenchymal stem cell transplantation prolongs survival in a rat model of amyotrophic lateral sclerosis. *J Neurosci Res* 2009; **87**: 2034-2046 [PMID: 19267424 DOI: 10.1002/jnr.22038]
- 76 **Kim SM**, Flaßkamp H, Hermann A, Araújo-Bravo MJ, Lee SC, Lee SH, Seo EH, Lee SH, Storch A, Lee HT, Schöler HR, Tapia N, Han DW. Direct conversion of mouse fibroblasts into induced neural stem cells. *Nat Protoc* 2014; **9**: 871-881 [PMID: 24651499 DOI: 10.1038/nprot.2014.056]
- 77 **Meyer K**, Ferraiuolo L, Miranda CJ, Likhite S, McElroy S, Rensch S, Ditsworth D, Lagier-Tourenne C, Smith RA, Ravits J, Burghes AH, Shaw PJ, Cleveland DW, Kolb SJ, Kaspar BK. Direct conversion of patient fibroblasts demonstrates non-cell autonomous toxicity of astrocytes to motor neurons in familial and sporadic ALS. *Proc Natl Acad Sci USA* 2014; **111**: 829-832 [PMID: 24379375 DOI: 10.1073/pnas.1314085111]
- 78 **Mitchell RR**, Szabo E, Benoit YD, Case DT, Michael R, Alamilla J, Lee JH, Fiebig-Comyn A, Gillespie DC, Bhatia M. Activation of neural cell fate programs toward direct conversion of adult human fibroblasts into tri-potent neural progenitors using OCT-4. *Stem Cells Dev* 2014; **23**: 1937-1946 [PMID: 24694094 DOI: 10.1089/scd.2014.0023]
- 79 **Nicaise C**, Bohl D, Pochet R. [Cellular transdifferentiation in amyotrophic lateral sclerosis]. *Med Sci (Paris)* 2011; **27**: 799-801 [PMID: 22027411 DOI: 10.1051/medsci/20112710002]
- 80 **Gordon PH**. Amyotrophic lateral sclerosis: pathophysiology, diagnosis and management. *CNS Drugs* 2011; **25**: 1-15 [PMID: 21128691 DOI: 10.2165/11586000-000000000-00000]
- 81 **Boillée S**, Vande Velde C, Cleveland DW. ALS: a disease of motor neurons and their nonneuronal neighbors. *Neuron* 2006; **52**: 39-59 [PMID: 17015226 DOI: 10.1016/j.neuron.2006.09.018]
- 82 **Garbuzova-Davis S**, Haller E, Saporta S, Kolomey I, Nicosia SV, Sanberg PR. Ultrastructure of blood-brain barrier and blood-spinal cord barrier in SOD1 mice modeling ALS. *Brain Res* 2007; **1157**: 126-137 [PMID: 17512910 DOI: 10.1016/j.brainres.2007.04.044]
- 83 **Garbuzova-Davis S**, Hernandez-Ontiveros DG, Rodrigues MC, Haller E, Frisina-Deyo A, Mirtyl S, Sallot S, Saporta S, Borlongan CV, Sanberg PR. Impaired blood-brain/spinal cord barrier in ALS patients. *Brain Res* 2012; **1469**: 114-128 [PMID: 22750125 DOI: 10.1016/j.brainres.2012.05.056]
- 84 **Nicaise C**, Mitrecic D, Demetter P, De Decker R, Authet M, Boom A, Pochet R. Impaired blood-brain and blood-spinal cord barriers in mutant SOD1-linked ALS rat. *Brain Res* 2009; **1301**: 152-162 [PMID: 19748495 DOI: 10.1016/j.brainres.2009.09.018]
- 85 **Andjus PR**, Bataveljić D, Vanhoutte G, Mitrecic D, Pizzolante F, Djogo N, Nicaise C, Gankam Kengne F, Gangitano C, Michetti F, van der Linden A, Pochet R, Bacić G. In vivo morphological changes in animal models of amyotrophic lateral sclerosis and Alzheimer's-like disease: MRI approach. *Anat Rec (Hoboken)* 2009; **292**: 1882-1892 [PMID: 19943341 DOI: 10.1002/ar.20995]
- 86 **Bataveljić D**, Djogo N, Zupunski L, Bajić A, Nicaise C, Pochet R, Bacić G, Andjus PR. Live monitoring of brain damage in the rat model of amyotrophic lateral sclerosis. *Gen Physiol Biophys* 2009;



- 28 Spec No: 212-218 [PMID: 19893103]
- 87 **Bataveljić D**, Nikolić L, Milosević M, Todorović N, Andjus PR. Changes in the astrocytic aquaporin-4 and inwardly rectifying potassium channel expression in the brain of the amyotrophic lateral sclerosis SOD1(G93A) rat model. *Glia* 2012; **60**: 1991-2003 [PMID: 22987392 DOI: 10.1002/glia.22414]
  - 88 **Bataveljić D**, Stamenković S, Bačić G, Andjus PR. Imaging cellular markers of neuroinflammation in the brain of the rat model of amyotrophic lateral sclerosis. *Acta Physiol Hung* 2011; **98**: 27-31 [PMID: 21388928 DOI: 10.1556/APhysiol.98.2011.1.4]
  - 89 **Brown RH**. Amyotrophic lateral sclerosis. Insights from genetics. *Arch Neurol* 1997; **54**: 1246-1250 [PMID: 9341570 DOI: 10.1001/archneur.1997.00550220050013]
  - 90 **Wang IF**, Wu LS, Shen CK. TDP-43: an emerging new player in neurodegenerative diseases. *Trends Mol Med* 2008; **14**: 479-485 [PMID: 18929508 DOI: 10.1016/j.molmed.2008.09.001]
  - 91 **Deng HX**, Chen W, Hong ST, Boycott KM, Gorrie GH, Siddique N, Yang Y, Fecto F, Shi Y, Zhai H, Jiang H, Hirano M, Rampersaud E, Jansen GH, Donkervoort S, Bigio EH, Brooks BR, Ajroud K, Suft RL, Haines JL, Mugnaini E, Pericak-Vance MA, Siddique T. Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia. *Nature* 2011; **477**: 211-215 [PMID: 21857683 DOI: 10.1038/nature10353]
  - 92 **Maruyama H**, Morino H, Ito H, Izumi Y, Kato H, Watanabe Y, Kinoshita Y, Kamada M, Nodera H, Suzuki H, Komure O, Matsuura S, Kobatake K, Morimoto N, Abe K, Suzuki N, Aoki M, Kawata A, Hirai T, Kato T, Ogasawara K, Hirano A, Takumi T, Kusaka H, Hagiwara K, Kaji R, Kawakami H. Mutations of optineurin in amyotrophic lateral sclerosis. *Nature* 2010; **465**: 223-226 [PMID: 20428114 DOI: 10.1038/nature08971]
  - 93 **Heutink P**, Jansen IE, Lynes EM. C9orf72; abnormal RNA expression is the key. *Exp Neurol* 2014; **262** Pt B: 102-110 [PMID: 24873727 DOI: 10.1016/j.expneurol.2014.05.020]
  - 94 **Boillee S**, Yamanaka K, Lobsiger CS, Copeland NG, Jenkins NA, Kassiotis G, Kollias G, Cleveland DW. Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* 2006; **312**: 1389-1392 [PMID: 16741123 DOI: 10.1126/science.1123511]
  - 95 **Papadeas ST**, Kraig SE, O'Banion C, Lepore AC, Maragakis NJ. Astrocytes carrying the superoxide dismutase 1 (SOD1G93A) mutation induce wild-type motor neuron degeneration in vivo. *Proc Natl Acad Sci USA* 2011; **108**: 17803-17808 [PMID: 21969586 DOI: 10.1073/pnas.1103141108]
  - 96 **Yamanaka K**, Boillee S, Roberts EA, Garcia ML, McAlonis-Downes M, Mikse OR, Cleveland DW, Goldstein LS. Mutant SOD1 in cell types other than motor neurons and oligodendrocytes accelerates onset of disease in ALS mice. *Proc Natl Acad Sci USA* 2008; **105**: 7594-7599 [PMID: 18492803 DOI: 10.1073/pnas.0802556105]
  - 97 **Yamanaka K**, Chun SJ, Boillee S, Fujimori-Tonou N, Yamashita H, Gutmann DH, Takahashi R, Misawa H, Cleveland DW. Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat Neurosci* 2008; **11**: 251-253 [PMID: 18246065 DOI: 10.1038/nn2047]
  - 98 **Van Damme P**, Bogaert E, Dewil M, Hersmus N, Kiraly D, Scheveneels W, Bockx I, Braeken D, Verpoorten N, Verhoeven K, Timmerman V, Herijgers P, Callewaert G, Carmeliet P, Van Den Bosch L, Robberecht W. Astrocytes regulate GluR2 expression in motor neurons and their vulnerability to excitotoxicity. *Proc Natl Acad Sci USA* 2007; **104**: 14825-14830 [PMID: 17804792 DOI: 10.1073/pnas.0705046104]
  - 99 **Liu D**, Thangnipon W, McAdoo DJ. Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord. *Brain Res* 1991; **547**: 344-348 [PMID: 1884213 DOI: 10.1016/0006-8993(91)90984-4]
  - 100 **Panter SS**, Yum SW, Faden AI. Alteration in extracellular amino acids after traumatic spinal cord injury. *Ann Neurol* 1990; **27**: 96-99 [PMID: 2301932 DOI: 10.1002/ana.410270115]
  - 101 **Domercq M**, Etchebarria E, Pérez-Samartín A, Matute C. Excitotoxic oligodendrocyte death and axonal damage induced by glutamate transporter inhibition. *Glia* 2005; **52**: 36-46 [PMID: 15892126 DOI: 10.1002/glia.20221]
  - 102 **Xu GY**, Hughes MG, Ye Z, Hulsebosch CE, McAdoo DJ. Concentrations of glutamate released following spinal cord injury kill oligodendrocytes in the spinal cord. *Exp Neurol* 2004; **187**: 329-336 [PMID: 15144859 DOI: 10.1016/j.expneurol.2004.01.029]
  - 103 **Lepore AC**, O'Donnell J, Kim AS, Yang EJ, Tuteja A, Haidet-Phillips A, O'Banion CP, Maragakis NJ. Reduction in expression of the astrocyte glutamate transporter, GLT1, worsens functional and histological outcomes following traumatic spinal cord injury. *Glia* 2011; **59**: 1996-2005 [PMID: 21882244 DOI: 10.1002/glia.21241]
  - 104 **Lepore AC**, Rauck B, Dejea C, Pardo AC, Rao MS, Rothstein JD, Maragakis NJ. Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. *Nat Neurosci* 2008; **11**: 1294-1301 [PMID: 18931666 DOI: 10.1038/nn.2210]
  - 105 **Li K**, Nicaise C, Sannie D, Hala TJ, Javed E, Parker JL, Putatunda R, Regan KA, Suain V, Brion JP, Rhoderick F, Wright MC, Poulsen DJ, Lepore AC. Overexpression of the astrocyte glutamate transporter GLT1 exacerbates phrenic motor neuron degeneration, diaphragm compromise, and forelimb motor dysfunction following cervical contusion spinal cord injury. *J Neurosci* 2014; **34**: 7622-7638 [PMID: 24872566 DOI: 10.1523/JNEUROSCI.4690-13.2014]
  - 106 **Putatunda R**, Hala TJ, Chin J, Lepore AC. Chronic at-level thermal hyperalgesia following rat cervical contusion spinal cord injury is accompanied by neuronal and astrocyte activation and loss of the astrocyte glutamate transporter, GLT1, in superficial dorsal horn. *Brain Res* 2014; **1581**: 64-79 [PMID: 24833066 DOI: 10.1016/j.brainres.2014.05.003]
  - 107 **Lepore AC**, O'Donnell J, Bonner JF, Paul C, Miller ME, Rauck B, Kushner RA, Rothstein JD, Fischer I, Maragakis NJ. Spatial and temporal changes in promoter activity of the astrocyte glutamate transporter GLT1 following traumatic spinal cord injury. *J Neurosci Res* 2011; **89**: 1001-1017 [PMID: 21488085 DOI: 10.1002/jnr.22624]
  - 108 **Verkhatsky A**, Sofroniew MV, Messing A, deLanerolle NC, Rempe D, Rodríguez JJ, Nedergaard M. Neurological diseases as primary gliopathies: a reassessment of neurocentrism. *ASN Neuro* 2012; **4**: [PMID: 22339481 DOI: 10.1042/AN20120010]
  - 109 **Dietrich J**, Noble M, Mayer-Pröschel M. Characterization of A2B5+ glial precursor cells from cryopreserved human fetal brain progenitor cells. *Glia* 2002; **40**: 65-77 [PMID: 12237844 DOI: 10.1002/glia.10116]
  - 110 **Gregori N**, Pröschel C, Noble M, Mayer-Pröschel M. The tripotential glial-restricted precursor (GRP) cell and glial development in the spinal cord: generation of bipotential oligodendrocyte-type-2 astrocyte progenitor cells and dorsal-ventral differences in GRP cell function. *J Neurosci* 2002; **22**: 248-256 [PMID: 11756508]
  - 111 **Herrera J**, Yang H, Zhang SC, Proschel C, Tresco P, Duncan ID, Luskin M, Mayer-Pröschel M. Embryonic-derived glial-restricted precursor cells (GRP cells) can differentiate into astrocytes and oligodendrocytes in vivo. *Exp Neurol* 2001; **171**: 11-21 [PMID: 11520117 DOI: 10.1006/exnr.2001.7729]
  - 112 **Han SS**, Liu Y, Tyler-Polsz C, Rao MS, Fischer I. Transplantation of glial-restricted precursor cells into the adult spinal cord: survival, glial-specific differentiation, and preferential migration in white matter. *Glia* 2004; **45**: 1-16 [PMID: 14648541 DOI: 10.1002/glia.10282]
  - 113 **Lepore AC**, Walczak P, Rao MS, Fischer I, Bulte JW. MR imaging of lineage-restricted neural precursors following transplantation into the adult spinal cord. *Exp Neurol* 2006; **201**: 49-59 [PMID: 16764862 DOI: 10.1016/j.expneurol.2006.03.032]
  - 114 **Maragakis NJ**, Dietrich J, Wong V, Xue H, Mayer-Pröschel M, Rao MS, Rothstein JD. Glutamate transporter expression and function in human glial progenitors. *Glia* 2004; **45**: 133-143 [PMID: 14730707 DOI: 10.1002/glia.10310]
  - 115 **Davies JE**, Pröschel C, Zhang N, Noble M, Mayer-Pröschel M, Davies SJ. Transplanted astrocytes derived from BMP- or CNTF-treated glial-restricted precursors have opposite effects on recovery and allodynia after spinal cord injury. *J Biol* 2008; **7**: 24 [PMID: 18803859 DOI: 10.1186/jbiol85]



- 116 **Davies SJ**, Shih CH, Noble M, Mayer-Proschel M, Davies JE, Proschel C. Transplantation of specific human astrocytes promotes functional recovery after spinal cord injury. *PLoS One* 2011; **6**: e17328 [PMID: 21407803 DOI: 10.1371/journal.pone.0017328]
- 117 **Cao Q**, Xu XM, Devries WH, Enzmann GU, Ping P, Tsoulfas P, Wood PM, Bunge MB, Whittemore SR. Functional recovery in traumatic spinal cord injury after transplantation of multineurotrophin-expressing glial-restricted precursor cells. *J Neurosci* 2005; **25**: 6947-6957 [PMID: 16049170 DOI: 10.1523/JNEUROSCI.1065-05.2005]
- 118 **Corti S**, Locatelli F, Papadimitriou D, Del Bo R, Nizzardo M, Nardini M, Donadoni C, Salani S, Fortunato F, Strazzer S, Bresolin N, Comi GP. Neural stem cells LewisX+ CXCR4+ modify disease progression in an amyotrophic lateral sclerosis model. *Brain* 2007; **130**: 1289-1305 [PMID: 17439986 DOI: 10.1093/brain/awm043]
- 119 **Klein SM**, Behrstock S, McHugh J, Hoffmann K, Wallace K, Suzuki M, Aebischer P, Svendsen CN. GDNF delivery using human neural progenitor cells in a rat model of ALS. *Hum Gene Ther* 2005; **16**: 509-521 [PMID: 15871682 DOI: 10.1089/hum.2005.16.509]
- 120 **Ogawa Y**, Sawamoto K, Miyata T, Miyao S, Watanabe M, Nakamura M, Bregman BS, Koike M, Uchiyama Y, Toyama Y, Okano H. Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. *J Neurosci Res* 2002; **69**: 925-933 [PMID: 12205685 DOI: 10.1002/jnr.10341]
- 121 **Suzuki M**, McHugh J, Tork C, Shelley B, Klein SM, Aebischer P, Svendsen CN. GDNF secreting human neural progenitor cells protect dying motor neurons, but not their projection to muscle, in a rat model of familial ALS. *PLoS One* 2007; **2**: e689 [PMID: 17668067 DOI: 10.1371/journal.pone.0000689]
- 122 **Xu L**, Ryugo DK, Pongstaporn T, Johe K, Koliatos VE. Human neural stem cell grafts in the spinal cord of SOD1 transgenic rats: differentiation and structural integration into the segmental motor circuitry. *J Comp Neurol* 2009; **514**: 297-309 [PMID: 19326469 DOI: 10.1002/cne.22022]
- 123 **Xu L**, Yan J, Chen D, Welsh AM, Hazel T, Johe K, Hatfield G, Koliatos VE. Human neural stem cell grafts ameliorate motor neuron disease in SOD-1 transgenic rats. *Transplantation* 2006; **82**: 865-875 [PMID: 17038899 DOI: 10.1097/01.tp.0000235532.00920.7a]
- 124 **Lepore AC**, O'Donnell J, Kim AS, Williams T, Tuteja A, Rao MS, Kelley LL, Campanelli JT, Maragakis NJ. Human glial-restricted progenitor transplantation into cervical spinal cord of the SOD1 mouse model of ALS. *PLoS One* 2011; **6**: e25968 [PMID: 21998733 DOI: 10.1371/journal.pone.0025968]
- 125 **Potts MB**, Silvestrini MT, Lim DA. Devices for cell transplantation into the central nervous system: Design considerations and emerging technologies. *Surg Neurol Int* 2013; **4**: S22-S30 [PMID: 23653887 DOI: 10.4103/2152-7806.109190]
- 126 **Danielyan L**, Schäfer R, von Ameln-Mayerhofer A, Buadze M, Geisler J, Klopfer T, Burkhardt U, Proksch B, Verleysdonk S, Ayturan M, Buniatian GH, Gleiter CH, Frey WH. Intranasal delivery of cells to the brain. *Eur J Cell Biol* 2009; **88**: 315-324 [PMID: 19324456 DOI: 10.1016/j.jecb.2009.02.001]
- 127 **Habisch HJ**, Janowski M, Binder D, Kuzma-Kozakiewicz M, Widmann A, Habich A, Schwalenstöcker B, Hermann A, Brenner R, Lukomska B, Domanska-Janik K, Ludolph AC, Storch A. Intrathecal application of neuroectodermally converted stem cells into a mouse model of ALS: limited intraparenchymal migration and survival narrows therapeutic effects. *J Neural Transm* 2007; **114**: 1395-1406 [PMID: 17510731 DOI: 10.1007/s00702-007-0748-y]
- 128 **Hwang DH**, Lee HJ, Park IH, Seok JI, Kim BG, Joo IS, Kim SU. Intrathecal transplantation of human neural stem cells overexpressing VEGF provide behavioral improvement, disease onset delay and survival extension in transgenic ALS mice. *Gene Ther* 2009; **16**: 1234-1244 [PMID: 19626053 DOI: 10.1038/gt.2009.80]
- 129 **Kim H**, Kim HY, Choi MR, Hwang S, Nam KH, Kim HC, Han JS, Kim KS, Yoon HS, Kim SH. Dose-dependent efficacy of ALS-human mesenchymal stem cells transplantation into cisterna magna in SOD1-G93A ALS mice. *Neurosci Lett* 2010; **468**: 190-194 [PMID: 19879334 DOI: 10.1016/j.neulet.2009.10.074]
- 130 **Corti S**, Locatelli F, Donadoni C, Guglieri M, Papadimitriou D, Strazzer S, Del Bo R, Comi GP. Wild-type bone marrow cells ameliorate the phenotype of SOD1-G93A ALS mice and contribute to CNS, heart and skeletal muscle tissues. *Brain* 2004; **127**: 2518-2532 [PMID: 15469951 DOI: 10.1093/brain/awh273]
- 131 **Suzuki M**, McHugh J, Tork C, Shelley B, Hayes A, Bellantuono I, Aebischer P, Svendsen CN. Direct muscle delivery of GDNF with human mesenchymal stem cells improves motor neuron survival and function in a rat model of familial ALS. *Mol Ther* 2008; **16**: 2002-2010 [PMID: 18797452 DOI: 10.1038/mt.2008.197]
- 132 **Akiyama Y**, Radtke C, Honmou O, Kocsis JD. Remyelination of the spinal cord following intravenous delivery of bone marrow cells. *Glia* 2002; **39**: 229-236 [PMID: 12203389 DOI: 10.1002/glia.10102]
- 133 **Chen J**, Li Y, Wang L, Zhang Z, Lu D, Lu M, Chopp M. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 2001; **32**: 1005-1011 [PMID: 11283404 DOI: 10.1161/01.STR.32.4.1005]
- 134 **Garbuzova-Davis S**, Willing AE, Zigova T, Saporta S, Justen EB, Lane JC, Hudson JE, Chen N, Davis CD, Sanberg PR. Intravenous administration of human umbilical cord blood cells in a mouse model of amyotrophic lateral sclerosis: distribution, migration, and differentiation. *J Hematother Stem Cell Res* 2003; **12**: 255-270 [PMID: 12857367 DOI: 10.1089/152581603322022990]
- 135 **Mitrečić D**, Nicaise C, Gajović S, Pochet R. Distribution, differentiation, and survival of intravenously administered neural stem cells in a rat model of amyotrophic lateral sclerosis. *Cell Transplant* 2010; **19**: 537-548 [PMID: 20350352 DOI: 10.3727/096368910X498269]
- 136 **Ohnishi S**, Ito H, Suzuki Y, Adachi Y, Wate R, Zhang J, Nakano S, Kusaka H, Ikehara S. Intra-bone marrow-bone marrow transplantation slows disease progression and prolongs survival in G93A mutant SOD1 transgenic mice, an animal model mouse for amyotrophic lateral sclerosis. *Brain Res* 2009; **1296**: 216-224 [PMID: 19686706 DOI: 10.1016/j.brainres.2009.08.012]
- 137 **Mitrečić D**. Current advances in intravascular administration of stem cells for neurological diseases: a new dose of rejuvenation injected. *Rejuvenation Res* 2011; **14**: 553-555 [PMID: 21951133 DOI: 10.1089/rej.2011.1209]
- 138 **Glover JC**, Boulland JL, Halasi G, Kasumacic N. Chimeric animal models in human stem cell biology. *ILAR J* 2009; **51**: 62-73 [PMID: 20075498 DOI: 10.1093/ilar.51.1.62]
- 139 **Lindvall O**, Kokaia Z. Stem cells in human neurodegenerative disorders--time for clinical translation? *J Clin Invest* 2010; **120**: 29-40 [PMID: 20051634 DOI: 10.1172/JCI40543]
- 140 **Allard J**, Li K, Lopez XM, Blanchard S, Barbot P, Rorive S, Decaestecker C, Pochet R, Bohl D, Lepore AC, Salmon I, Nicaise C. Immunohistochemical toolkit for tracking and quantifying xenotransplanted human stem cells. *Regen Med* 2014; **9**: 437-452 [PMID: 25159062 DOI: 10.2217/rme.14.26]
- 141 **Nagai M**, Re DB, Nagata T, Chalazonitis A, Jessell TM, Wichterle H, Przedborski S. Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat Neurosci* 2007; **10**: 615-622 [PMID: 17435755 DOI: 10.1038/nrn1876]
- 142 **Clement AM**, Nguyen MD, Roberts EA, Garcia ML, Boillée S, Rule M, McMahon AP, Doucette W, Siwek D, Ferrante RJ, Brown RH, Julien JP, Goldstein LS, Cleveland DW. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science* 2003; **302**: 113-117 [PMID: 14526083 DOI: 10.1126/science.1086071]
- 143 **Rothstein JD**, Van Kammen M, Levey AI, Martin LJ, Kuncel RW. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Ann Neurol* 1995; **38**: 73-84 [PMID: 7611729 DOI: 10.1002/ana.410380114]
- 144 **Boido M**, Piras A, Valsecchi V, Spigolon G, Mareschi K, Ferrero I, Vizzini A, Temi S, Mazzini L, Fagioli F, Vercelli A. Human mesenchymal stromal cell transplantation modulates neuroinflammatory milieu in a mouse model of amyotrophic lateral sclerosis. *Cytotherapy* 2014; **16**: 1059-1072 [PMID: 24794182 DOI: 10.1016/j.jcyt.2014.02.003]



- 145 **Nizzardo M**, Simone C, Rizzo F, Ruggieri M, Salani S, Riboldi G, Faravelli I, Zanetta C, Bresolin N, Comi GP, Corti S. Minimally invasive transplantation of iPSC-derived ALDHhiSSCcloVLA4+ neural stem cells effectively improves the phenotype of an amyotrophic lateral sclerosis model. *Hum Mol Genet* 2014; **23**: 342-354 [PMID: 24006477 DOI: 10.1093/hmg/ddt425]
- 146 **Rizvanov AA**, Guseva DS, Salafutdinov II, Kudryashova NV, Bashirov FV, Kiyasov AP, Yalvaç ME, Gazizov IM, Kaligin MS, Sahin F, Mukhamedyarov MA, Palotás A, Islamov RR. Genetically modified human umbilical cord blood cells expressing vascular endothelial growth factor and fibroblast growth factor 2 differentiate into glial cells after transplantation into amyotrophic lateral sclerosis transgenic mice. *Exp Biol Med* (Maywood) 2011; **236**: 91-98 [PMID: 21163822 DOI: 10.1258/ebm.2010.010172]
- 147 **Park S**, Kim HT, Yun S, Kim IS, Lee J, Lee IS, Park KI. Growth factor-expressing human neural progenitor cell grafts protect motor neurons but do not ameliorate motor performance and survival in ALS mice. *Exp Mol Med* 2009; **41**: 487-500 [PMID: 19322031 DOI: 10.3858/emmm.2009.41.7.054]
- 148 **Lunn JS**, Sakowski SA, Feldman EL. Concise review: Stem cell therapies for amyotrophic lateral sclerosis: recent advances and prospects for the future. *Stem Cells* 2014; **32**: 1099-1109 [PMID: 24448926 DOI: 10.1002/stem.1628]
- 149 **Smith GM**, Miller RH, Silver J. Changing role of forebrain astrocytes during development, regenerative failure, and induced regeneration upon transplantation. *J Comp Neurol* 1986; **251**: 23-43 [PMID: 3760257 DOI: 10.1002/cne.902510103]
- 150 **Smith GM**, Rutishauser U, Silver J, Miller RH. Maturation of astrocytes in vitro alters the extent and molecular basis of neurite outgrowth. *Dev Biol* 1990; **138**: 377-390 [PMID: 2318341]
- 151 **Smith GM**, Miller RH. Immature type-I astrocytes suppress glial scar formation, are motile and interact with blood vessels. *Brain Res* 1991; **543**: 111-122 [PMID: 2054666]
- 152 **Joosten EA**, Veldhuis WB, Hamers FP. Collagen containing neonatal astrocytes stimulates regrowth of injured fibers and promotes modest locomotor recovery after spinal cord injury. *J Neurosci Res* 2004; **77**: 127-142 [PMID: 15197746 DOI: 10.1002/jnr.20088]
- 153 **Cao QL**, Zhang YP, Howard RM, Walters WM, Tsoulfas P, Whittemore SR. Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. *Exp Neurol* 2001; **167**: 48-58 [PMID: 11161592 DOI: 10.1006/exnr.2000.7536]
- 154 **Hofstetter CP**, Holmström NA, Lilja JA, Schweinhardt P, Hao J, Spenger C, Wiesenfeld-Hallin Z, Kurpad SN, Frisén J, Olson L. Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. *Nat Neurosci* 2005; **8**: 346-353 [PMID: 15711542 DOI: 10.1038/nn1405]
- 155 **Hill CE**, Proschel C, Noble M, Mayer-Proschel M, Gensel JC, Beattie MS, Bresnahan JC. Acute transplantation of glial-restricted precursor cells into spinal cord contusion injuries: survival, differentiation, and effects on lesion environment and axonal regeneration. *Exp Neurol* 2004; **190**: 289-310 [PMID: 15530870 DOI: 10.1016/j.expneurol.2004.05.043]
- 156 **Davies JE**, Huang C, Proschel C, Noble M, Mayer-Proschel M, Davies SJ. Astrocytes derived from glial-restricted precursors promote spinal cord repair. *J Biol* 2006; **5**: 7 [PMID: 16643674 DOI: 10.1186/jbiol35]
- 157 **Haas C**, Neuhuber B, Yamagami T, Rao M, Fischer I. Phenotypic analysis of astrocytes derived from glial restricted precursors and their impact on axon regeneration. *Exp Neurol* 2012; **233**: 717-732 [PMID: 22101004 DOI: 10.1016/j.expneurol.2011.11.002]
- 158 **Shih CH**, Lacagnina M, Leuer-Biscioti K, Proschel C. Astroglial-derived periostin promotes axonal regeneration after spinal cord injury. *J Neurosci* 2014; **34**: 2438-2443 [PMID: 24523534 DOI: 10.1523/JNEUROSCI.2947-13.2014]
- 159 **Jin Y**, Neuhuber B, Singh A, Bouyer J, Lepore A, Bonner J, Himes T, Campanelli JT, Fischer I. Transplantation of human glial restricted progenitors and derived astrocytes into a contusion model of spinal cord injury. *J Neurotrauma* 2011; **28**: 579-594 [PMID: 21222572 DOI: 10.1089/neu.2010.1626]
- 160 **Haas C**, Fischer I. Human astrocytes derived from glial restricted progenitors support regeneration of the injured spinal cord. *J Neurotrauma* 2013; **30**: 1035-1052 [PMID: 23635322 DOI: 10.1089/neu.2013.2915]
- 161 **Emdad L**, D'Souza SL, Kothari HP, Qadeer ZA, Germano IM. Efficient differentiation of human embryonic and induced pluripotent stem cells into functional astrocytes. *Stem Cells Dev* 2012; **21**: 404-410 [PMID: 21631388 DOI: 10.1089/scd.2010.0560]
- 162 **Juopperi TA**, Kim WR, Chiang CH, Yu H, Margolis RL, Ross CA, Ming GL, Song H. Astrocytes generated from patient induced pluripotent stem cells recapitulate features of Huntington's disease patient cells. *Mol Brain* 2012; **5**: 17 [PMID: 22613578 DOI: 10.1186/1756-6606-5-17]
- 163 **Roybon L**, Lamas NJ, Garcia-Diaz A, Yang EJ, Sattler R, Jackson-Lewis V, Kim YA, Kachel CA, Rothstein JD, Przedborski S, Wichterle H, Henderson CE. Human stem cell-derived spinal cord astrocytes with defined mature or reactive phenotypes. *Cell Rep* 2013; **4**: 1035-1048 [PMID: 23994478 DOI: 10.1016/j.celrep.2013.06.021]
- 164 **Haidet-Phillips AM**, Roybon L, Gross SK, Tuteja A, Donnelly CJ, Richard JP, Ko M, Sherman A, Eggan K, Henderson CE, Maragakis NJ. Gene profiling of human induced pluripotent stem cell-derived astrocyte progenitors following spinal cord engraftment. *Stem Cells Transl Med* 2014; **3**: 575-585 [PMID: 24604284 DOI: 10.5966/sctm.2013-0153]
- 165 **Nori S**, Okada Y, Yasuda A, Tsuji O, Takahashi Y, Kobayashi Y, Fujiyoshi K, Koike M, Uchiyama Y, Ikeda E, Toyama Y, Yamanaka S, Nakamura M, Okano H. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. *Proc Natl Acad Sci USA* 2011; **108**: 16825-16830 [PMID: 21949375 DOI: 10.1073/pnas.1108077108]
- 166 **Tsuji O**, Miura K, Okada Y, Fujiyoshi K, Mukaino M, Nagoshi N, Kitamura K, Kumagai G, Nishino M, Tomisato S, Higashi H, Nagai T, Katoh H, Kohda K, Matsuzaki Y, Yuzaki M, Ikeda E, Toyama Y, Nakamura M, Yamanaka S, Okano H. Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. *Proc Natl Acad Sci USA* 2010; **107**: 12704-12709 [PMID: 20615974 DOI: 10.1073/pnas.0910106107]
- 167 **Geffner LF**, Santacruz P, Izurieta M, Flor L, Maldonado B, Auad AH, Montenegro X, Gonzalez R, Silva F. Administration of autologous bone marrow stem cells into spinal cord injury patients via multiple routes is safe and improves their quality of life: comprehensive case studies. *Cell Transplant* 2008; **17**: 1277-1293 [PMID: 19364066 DOI: 10.3727/096368908787648074]
- 168 **Jiang PC**, Xiong WP, Wang G, Ma C, Yao WQ, Kendell SF, Mehling BM, Yuan XH, Wu DC. A clinical trial report of autologous bone marrow-derived mesenchymal stem cell transplantation in patients with spinal cord injury. *Exp Ther Med* 2013; **6**: 140-146 [PMID: 23935735 DOI: 10.3892/etm.2013.1083]
- 169 **Karamouzian S**, Nematollahi-Mahani SN, Nakhaee N, Eskandary H. Clinical safety and primary efficacy of bone marrow mesenchymal cell transplantation in subacute spinal cord injured patients. *Clin Neurol Neurosurg* 2012; **114**: 935-939 [PMID: 22464434 DOI: 10.1016/j.clineuro.2012.02.003]
- 170 **Moviglia GA**, Fernandez Viña R, Brizuela JA, Saslavsky J, Vrsalovic F, Varela G, Bastos F, Farina P, Etchegaray G, Barbieri M, Martinez G, Picasso F, Schmidt Y, Brizuela P, Gaeta CA, Costanzo H, Moviglia Brandolino MT, Merino S, Pes ME, Veloso MJ, Rugilo C, Tamer I, Shuster GS. Combined protocol of cell therapy for chronic spinal cord injury. Report on the electrical and functional recovery of two patients. *Cytotherapy* 2006; **8**: 202-209 [PMID: 16793729 DOI: 10.1080/14653240600736048]
- 171 **Moviglia GA**, Varela G, Brizuela JA, Moviglia Brandolino MT, Farina P, Etchegaray G, Piccone S, Hirsch J, Martinez G, Marino S, Deffain S, Coria N, Gonzáles A, Sztanko M, Salas-Zamora P, Previgliano I, Aingel V, Farias J, Gaeta CA, Saslavsky J, Blassetti N. Case report on the clinical results of a combined cellular therapy for chronic spinal cord injured patients. *Spinal Cord* 2009; **47**: 499-503 [PMID: 19223861 DOI: 10.1038/sc.2008.164]
- 172 **Vargas MR**, Johnson DA, Sirkis DW, Messing A, Johnson JA. Nrf2 activation in astrocytes protects against neurodegeneration in mouse models of familial amyotrophic lateral sclerosis. *J*



- Neurosci* 2008; **28**: 13574-13581 [PMID: 19074031 DOI: 10.1523/JNEUROSCI.4099-08.2008]
- 173 **Chen Y**, Vartiainen NE, Ying W, Chan PH, Koistinaho J, Swanson RA. Astrocytes protect neurons from nitric oxide toxicity by a glutathione-dependent mechanism. *J Neurochem* 2001; **77**: 1601-1610 [PMID: 11413243 DOI: 10.1046/j.1471-4159.2001.00374.x]
  - 174 **Sarafian TA**, Montes C, Imura T, Qi J, Coppola G, Geschwind DH, Sofroniew MV. Disruption of astrocyte STAT3 signaling decreases mitochondrial function and increases oxidative stress in vitro. *PLoS One* 2010; **5**: e9532 [PMID: 20224768 DOI: 10.1371/journal.pone.0009532]
  - 175 **Okada S**, Nakamura M, Katoh H, Miyao T, Shimazaki T, Ishii K, Yamane J, Yoshimura A, Iwamoto Y, Toyama Y, Okano H. Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. *Nat Med* 2006; **12**: 829-834 [PMID: 16783372 DOI: 10.1038/nm1425]
  - 176 **Jung G**, Sun J, Petrowitz B, Riecken K, Kruszwski K, Jankowiak W, Kunst F, Skevas C, Richard G, Fehse B, Bartsch U. Genetically modified neural stem cells for a local and sustained delivery of neuroprotective factors to the dystrophic mouse retina. *Stem Cells Transl Med* 2013; **2**: 1001-1010 [PMID: 24167317 DOI: 10.5966/sctm.2013-0013]
  - 177 **Lee HJ**, Lim IJ, Park SW, Kim YB, Ko Y, Kim SU. Human neural stem cells genetically modified to express human nerve growth factor (NGF) gene restore cognition in the mouse with ibotenic acid-induced cognitive dysfunction. *Cell Transplant* 2012; **21**: 2487-2496 [PMID: 22526467 DOI: 10.3727/096368912X638964]
  - 178 **Gowing G**, Shelley B, Staggenborg K, Hurley A, Avalos P, Victoroff J, Latter J, Garcia L, Svendsen CN. Glial cell line-derived neurotrophic factor-secreting human neural progenitors show long-term survival, maturation into astrocytes, and no tumor formation following transplantation into the spinal cord of immunocompromised rats. *Neuroreport* 2014; **25**: 367-372 [PMID: 24284956 DOI: 10.1097/WNR.0000000000000092]
  - 179 **Ericson C**, Georgievskia B, Lundberg C. Ex vivo gene delivery of GDNF using primary astrocytes transduced with a lentiviral vector provides neuroprotection in a rat model of Parkinson's disease. *Eur J Neurosci* 2005; **22**: 2755-2764 [PMID: 16324109 DOI: 10.1111/j.1460-9568.2005.04503.x]
  - 180 **Castillo B**, del Cerro M, Breakefield XO, Frim DM, Barnstable CJ, Dean DO, Bohn MC. Retinal ganglion cell survival is promoted by genetically modified astrocytes designed to secrete brain-derived neurotrophic factor (BDNF). *Brain Res* 1994; **647**: 30-36 [PMID: 8069702 DOI: 10.1016/0006-8993(94)91395-1]
  - 181 **Fan C**, Zheng Y, Cheng X, Qi X, Bu P, Luo X, Kim DH, Cao Q. Transplantation of D15A-expressing glial-restricted-precursor-derived astrocytes improves anatomical and locomotor recovery after spinal cord injury. *Int J Biol Sci* 2013; **9**: 78-93 [PMID: 23289019 DOI: 10.7150/ijbs.5626]
  - 182 **Nicaise C**, Frank DM, Hala TJ, Authalet M, Pochet R, Adriaens D, Brion JP, Wright MC, Lepore AC. Early phrenic motor neuron loss and transient respiratory abnormalities after unilateral cervical spinal cord contusion. *J Neurotrauma* 2013; **30**: 1092-1099 [PMID: 23534670 DOI: 10.1089/neu.2012.2728]
  - 183 **Nicaise C**, Hala TJ, Frank DM, Parker JL, Authalet M, Leroy K, Brion JP, Wright MC, Lepore AC. Phrenic motor neuron degeneration compromises phrenic axonal circuitry and diaphragm activity in a unilateral cervical contusion model of spinal cord injury. *Exp Neurol* 2012; **235**: 539-552 [PMID: 22465264 DOI: 10.1016/j.expneurol.2012.03.007]
  - 184 **Bernstein JJ**, Goldberg WJ. Grafted fetal astrocyte migration can prevent host neuronal atrophy: comparison of astrocytes from cultures and whole piece donors. *Restor Neurol Neurosci* 1991; **2**: 261-270 [PMID: 21551612 DOI: 10.3233/RNN-1991-245615]
  - 185 **Wang JJ**, Chuah MI, Yew DT, Leung PC, Tsang DS. Effects of astrocyte implantation into the hemisectioned adult rat spinal cord. *Neuroscience* 1995; **65**: 973-981 [PMID: 7617172 DOI: 10.1016/0306-4522(94)00519-B]
  - 186 **Olby NJ**, Blakemore WF. Reconstruction of the glial environment of a photochemically induced lesion in the rat spinal cord by transplantation of mixed glial cells. *J Neurocytol* 1996; **25**: 481-498 [PMID: 8899569 DOI: 10.1007/BF02284817]
  - 187 **Mitsui T**, Shumsky JS, Lepore AC, Murray M, Fischer I. Transplantation of neuronal and glial restricted precursors into contused spinal cord improves bladder and motor functions, decreases thermal hypersensitivity, and modifies intraspinal circuitry. *J Neurosci* 2005; **25**: 9624-9636 [PMID: 16237167 DOI: 10.1523/JNEUROSCI.2175-05.2005]
  - 188 **Pencalet P**, Serguera C, Corti O, Privat A, Mallet J, Giménez y Ribotta M. Integration of genetically modified adult astrocytes into the lesioned rat spinal cord. *J Neurosci Res* 2006; **83**: 61-67 [PMID: 16294335 DOI: 10.1002/jnr.20697]
  - 189 **Hayashi K**, Hashimoto M, Koda M, Naito AT, Murata A, Okawa A, Takahashi K, Yamazaki M. Increase of sensitivity to mechanical stimulus after transplantation of murine induced pluripotent stem cell-derived astrocytes in a rat spinal cord injury model. *J Neurosurg Spine* 2011; **15**: 582-593 [PMID: 21854127 DOI: 10.3171/2011.7.SPINE10775]
  - 190 **Wu L**, Li J, Chen L, Zhang H, Yuan L, Davies SJ. Combined transplantation of GDAs(BMP) and hr-decorin in spinal cord contusion repair. *Neural Regen Res* 2013; **8**: 2236-2248 [PMID: 25206533 DOI: 10.3969/j.issn.1673-5374.2013.24.003]

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## Tooth-derived stem cells: Update and perspectives

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stem cells (SCs) present the characteristics of self-renewal and differentiation capacity, which make them promising candidates for tissue engineering. Although they present some common markers, such as cluster of differentiation (CD)105, CD146 and STRO-1, SCs derived from various tissues have different patterns in relation to proliferation, clonogenicity, and differentiation abilities *in vitro* and *in vivo*. Tooth-derived tissues have been proposed as an accessible source to obtain SCs with limited morbidity, and various tooth-derived SCs (TDSCs) have been isolated and characterized, such as dental pulp SCs, SCs from human exfoliated deciduous teeth, periodontal ligament SCs, dental follicle progenitor cells, SCs from apical papilla, and periodontal ligament of deciduous teeth SCs. However, heterogeneity among these populations has been observed, and the best method to select the most appropriate TDSCs for regeneration approaches has not yet been established. The objective of this review is to outline the current knowledge concerning the various types of TDSCs, and discuss the perspectives for their use in regenerative approaches.

**Key words:** Cell-based therapy; Mesenchymal stem cells; Dental stem cells; Differentiation; Tissue engineering

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**Core tip:** Stem cells (SCs) present the characteristics of self-renewal and differentiation capacity, which make them promising candidates for regenerative approaches. Although they present some common markers, SCs derived from various tissues have different patterns of proliferation, clonogenicity, and differentiation. Tooth-derived tissues are an accessible source of SCs with limited morbidity. However, heterogeneity within populations of tooth-derived SCs has been observed, and the best method to select the most appropriate SCs for regenerative approaches has not yet been established.

### Abstract

Tissue engineering is an emerging field of science that focuses on creating suitable conditions for the regeneration of tissues. The basic components for tissue engineering involve an interactive triad of scaffolds, signaling molecules, and cells. In this context,



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## INTRODUCTION

Stem cells (SCs) are cells that present two distinctive characteristics: they are able to continuously self-renew, and they can be induced to differentiate into multiple specialized cell types<sup>[1]</sup>. SCs have therefore been a subject of interest to researchers and the general public, as a way to regenerate damaged tissues and improve the resolution of some illnesses, such as Parkinson's disease<sup>[2]</sup> and diabetes<sup>[3]</sup>, that current approaches in the medical field have not yet achieved. In this context, many studies have been conducted to identify and isolate SCs and to understand their biologic aspects.

SCs can be isolated in the earliest stages of embryogenesis (embryonic SCs)<sup>[4-9]</sup> or in various postnatal tissues (adult SCs)<sup>[2,10,11]</sup> (Table 1). Although embryonic SCs present interesting properties, such as the ability to differentiate into hundreds of other cell types, the bioethical aspects involved in the study of these cells, especially for human embryos, have hindered advances in this field and research has thus been focused on adult SCs<sup>[1,10-12]</sup>. Adult SCs can be obtained from adult specialized tissues, such as bone marrow<sup>[13-16]</sup>, skin<sup>[17,18]</sup> and fat<sup>[19-23]</sup>, where they likely act to renew cell populations and maintain tissue homeostasis, or help to repair the tissue in case of injury<sup>[18,24,25]</sup>. Even though adult SCs can be obtained from less ethically concerning sources, they have some limitations compared to embryonic SCs, such as more limited lifespan and differentiation potential<sup>[1,11,24,26]</sup>. In order to overcome these drawbacks, adult SCs can be reprogrammed by the insertion of SC-associated genes, forming induced pluripotent SCs (iPSCs)<sup>[3,27-31]</sup>.

Within the medical field, mesenchymal SCs (MSCs) have been widely studied to understand their role in skeletal tissue development, physiology and repair<sup>[14]</sup>, and because of their promising therapeutic potential<sup>[2]</sup>. MSCs are characterized by the capacity to differentiate into multiple types of skeletal tissues<sup>[14,32-36]</sup>. They were first described as adherent, clonogenic, self-renewing, fibroblast-like cells (colony-forming unit fibroblasts) obtained from bone marrow<sup>[35,37,38]</sup>. Subsequently, several studies were performed to identify other sources and to understand how these cells can give rise to distinct cell types, for the purpose of using these cells in regenerative procedures<sup>[39-43]</sup>.

In this context, dental tissues have also been investigated as niches of MSCs, and many tooth-derived SCs (TDSCs) have been identified and characterized, including dental pulp SCs (DPSCs)<sup>[44-48]</sup>, SCs from human exfoliated deciduous teeth (SHED)<sup>[49-53]</sup>,

periodontal ligament SCs (PDLSCs), dental follicle progenitor cells (DFPCs)<sup>[54-56]</sup>, SCs from apical papilla (SCAP)<sup>[19,56-59]</sup>, and periodontal ligament of deciduous teeth SCs (DePDL)<sup>[50,51,60-62]</sup> (Figure 1). Dental tissues are an accessible source of MSCs that can be obtained with limited morbidity and without additional risks to the donor, as extracted/exfoliated teeth represent a waste product of dental procedures<sup>[13,63-65]</sup>. However, the properties of these TDSCs and their feasibility for regenerating tissues still need to be investigated in greater detail. Thus, the aim of the present review is to describe the current knowledge concerning TDSCs, and to consider the perspectives for their use in regenerative approaches.

## ISOLATION, CHARACTERIZATION, AND DIFFERENTIATION POTENTIAL OF TDSCs

Because of the variety of methodologies used to isolate and characterize MSCs, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define human bone marrow SCs (BMSCs) and other types of MSCs *in vitro*<sup>[32]</sup>. Briefly, MSCs must adhere to plastic under standard culture conditions, express cluster of differentiation (CD)105, CD73 and CD90, but not CD45, CD34, CD14, CD11b, CD79a, CD19, or human leukocyte antigen-DR surface molecules, and have the potential to differentiate along osteogenic, chondrogenic and adipogenic lineages<sup>[32]</sup>. Therefore, studies characterizing TDSCs usually evaluate these criteria, as well as clonogenicity (capacity to form adherent colonies derived from one single cell) and differentiation potency, in order to compare them to each other and to BMSCs<sup>[66,67]</sup> (Table 2).

### DPSCs

DPSCs were the first TDSCs isolated and characterized in 2000<sup>[44]</sup>. Obtained from permanent third molars, these cells were found to be more proliferative than BMSCs, and had the capacity to form mineral deposits *in vitro*, though in reduced amounts compared to BMSCs<sup>[44]</sup>. DPSCs also failed to form lipid-laden adipocytes *in vitro*, whereas BMSCs are capable of differentiating into adipocytes<sup>[44]</sup>. However, more recent studies demonstrate that DPSCs can differentiate into adipocyte cells when other supplements are added to the adipogenic induction medium<sup>[46,65]</sup>.

When transplanted *in vivo*, some DPSC clones differentiate into aligned odontoblast-like cells, with prolonged processes oriented into newly formed dentin-like structures<sup>[44,46]</sup>, whereas BMSCs form distinct lamellae of bone<sup>[44]</sup>. DPSCs can also form reparative dentin-like tissue on the surface of human dentin *in vivo*<sup>[68]</sup>.

### SHED

In 2003, progenitor cells were isolated from the remnant pulp of exfoliated deciduous teeth<sup>[49]</sup>. SHED



**Table 1** Classification of stem cells according to their plasticity<sup>[1,2,41,66]</sup>

Stem cell type	Description
Totipotent	Stem cells able to differentiate into cells of all three germ layers (ectoderm, mesoderm and endoderm) and extra-embryonic tissues ( <i>e.g.</i> , zygote)
Pluripotent	Stem cells able to differentiate into all cells of the body, but that cannot form extra-embryonic tissues ( <i>e.g.</i> , embryonic stem cells and induced pluripotent stem cells)
Multipotent	Stem cells that have differentiation abilities restricted to some cell types, usually from the germ layer they are derived from ( <i>e.g.</i> , mesenchymal stem cells)

**Table 2** Characterization of tooth-derived stem cells

TDSCs	Ref.	Location	Expression markers		Differentiation capacity	
			Positive	Negative	<i>In vitro</i>	<i>In vivo</i>
DPSCs	[13,44,46,51,65]	Permanent tooth pulp	CD29, CD44, CD73, CD90, CD105, CD146, STRO-1, Oct-3/4, Sox-2, nanog	CD14, CD34, CD45	Osteoblast, adipocyte, chondrocyte, hepatocyte, neuron	Dentin-like structures
SHED	[49,51,53,69,70]	Deciduous tooth pulp	CD29, CD105, CD146, STRO-1	CD31, CD34	Osteoblast, odontoblast, adipocyte, neural cell	Dentin formation, induce bone formation by murine host cells
SCAP	[13,56,57,65]	Apical papilla of developing tooth	CD24, CD29, CD31, CD44, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, Oct-3/4, Sox-2, nanog, survivin	CD14, CD18, CD34, CD45, CD150	Osteoblast, adipocyte, chondrocyte, hepatocyte, neuron	Dentin-like tissue
DFPCs	[13,54,56,65,72]	Dental follicle of developing tooth	CD29, CD44, CD73, CD90, CD105, nestin	CD14, CD31, CD34, CD45, CD117	Osteoblast, adipocyte, chondrocyte, hepatocyte, neuron	Bone/cementum-like tissue
PDLSCs	[13,57,60,73]	Permanent tooth periodontal ligament	CD44, CD90, CD105, CD166, CD146, STRO-1, Oct-3/4, Sox2, nanog, nestin	CD14, CD34, CD34, CD45	Osteoblast/ cementoblast, adipocyte, neuron	Periodontal ligament/ cementum-like tissue
DePDL	[60]	Deciduous tooth periodontal ligament	CD105, CD166, STRO-Oct-4	CD34, CD45	Osteoblast, adipocyte	

CD: Cluster of differentiation; DePDL: Periodontal ligament of deciduous teeth stem cells; DFPCs: Dental follicle progenitor cells; DPSCs: Dental pulp stem cells; Oct: Octamer; PDLSCs: Periodontal ligament stem cells; SCAP: Stem cells from apical papilla; SHED: Stem cells from human exfoliated deciduous teeth; Sox2: SRY-box containing gene 2.

were found to be more proliferative than BMSCs and DPSCs<sup>[49,51]</sup>, and showed higher capability for osteogenic and adipogenic differentiation than DPSCs *in vitro*<sup>[51]</sup>. SHED can also differentiate into neural cells<sup>[69]</sup>. When 12 single-colony-derived SHED clones were transplanted into immunocompromised mice, only three clones demonstrated the potential to generate ectopic dentin-like tissue on the hydroxyapatite/tricalcium phosphate (HA/TCP) carrier equivalent to that generated by multiclonal-derived SHED<sup>[49]</sup>. When SHED were seeded into human tooth slices and transplanted into immunodeficient mice, they were also able to form a dentin-like structure<sup>[70]</sup>. Although some researchers claim that SHED have the ability to differentiate into osteoblasts *in vivo*<sup>[51]</sup>, Miura *et al.*<sup>[49]</sup> reported that, in fact, SHED act as an osteoinductive factor, inducing the host cells to form bone.

### SCAP

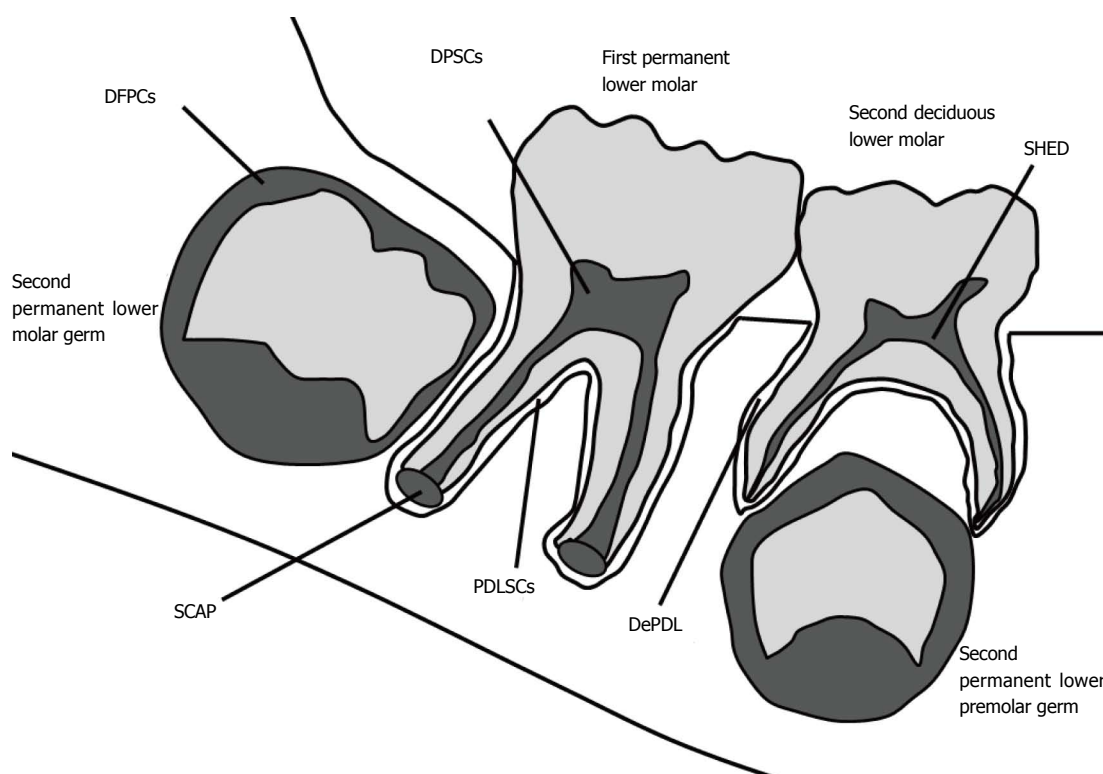
The apical papilla is the tissue located at the apex of the root of developing teeth<sup>[66]</sup>, and is distinct from the pulp<sup>[71]</sup>. As this tissue is associated with root formation, it potentially provides a source of MSCs for

this purpose. SCAP are the cells isolated from this tissue that present characteristics of MSCs, and can give rise to odontoblastic, osteoblastic and adipocyte-like cells when cultured under appropriate conditions<sup>[57]</sup>. SCAP also have the capability to form a dentin-like structure when transplanted into immunocompromised mice, using HA/TCP as a scaffold<sup>[57]</sup>. Sonoyama *et al.*<sup>[57]</sup> evaluated whether SCAP and DPSCs are the same or distinct MSC populations based on their cDNA microarray profile, and observed that many genes were differentially expressed by these MSC populations. In particular, CD24 and survivin were highly expressed in the SCAP population. Additionally, SCAP also showed other favorable characteristics, such as higher proliferative rate and telomerase activity, and improved migration capacity<sup>[57]</sup>.

### DFPCs

The dental follicle is a condensation of ectomesenchymal cells that surrounds the tooth germ in early stages of tooth formation and contains cells that form the three tissues that constitute the periodontium: periodontal ligament, cementum and alveolar bone<sup>[54,72]</sup>. When the





**Figure 1 Sources of tooth-derived stem cells.** DePDL: Periodontal ligament of deciduous teeth stem cells; DFPCs: Dental follicle progenitor cells; DPSCs: Dental pulp stem cells; PDLSCs: Periodontal ligament stem cells; SCAP: Stem cells from apical papilla; SHED: Stem cells from human exfoliated deciduous teeth.

heterogeneity of DPFCs was analyzed, it was observed that, although all cloned cell lines were positive for MSC-related surface markers (CD105, CD44, CD29) and negative for hematopoietic markers (CD34, CD117), they were different in terms of proliferation and mineralization patterns, indicating that they could be committed to distinct lineages<sup>[72]</sup>.

In order to avoid donor variability, TDSCs from follicle, pulp and papilla were isolated from a single donor tooth and the morphology, proliferation rate, expression of MSC-specific and pluripotency markers, and *in vitro* differentiation into osteoblasts, adipocytes, chondrocytes and hepatocyte-like cells were compared<sup>[65]</sup>. Adherent, fibroblast-like morphology was observed in all TDSCs cultured under the same standard conditions, and DFPCs were more proliferative than DPSCs and SCAP<sup>[65]</sup>. Although all three cell types were able to differentiate into the osteoblast lineage, DFPCs and DPSCs showed higher potentials than SCAP to form mineralized nodules *in vitro*<sup>[65]</sup>. Additionally, when cultivated under chondrogenic-inducing conditions, DFPCs expressed all three chondrogenic-specific markers (aggrecan, and type I and type III collagen), whereas DPSCs and SCAP only expressed aggrecan<sup>[65]</sup>.

### PDLSCs

The periodontal ligament harbors a heterogeneous cell population, with subsets of cells in various stages of commitment to fibroblastic and osteoblastic/cementoblastic lineages<sup>[34,73,74]</sup>. Within these subsets,

it was supposed that putative MSCs would be present in the periodontal ligament, which was confirmed in 2004<sup>[73]</sup>. PDLSCs show expression of STRO-1 and CD146, SC markers previously reported to be expressed in BMSCs and DPSCs, and also express scleraxis, a tendon-specific transcription factor<sup>[73]</sup>.

PDLSCs are more proliferative and clonogenic than BMSCs<sup>[34]</sup>. PDLSCs can also differentiate into adipocytes<sup>[34,73]</sup> and chondrocytes<sup>[34]</sup>, as well as osteoblasts/cementoblasts<sup>[34,60,73]</sup>. Although PDLSCs are able to form mineralized tissue *in vitro* when osteoblastic/cementoblastic differentiation is induced, they form fewer mineralized nodules than BMSCs<sup>[34,73]</sup>. Additionally, when transplanted into immunocompromised mice, some clones of PDLSCs have been shown to form periodontal ligament-like structures *in vivo*<sup>[73]</sup>.

### DePDL

As MSCs can be isolated from the pulp of deciduous teeth, it was thought that the periodontal ligament of deciduous teeth may also harbor MSCs<sup>[60]</sup>. In 2010, DePDL were isolated and compared with their permanent counterparts and found to be more proliferative than PDLSCs<sup>[60]</sup>. Moreover, it was observed that, although DePDL and PDLSCs have the ability to differentiate into both adipocyte-like and osteoblast-like cells *in vitro*, DePDL show a higher potential for adipogenic commitment, and PDLSCs have a higher potential for osteogenic commitment<sup>[60]</sup>.



### iPSCs from TDSCs

Following reports of reprogramming of dermal fibroblasts to behave like embryonic SCs<sup>[27,28]</sup>, studies were conducted to evaluate if other cell types could also be reprogrammed, including TDSCs<sup>[29,30]</sup>. It was reported that human gingival and periodontal ligament fibroblasts<sup>[29]</sup>, SHED, SCAP and DPSCs<sup>[30]</sup> can be reprogrammed as iPSCs, with formation of teratomas after implantation in immunocompromised mice<sup>[29,30]</sup>.

## PERSPECTIVES ON TDSCs IN REGENERATIVE APPROACHES

Tissue engineering is an emerging field based on basic science and engineering technology, designed to create suitable conditions to regenerate damaged tissues<sup>[75-78]</sup>. The basic components for tissue engineering involve an interactive triad of scaffolds<sup>[79-81]</sup>, signaling molecules<sup>[82-84]</sup> and cells<sup>[24,33,41,85]</sup>, which play a fundamental role in the regeneration process<sup>[24,76,86,87]</sup>. Scaffolds serve as a three-dimensional template mimicking the extracellular matrix; signaling molecules enhance this cellular activity by stimulating cells to migrate, proliferate and differentiate; and cells provide the machinery synthesis of the extracellular matrix and tissue regeneration<sup>[24,75,76,88,89]</sup>. Due to their interesting properties, including self-renewal and differentiation abilities, MSCs are considered important for tissue maintenance and renewal, and, therefore, a promising candidate for tissue engineering<sup>[24,90-94]</sup>.

Some studies demonstrated that cell-based therapies are able to regenerate dental tissues<sup>[48,57,70,95-98]</sup>. In a study in dogs, complete pulp regeneration was achieved when CD105<sup>+</sup> DPSCs with stromal cell-derived factor-1 were transplanted into pulp, and this was not observed when total pulp cells or CD105<sup>+</sup> adipose-derived cells were used<sup>[48]</sup>. Supplementation of guided tissue regeneration with periodontal ligament cells for the treatment of class II and III furcation defects in dogs enhances periodontal regeneration<sup>[95,97]</sup>. Twelve weeks after PDLSCs with an HA/TCP scaffold were transplanted into periodontal defects in a minipig model, new bone, cementum and periodontal ligament formation was observed<sup>[98]</sup>. Sonoyama *et al.*<sup>[57]</sup> also explored the potential of human PDLSCs and SCAP to generate a root-periodontal ligament complex in minipigs. They were able to obtain engineered roots capable of supporting porcelain, though with lower compressive strength. Nakahara<sup>[96]</sup> reported the formation of root and periodontal ligament in a new culture system using one tooth crown collected from a neonatal mouse, which was referred to as a "test-tube dental implant". The author stated that cell therapy will be the next generation of dental medicine, but further information regarding human SCs is necessary for safe and reliable clinical applications<sup>[96]</sup>.

In regard to human clinical trials, autologous progenitor cells obtained from periodontal ligament have been used to treat intrabony defects<sup>[99]</sup>. These progenitor cells were of a later cell lineage with

decreased capacity for osteogenic and adipogenic differentiation compared to PDLSCs *in vitro*. Despite this, these progenitors were able to promote improvement in clinical and radiographic parameters. Another study reported the cultivation of periodontal ligament cells on titanium pins that were subsequently implanted in patients and in dogs<sup>[91]</sup>. Clinical evaluation of the implants placed in the patients showed satisfactory mechanical function, and radiographs revealed bone filling and formation of a lamina-dura around the implants. Additionally, histologic evaluation of the implants placed in dogs revealed a ligament-like formation. Although these studies are not directly related to MSCs, they indicate that cell therapy can be a feasible clinical approach in the near future.

Despite these promising studies in cell-based tissue engineering, it is important to highlight that the best method to select the most appropriate MSC type for regenerating dental tissues is not yet clear. Although BMSCs, DPSCs, SHED, SCAP, DFPCs, PDLSCs, and DePDL present a common marker profile, they differ in their clonogenicity, proliferative ability, and differentiation potential *in vitro* and *in vivo*, suggesting that these properties are related to the microenvironments of origin of each cell lineage<sup>[13,24,34,44,60,65,67,73]</sup>. Additionally, it has been noted that, even in the same population of MSCs, there are heterogeneous cell subpopulations with distinct differentiation potentials<sup>[100]</sup>. This heterogeneity in relation to the ability to differentiate *in vitro* and to form dental tissues *in vivo* has also been reported in some TDSCs lineages, including DPSCs<sup>[44,46,67]</sup>, SHED<sup>[49]</sup>, DFPCs<sup>[72]</sup>, and PDLSCs<sup>[34,73,74,101-104]</sup>. Therefore, it can be concluded that, although there are MSC-related surface markers, such as STRO-1, CD146 and CD105, specific surface markers associated with the hierarchical commitment to differentiation pathways of TDSCs are not yet well established. In this context, further advances in understanding the regulation of MSCs during differentiation and dental development are required in order to develop new approaches for dental tissue regeneration with predictable outcomes<sup>[19,26,67,89]</sup>.

## CONCLUSION

The interest in organ regeneration using SCs has increased in the last decade. In this context, TDSCs are promising candidates, as they are readily available, highly proliferative, and present multi-differentiation abilities. Research on cell therapy for regenerating dental tissues has already been done, and shows promising results. Nevertheless, further research is needed to better characterize TDSCs and to understand their differentiation pathways in order to develop the most appropriate approaches for SC-based tissue engineering-therapies in dental practice.

## REFERENCES

- 1 Fischbach GD, Fischbach RL. Stem cells: science, policy, and



- ethics. *J Clin Invest* 2004; **114**: 1364-1370 [PMID: 15545983 DOI: 10.1172/JCI23549]
- 2 **Watt FM**, Driskell RR. The therapeutic potential of stem cells. *Philos Trans R Soc Lond B Biol Sci* 2010; **365**: 155-163 [PMID: 20008393 DOI: 10.1098/rstb.2009.0149]
- 3 **Vogel G**. Stem cells. Therapeutic cloning reaches milestone. *Science* 2014; **344**: 462-463 [PMID: 24786057 DOI: 10.1126/science.344.6183.462]
- 4 **Kaukua N**, Shahidi MK, Konstantinidou C, Dyachuk V, Kauka M, Furlan A, An Z, Wang L, Hultman I, Ahrlund-Richter L, Blom H, Brismar H, Lopes NA, Pachnis V, Suter U, Clevers H, Thesleff I, Sharpe P, Ernfors P, Fried K, Adameyko I. Glial origin of mesenchymal stem cells in a tooth model system. *Nature* 2014; **513**: 551-554 [PMID: 25079316 DOI: 10.1038/nature13536]
- 5 **Gothard D**, Roberts SJ, Shakesheff KM, Buttery LD. Engineering embryonic stem-cell aggregation allows an enhanced osteogenic differentiation in vitro. *Tissue Eng Part C Methods* 2010; **16**: 583-595 [PMID: 19751101 DOI: 10.1089/ten.TEC.2009.0462]
- 6 **Lock LT**, Tzanakakis ES. Expansion and differentiation of human embryonic stem cells to endoderm progeny in a microcarrier stirred-suspension culture. *Tissue Eng Part A* 2009; **15**: 2051-2063 [PMID: 19196140 DOI: 10.1089/ten.tea.2008.0455]
- 7 **Wang X**, Ye K. Three-dimensional differentiation of embryonic stem cells into islet-like insulin-producing clusters. *Tissue Eng Part A* 2009; **15**: 1941-1952 [PMID: 19196138 DOI: 10.1089/ten.tea.2008.0181]
- 8 **Hillel AT**, Varghese S, Petsche J, Shambloft MJ, Elisseeff JH. Embryonic germ cells are capable of adipogenic differentiation in vitro and in vivo. *Tissue Eng Part A* 2009; **15**: 479-486 [PMID: 18673089 DOI: 10.1089/ten.tea.2007.0352]
- 9 **Inanç B**, Elçin AE, Elçin YM. Human embryonic stem cell differentiation on tissue engineering scaffolds: effects of NGF and retinoic acid induction. *Tissue Eng Part A* 2008; **14**: 955-964 [PMID: 19230122 DOI: 10.1089/tea.2007.0213]
- 10 **Hyun I**. The bioethics of stem cell research and therapy. *J Clin Invest* 2010; **120**: 71-75 [PMID: 20051638 DOI: 10.1172/JCI40435]
- 11 **Hyvid Nielsen T**. What happened to the stem cells? *J Med Ethics* 2008; **34**: 852-857 [PMID: 19043108 DOI: 10.1136/jme.2007.022236]
- 12 **de Vries RB**, Oerlemans A, Trommelmans L, Dierckx K, Gordijn B. Ethical aspects of tissue engineering: a review. *Tissue Eng Part B Rev* 2008; **14**: 367-375 [PMID: 18834330 DOI: 10.1089/ten.teb.2008.0199]
- 13 **Tamaki Y**, Nakahara T, Ishikawa H, Sato S. In vitro analysis of mesenchymal stem cells derived from human teeth and bone marrow. *Odontology* 2013; **101**: 121-132 [PMID: 22772774 DOI: 10.1007/s10266-012-0075-0]
- 14 **Caplan AI**. Mesenchymal stem cells. *J Orthop Res* 1991; **9**: 641-650 [PMID: 1870029 DOI: 10.1002/jor.1100090504]
- 15 **Menicanin D**, Bartold PM, Zannettino AC, Gronthos S. Identification of a common gene expression signature associated with immature clonal mesenchymal cell populations derived from bone marrow and dental tissues. *Stem Cells Dev* 2010; **19**: 1501-1510 [PMID: 20128661 DOI: 10.1089/scd.2009.0492]
- 16 **D'Angelo F**, Armentano I, Cacciotti I, Tiribuzi R, Quattrocchi M, Del Gaudio C, Fortunati E, Saino E, Caraffa A, Cerulli GG, Visai L, Kenny JM, Sampaioles M, Bianco A, Martino S, Orlicchio A. Tuning multi/pluri-potent stem cell fate by electrospun poly(L-lactic acid)-calcium-deficient hydroxyapatite nanocomposite mats. *Biomacromolecules* 2012; **13**: 1350-1360 [PMID: 22449037 DOI: 10.1021/bm3000716]
- 17 **Voigt M**, Schauer M, Schaefer DJ, Andree C, Horch R, Stark GB. Cultured epidermal keratinocytes on a microspherical transport system are feasible to reconstitute the epidermis in full-thickness wounds. *Tissue Eng* 1999; **5**: 563-572 [PMID: 10611548 DOI: 10.1089/ten.1999.5.563]
- 18 **Blanpain C**, Fuchs E. Stem cell plasticity. Plasticity of epithelial stem cells in tissue regeneration. *Science* 2014; **344**: 1242281 [PMID: 24926024 DOI: 10.1126/science.1242281]
- 19 **Huang GT**, Al-Habib M, Gauthier P. Challenges of stem cell-based pulp and dentin regeneration: a clinical perspective. *Endod Topics* 2013; **28**: 51-60 [PMID: 23914150 DOI: 10.1111/etp.12035]
- 20 **Rada T**, Reis RL, Gomes ME. Distinct stem cells subpopulations isolated from human adipose tissue exhibit different chondrogenic and osteogenic differentiation potential. *Stem Cell Rev* 2011; **7**: 64-76 [PMID: 20396979 DOI: 10.1007/s12015-010-9147-0]
- 21 **Okura H**, Komoda H, Saga A, Kakuta-Yamamoto A, Hamada Y, Fumimoto Y, Lee CM, Ichinose A, Sawa Y, Matsuyama A. Properties of hepatocyte-like cell clusters from human adipose tissue-derived mesenchymal stem cells. *Tissue Eng Part C Methods* 2010; **16**: 761-770 [PMID: 19839740 DOI: 10.1089/ten.TEC.2009.0208]
- 22 **Hildner F**, Concaro S, Peterbauer A, Wolbank S, Danzer M, Lindahl A, Gatenholm P, Redl H, van Griensven M. Human adipose-derived stem cells contribute to chondrogenesis in coculture with human articular chondrocytes. *Tissue Eng Part A* 2009; **15**: 3961-3969 [PMID: 19586318 DOI: 10.1089/ten.TEA.2009.0002]
- 23 **Tsuji W**, Rubin JP, Marra KG. Adipose-derived stem cells: Implications in tissue regeneration. *World J Stem Cells* 2014; **6**: 312-321 [PMID: 25126381 DOI: 10.4252/wjsc.v6.i3.312]
- 24 **Han J**, Menicanin D, Gronthos S, Bartold PM. Stem cells, tissue engineering and periodontal regeneration. *Aust Dent J* 2014; **59** Suppl 1: 117-130 [PMID: 24111843 DOI: 10.1111/adj.12100]
- 25 **Tolar J**, Le Blanc K, Keating A, Blazar BR. Concise review: hitting the right spot with mesenchymal stromal cells. *Stem Cells* 2010; **28**: 1446-1455 [PMID: 20597105 DOI: 10.1002/stem.459]
- 26 **Silvério KG**, Benatti BB, Casati MZ, Sallum EA, Nociti FH. Stem cells: potential therapeutics for periodontal regeneration. *Stem Cell Rev* 2008; **4**: 13-19 [PMID: 18278569 DOI: 10.1007/s12015-008-9011-7]
- 27 **Takahashi K**, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]
- 28 **Park IH**, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 2008; **451**: 141-146 [PMID: 18157115 DOI: 10.1038/nature06534]
- 29 **Wada N**, Wang B, Lin NH, Laslett AL, Gronthos S, Bartold PM. Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts. *J Periodontol Res* 2011; **46**: 438-447 [PMID: 21443752 DOI: 10.1111/j.1600-0765.2011.01358.x]
- 30 **Yan X**, Qin H, Qu C, Tuan RS, Shi S, Huang GT. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. *Stem Cells Dev* 2010; **19**: 469-480 [PMID: 19795982 DOI: 10.1089/scd.2009.0314]
- 31 **Stadtfeld M**, Hochedlinger K. Induced pluripotency: history, mechanisms, and applications. *Genes Dev* 2010; **24**: 2239-2263 [PMID: 20952534 DOI: 10.1101/gad.1963910]
- 32 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]
- 33 **P M**, S H, R M, M G, W S K. Adult mesenchymal stem cells and cell surface characterization - a systematic review of the literature. *Open Orthop J* 2011; **5**: 253-260 [PMID: 21966340 DOI: 10.2174/1874325001105010253]
- 34 **Gay IC**, Chen S, MacDougall M. Isolation and characterization of multipotent human periodontal ligament stem cells. *Orthod Craniofac Res* 2007; **10**: 149-160 [PMID: 17651131 DOI: 10.1111/j.1601-6343.2007.00399.x]
- 35 **Pittenger MF**, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-147 [PMID: 10102814 DOI: 10.1126/science.284.5411.143]
- 36 **Greco SJ**, Liu K, Rameshwar P. Functional similarities among



- genes regulated by OCT4 in human mesenchymal and embryonic stem cells. *Stem Cells* 2007; **25**: 3143-3154 [PMID: 17761754 DOI: 10.1634/stemcells.2007-0351]
- 37 **Friedenstein AJ**, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976; **4**: 267-274 [PMID: 976387]
  - 38 **Friedenstein AJ**, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: in vitro cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet* 1987; **20**: 263-272 [PMID: 3690622]
  - 39 **Jurga M**, Lipkowski AW, Lukomska B, Buzanska L, Kurzepa K, Sobanski T, Habich A, Coecke S, Gajkowska B, Domanska-Janik K. Generation of functional neural artificial tissue from human umbilical cord blood stem cells. *Tissue Eng Part C Methods* 2009; **15**: 365-372 [PMID: 19719393 DOI: 10.1089/ten.tec.2008.0485]
  - 40 **He F**, Chen X, Pei M. Reconstruction of an in vitro tissue-specific microenvironment to rejuvenate synovium-derived stem cells for cartilage tissue engineering. *Tissue Eng Part A* 2009; **15**: 3809-3821 [PMID: 19545204 DOI: 10.1089/ten.TEA.2009.0188]
  - 41 **Reinke S**, Dienelt A, Blankenstein A, Duda GN, Geissler S. Qualifying stem cell sources: how to overcome potential pitfalls in regenerative medicine? *J Tissue Eng Regen Med* 2014 Jun 12; Epub ahead of print [PMID: 24919850 DOI: 10.1002/term.1923]
  - 42 **Olson HE**, Rooney GE, Gross L, Nesbitt JJ, Galvin KE, Knight A, Chen B, Yaszemski MJ, Windebank AJ. Neural stem cell- and Schwann cell-loaded biodegradable polymer scaffolds support axonal regeneration in the transected spinal cord. *Tissue Eng Part A* 2009; **15**: 1797-1805 [PMID: 19191513 DOI: 10.1089/ten.tea.2008.0364]
  - 43 **Vellasamy S**, Sandrasaigaran P, Vidyadaran S, George E, Ramasamy R. Isolation and characterisation of mesenchymal stem cells derived from human placenta tissue. *World J Stem Cells* 2012; **4**: 53-61 [PMID: 22993662 DOI: 10.4252/wjsc.v4.i6.53]
  - 44 **Gronthos S**, Mankani M, Brahimi J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci USA* 2000; **97**: 13625-13630 [PMID: 11087820 DOI: 10.1073/pnas.240309797]
  - 45 **Yang X**, Walboomers XF, van den Beucken JJ, Bian Z, Fan M, Jansen JA. Hard tissue formation of STRO-1-selected rat dental pulp stem cells in vivo. *Tissue Eng Part A* 2009; **15**: 367-375 [PMID: 18652538 DOI: 10.1089/ten.tea.2008.0133]
  - 46 **Gronthos S**, Brahimi J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002; **81**: 531-535 [PMID: 12147742 DOI: 10.1177/154405910208100806]
  - 47 **Zhang W**, Walboomers XF, Wolke JG, Bian Z, Fan MW, Jansen JA. Differentiation ability of rat postnatal dental pulp cells in vitro. *Tissue Eng* 2005; **11**: 357-368 [PMID: 15869416 DOI: 10.1089/ten.2005.11.357]
  - 48 **Iohara K**, Imabayashi K, Ishizaka R, Watanabe A, Nabekura J, Ito M, Matsushita K, Nakamura H, Nakashima M. Complete pulp regeneration after pulpectomy by transplantation of CD105+ stem cells with stromal cell-derived factor-1. *Tissue Eng Part A* 2011; **17**: 1911-1920 [PMID: 21417716 DOI: 10.1089/ten.TEA.2010.0615]
  - 49 **Miura M**, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* 2003; **100**: 5807-5812 [PMID: 12716973 DOI: 10.1073/pnas.0937635100]
  - 50 **Fu X**, Jin L, Ma P, Fan Z, Wang S. Allogeneic stem cells from deciduous teeth in treatment for periodontitis in miniature swine. *J Periodontol* 2014; **85**: 845-851 [PMID: 24001042 DOI: 10.1902/jop.2013.130254]
  - 51 **Wang X**, Sha XJ, Li GH, Yang FS, Ji K, Wen LY, Liu SY, Chen L, Ding Y, Xuan K. Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells. *Arch Oral Biol* 2012; **57**: 1231-1240 [PMID: 22455989 DOI: 10.1016/j.archoralbio.2012.02.014]
  - 52 **Zheng Y**, Liu Y, Zhang CM, Zhang HY, Li WH, Shi S, Le AD, Wang SL. Stem cells from deciduous tooth repair mandibular defect in swine. *J Dent Res* 2009; **88**: 249-254 [PMID: 19329459 DOI: 10.1177/0022034509333804]
  - 53 **Behnia A**, Haghighat A, Talebi A, Nourbakhsh N, Heidari F. Transplantation of stem cells from human exfoliated deciduous teeth for bone regeneration in the dog mandibular defect. *World J Stem Cells* 2014; **6**: 505-510 [PMID: 25258673 DOI: 10.4252/wjsc.v6.i4.505]
  - 54 **Morsczech C**, Götz W, Schierholz J, Zeilhofer F, Kühn U, Möhl C, Sippel C, Hoffmann KH. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biol* 2005; **24**: 155-165 [PMID: 15890265 DOI: 10.1016/j.matbio.2004.12.004]
  - 55 **Silvério KG**, Davidson KC, James RG, Adams AM, Foster BL, Nociti FH, Somerman MJ, Moon RT. Wnt/ $\beta$ -catenin pathway regulates bone morphogenetic protein (BMP2)-mediated differentiation of dental follicle cells. *J Periodontol Res* 2012; **47**: 309-319 [PMID: 22150562 DOI: 10.1111/j.1600-0765.2011.01433.x]
  - 56 **Guo L**, Li J, Qiao X, Yu M, Tang W, Wang H, Guo W, Tian W. Comparison of odontogenic differentiation of human dental follicle cells and human dental papilla cells. *PLoS One* 2013; **8**: e62332 [PMID: 23620822 DOI: 10.1371/journal.pone.0062332]
  - 57 **Sonoyama W**, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Wang S, Shi S. Mesenchymal stem cell-mediated functional tooth regeneration in swine. *PLoS One* 2006; **1**: e79 [PMID: 17183711 DOI: 10.1371/journal.pone.0000079]
  - 58 **Zhang W**, Zhang X, Ling J, Liu W, Zhang X, Ma J, Zheng J. Proliferation and odontogenic differentiation of BMP2 gene-transfected stem cells from human tooth apical papilla: an in vitro study. *Int J Mol Med* 2014; **34**: 1004-1012 [PMID: 25070743 DOI: 10.3892/ijmm.2014.1862]
  - 59 **Wang J**, Liu B, Gu S, Liang J. Effects of Wnt/ $\beta$ -catenin signalling on proliferation and differentiation of apical papilla stem cells. *Cell Prolif* 2012; **45**: 121-131 [PMID: 22288815 DOI: 10.1111/j.1365-2184.2012.00806.x]
  - 60 **Silvério KG**, Rodrigues TL, Coletta RD, Benevides L, Da Silva JS, Casati MZ, Sallum EA, Nociti FH. Mesenchymal stem cell properties of periodontal ligament cells from deciduous and permanent teeth. *J Periodontol* 2010; **81**: 1207-1215 [PMID: 20476882 DOI: 10.1902/jop.2010.090729]
  - 61 **Fukushima H**, Kawanabe N, Murata S, Ishihara Y, Yanagita T, Balam TA, Yamashiro T. SSEA-4 is a marker of human deciduous periodontal ligament stem cells. *J Dent Res* 2012; **91**: 955-960 [PMID: 22895512 DOI: 10.1177/0022034512458123]
  - 62 **Ji K**, Liu Y, Lu W, Yang F, Yu J, Wang X, Ma Q, Yang Z, Wen L, Xuan K. Periodontal tissue engineering with stem cells from the periodontal ligament of human retained deciduous teeth. *J Periodontol Res* 2013; **48**: 105-116 [PMID: 22881344 DOI: 10.1111/j.1600-0765.2012.01509.x]
  - 63 **Chen FM**, Sun HH, Lu H, Yu Q. Stem cell-delivery therapeutics for periodontal tissue regeneration. *Biomaterials* 2012; **33**: 6320-6344 [PMID: 22695066 DOI: 10.1016/j.biomaterials.2012.05.048]
  - 64 **Morad G**, Kheiri L, Khojasteh A. Dental pulp stem cells for in vivo bone regeneration: a systematic review of literature. *Arch Oral Biol* 2013; **58**: 1818-1827 [PMID: 24095289 DOI: 10.1016/j.archoralbio.2013.08.011]
  - 65 **Patil R**, Kumar BM, Lee WJ, Jeon RH, Jang SJ, Lee YM, Park BW, Byun JH, Ahn CS, Kim JW, Rho GJ. Multilineage potential and proteomic profiling of human dental stem cells derived from a single donor. *Exp Cell Res* 2014; **320**: 92-107 [PMID: 24162002 DOI: 10.1016/j.yexcr.2013.10.005]
  - 66 **Sedgley CM**, Botero TM. Dental stem cells and their sources. *Dent Clin North Am* 2012; **56**: 549-561 [PMID: 22835537 DOI: 10.1016/j.cden.2012.05.004]
  - 67 **Huang GT**, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res* 2009; **88**: 792-806 [PMID: 19767575 DOI: 10.1177/0022034509340867]
  - 68 **Batouli S**, Miura M, Brahimi J, Tsutsui TW, Fisher LW, Gronthos S, Robey PG, Shi S. Comparison of stem-cell-mediated osteogenesis and dentinogenesis. *J Dent Res* 2003; **82**: 976-981 [PMID: 14630898 DOI: 10.1177/154405910308201208]
  - 69 **Nourbakhsh N**, Soleimani M, Taghipour Z, Karbalaie K, Mousavi SB, Talebi A, Nadali F, Tanhaei S, Kiyani GA, Nematollahi M,



- Rabiei F, Mardani M, Bahramiyan H, Torabinejad M, Nasr-Esfahani MH, Baharvand H. Induced in vitro differentiation of neural-like cells from human exfoliated deciduous teeth-derived stem cells. *Int J Dev Biol* 2011; **55**: 189-195 [PMID: 21671222 DOI: 10.1387/ijdb.103090nn]
- 70 Cordeiro MM, Dong Z, Kaneko T, Zhang Z, Miyazawa M, Shi S, Smith AJ, Nör JE. Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. *J Endod* 2008; **34**: 962-969 [PMID: 18634928 DOI: 10.1016/j.joen.2008.04.009]
- 71 Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, Huang GT. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod* 2008; **34**: 166-171 [PMID: 18215674 DOI: 10.1016/j.joen.2007.11.021]
- 72 Luan X, Ito Y, Dangaria S, Diekwisch TG. Dental follicle progenitor cell heterogeneity in the developing mouse periodontium. *Stem Cells Dev* 2006; **15**: 595-608 [PMID: 16978062 DOI: 10.1089/scd.2006.15.595]
- 73 Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahimi J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004; **364**: 149-155 [PMID: 15246727 DOI: 10.1016/S0140-6736(04)16627-0]
- 74 Saito MT, Salmon CR, Amorim BR, Ambrosano GM, Casati MZ, Sallum EA, Nociti FH, Silvério KG. Characterization of highly osteoblast/cementoblast cell clones from a CD105-enriched periodontal ligament progenitor cell population. *J Periodontol* 2014; **85**: e205-e211 [PMID: 24579765 DOI: 10.1902/jop.2014.130461]
- 75 Scheller EL, Krebsbach PH, Kohn DH. Tissue engineering: state of the art in oral rehabilitation. *J Oral Rehabil* 2009; **36**: 368-389 [PMID: 19228277 DOI: 10.1111/j.1365-2842.2009.01939.x]
- 76 Bartold PM, McCulloch CA, Narayanan AS, Pitaru S. Tissue engineering: a new paradigm for periodontal regeneration based on molecular and cell biology. *Periodontol 2000* 2000; **24**: 253-269 [PMID: 11276871 DOI: 10.1034/j.1600-0757.2000.2240113.x]
- 77 Slavkin HC, Bartold PM. Challenges and potential in tissue engineering. *Periodontol 2000* 2006; **41**: 9-15 [PMID: 16686923 DOI: 10.1111/j.1600-0757.2006.00172.x]
- 78 Benatti BB, Silvério KG, Casati MZ, Sallum EA, Nociti FH. Physiological features of periodontal regeneration and approaches for periodontal tissue engineering utilizing periodontal ligament cells. *J Biosci Bioeng* 2007; **103**: 1-6 [PMID: 17298893 DOI: 10.1263/jbb.103.1]
- 79 Liao F, Chen Y, Li Z, Wang Y, Shi B, Gong Z, Cheng X. A novel bioactive three-dimensional beta-tricalcium phosphate/chitosan scaffold for periodontal tissue engineering. *J Mater Sci Mater Med* 2010; **21**: 489-496 [PMID: 19908128 DOI: 10.1007/s10856-009-3931-x]
- 80 Cunniffe GM, Dickson GR, Partap S, Stanton KT, O'Brien FJ. Development and characterization of a collagen nano-hydroxyapatite composite scaffold for bone tissue engineering. *J Mater Sci Mater Med* 2010; **21**: 2293-2298 [PMID: 20091099 DOI: 10.1007/s10856-009-3964-1]
- 81 Ahn S, Yoon H, Kim G, Kim Y, Lee S, Chun W. Designed three-dimensional collagen scaffolds for skin tissue regeneration. *Tissue Eng Part C Methods* 2010; **16**: 813-820 [PMID: 20001740 DOI: 10.1089/ten.tec.2009.0511]
- 82 Yang L, Zhang Y, Dong R, Peng L, Liu X, Wang Y, Cheng X. Effects of adenoviral-mediated coexpression of bone morphogenetic protein-7 and insulin-like growth factor-1 on human periodontal ligament cells. *J Periodontol Res* 2010; **45**: 532-540 [PMID: 20412417 DOI: 10.1111/j.1600-0765.2009.01268.x]
- 83 Kao RT, Murakami S, Beirne OR. The use of biologic mediators and tissue engineering in dentistry. *Periodontol 2000* 2009; **50**: 127-153 [PMID: 19388957 DOI: 10.1111/j.1600-0757.2008.00287.x]
- 84 Saito A, Saito E, Handa R, Honma Y, Kawanami M. Influence of residual bone on recombinant human bone morphogenetic protein-2-induced periodontal regeneration in experimental periodontitis in dogs. *J Periodontol* 2009; **80**: 961-968 [PMID: 19485827 DOI: 10.1902/jop.2009.080568]
- 85 Egusa H, Sonoyama W, Nishimura M, Atsuta I, Akiyama K. Stem cells in dentistry--part I: stem cell sources. *J Prosthodont Res* 2012; **56**: 151-165 [PMID: 22796367 DOI: 10.1016/j.jpor.2012.06.001]
- 86 Zhang Y, Wang Y, Shi B, Cheng X. A platelet-derived growth factor releasing chitosan/coral composite scaffold for periodontal tissue engineering. *Biomaterials* 2007; **28**: 1515-1522 [PMID: 17169421 DOI: 10.1016/j.biomaterials.2006.11.040]
- 87 Nakahara T, Nakamura T, Kobayashi E, Inoue M, Shigeno K, Tabata Y, Eto K, Shimizu Y. Novel approach to regeneration of periodontal tissues based on in situ tissue engineering: effects of controlled release of basic fibroblast growth factor from a sandwich membrane. *Tissue Eng* 2003; **9**: 153-162 [PMID: 12625964 DOI: 10.1089/107632703762687636]
- 88 Tabata M, Jin Q, Sugai JV, Giannobile WV. Current concepts in periodontal bioengineering. *Orthod Craniofac Res* 2005; **8**: 292-302 [PMID: 16238610 DOI: 10.1111/j.1601-6343.2005.00352.x]
- 89 Feng R, Lengner C. Application of Stem Cell Technology in Dental Regenerative Medicine. *Adv Wound Care (New Rochelle)* 2013; **2**: 296-305 [PMID: 24527351 DOI: 10.1089/wound.2012.0375]
- 90 Egusa H, Sonoyama W, Nishimura M, Atsuta I, Akiyama K. Stem cells in dentistry--Part II: Clinical applications. *J Prosthodont Res* 2012; **56**: 229-248 [PMID: 23137671 DOI: 10.1016/j.jpor.2012.10.001]
- 91 Gault P, Black A, Romette JL, Fuente F, Schroeder K, Thillou F, Brune T, Berdal A, Wurtz T. Tissue-engineered ligament: implant constructs for tooth replacement. *J Clin Periodontol* 2010; **37**: 750-758 [PMID: 20546087 DOI: 10.1111/j.1600-051X.2010.01588.x]
- 92 Koch L, Kuhn S, Sorg H, Gruene M, Schlie S, Gaebel R, Polchow B, Reimers K, Stoelting S, Ma N, Vogt PM, Steinhoff G, Chichkov B. Laser printing of skin cells and human stem cells. *Tissue Eng Part C Methods* 2010; **16**: 847-854 [PMID: 19883209 DOI: 10.1089/ten.tec.2009.0397]
- 93 Lin NH, Gronthos S, Bartold PM. Stem cells and future periodontal regeneration. *Periodontol 2000* 2009; **51**: 239-251 [PMID: 19878478 DOI: 10.1111/j.1600-0757.2009.00303.x]
- 94 Geuze RE, Wegman F, Oner FC, Dhert WJ, Alblas J. Influence of endothelial progenitor cells and platelet gel on tissue-engineered bone ectopically in goats. *Tissue Eng Part A* 2009; **15**: 3669-3677 [PMID: 19499998 DOI: 10.1089/ten.TEA.2009.0289]
- 95 Suaid FF, Ribeiro FV, Rodrigues TL, Silvério KG, Carvalho MD, Nociti FH, Casati MZ, Sallum EA. Autologous periodontal ligament cells in the treatment of class II furcation defects: a study in dogs. *J Clin Periodontol* 2011; **38**: 491-498 [PMID: 21392047 DOI: 10.1111/j.1600-051X.2011.01715.x]
- 96 Nakahara T. Potential feasibility of dental stem cells for regenerative therapies: stem cell transplantation and whole-tooth engineering. *Odontology* 2011; **99**: 105-111 [PMID: 21805289 DOI: 10.1007/s10266-011-0037-y]
- 97 Suaid FF, Ribeiro FV, Gomes TR, Silvério KG, Carvalho MD, Nociti FH, Casati MZ, Sallum EA. Autologous periodontal ligament cells in the treatment of Class III furcation defects: a study in dogs. *J Clin Periodontol* 2012; **39**: 377-384 [PMID: 22332838 DOI: 10.1111/j.1600-051X.2012.01858.x]
- 98 Liu Y, Zheng Y, Ding G, Fang D, Zhang C, Bartold PM, Gronthos S, Shi S, Wang S. Periodontal ligament stem cell-mediated treatment for periodontitis in miniature swine. *Stem Cells* 2008; **26**: 1065-1073 [PMID: 18238856 DOI: 10.1634/stemcells.2007-0734]
- 99 Feng F, Akiyama K, Liu Y, Yamaza T, Wang TM, Chen JH, Wang BB, Huang GT, Wang S, Shi S. Utility of PDL progenitors for in vivo tissue regeneration: a report of 3 cases. *Oral Dis* 2010; **16**: 20-28 [PMID: 20355278 DOI: 10.1111/j.1601-0825.2009.01593.x]
- 100 Okamoto T, Aoyama T, Nakayama T, Nakamata T, Hosaka T, Nishijo K, Nakamura T, Kiyono T, Toguchida J. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem Biophys Res Commun* 2002; **295**: 354-361 [PMID: 12150956 DOI: 10.1016/S0006-291X(02)00661-7]
- 101 Singhatanadgit W, Donos N, Olsen I. Isolation and characterization of stem cell clones from adult human ligament. *Tissue Eng Part A* 2009; **15**: 2625-2636 [PMID: 19207044 DOI: 10.1089/ten.TEA.2008.0442]



- 102 **Fujii S**, Maeda H, Wada N, Tomokiyo A, Saito M, Akamine A. Investigating a clonal human periodontal ligament progenitor/stem cell line in vitro and in vivo. *J Cell Physiol* 2008; **215**: 743-749 [PMID: 18181171 DOI: 10.1002/jcp.21359]
- 103 **Wang L**, Shen H, Zheng W, Tang L, Yang Z, Gao Y, Yang Q, Wang C, Duan Y, Jin Y. Characterization of stem cells from alveolar periodontal ligament. *Tissue Eng Part A* 2011; **17**: 1015-1026 [PMID: 21186958 DOI: 10.1089/ten.tea.2010.0140]
- 104 **Sununliganon L**, Singhatanadgit W. Highly osteogenic PDL stem cell clones specifically express elevated levels of ICAM1, ITGB1 and TERT. *Cytotechnology* 2012; **64**: 53-63 [PMID: 21866310 DOI: 10.1007/s10616-011-9390-5]

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## Could cancer and infection be adverse effects of mesenchymal stromal cell therapy?

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article reviews the effect of MSCs on tumor establishment, growth and metastasis and also susceptibility to infection and its progression. Data published to date shows a paradoxical effect regarding MSCs, which seems to depend on isolation and expansion, cells source and dose and the route and timing of administration. Cancer and infection may thus be adverse or therapeutic effects arising from MSC administration.

**Key words:** Cancer; Infection; Mesenchymal stem cells; Therapy; Biosafety

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**Core tip:** Mesenchymal stem cells (MSCs) derived from different origins have recently received much attention as potential therapeutic. However, such cells also appear to have essential functions in building and supporting tumor microenvironments. Here, we review the effect of MSCs on tumor establishment, as also susceptibility to infection and its progression. The literature reveals incongruity regarding the impact of MSCs on the development of cancer and infection; such paradoxical effect might be attributed to differences in isolation and expansion conditions, the source and dose of the cells, the administration route and its timing and host characteristics. MSCs immunomodulatory potential seems to be the leading mechanism responsible for such effects.

### Abstract

Multipotent mesenchymal stromal cells [also referred to as mesenchymal stem cells (MSCs)] are a heterogeneous subset of stromal cells. They can be isolated from bone marrow and many other types of tissue. MSCs are currently being tested for therapeutic purposes (*i.e.*, improving hematopoietic stem cell engraftment, managing inflammatory diseases and regenerating damaged organs). Their tropism for tumors and inflamed sites and their context-dependent potential for producing trophic and immunomodulatory factors raises the question as to whether MSCs promote cancer and/or infection. This

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### INTRODUCTION

Multipotent mesenchymal stromal cells, also referred



to as mesenchymal stem cells (MSCs), were described for the first time half a century ago<sup>[1]</sup>. Such cells are distributed throughout the stroma of several organs *in vivo* whilst MSCs adhere to plastic *in vitro* and proliferate when stimulated by fetal bovine serum<sup>[1]</sup>. MSCs differentiate into mesodermal cells *in vitro* and *in vivo* (*i.e.*, adipocytes, chondrocytes, osteocytes and myocytes)<sup>[2]</sup>. They can also cross the germ line barrier and produce cells from endo- and ectodermal lineages, such property being known as cell plasticity<sup>[3]</sup>.

MSCs are an ideal tool for cell therapy because they are easily procured from live donors<sup>[4]</sup> and can be efficiently expanded *ex vivo*<sup>[5]</sup>. The receptors do not need to have been conditioned before cell administration transplant<sup>[6]</sup>, as in total bone marrow or hematopoietic stem cell transplant. Once administered intravenously, they are able to home onto and engraft into damaged tissue where they could become differentiated into tissue-specific cells, release trophic factors, promote neovascularization, manage oxidative stress and fibrosis, or trigger an anti-inflammatory response<sup>[7-11]</sup>.

MSCs from the same individual (autologous) were administered into a human for the first time in 1995<sup>[12]</sup>; MSCs were safely allogeneically transplanted seven years later<sup>[13]</sup>. More than 350 clinical trials involving the use of MSCs are currently under way ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)) and no serious adverse events have been reported to date. Nevertheless, MSCs biosafety is still a major concern, particularly regarding the development of adverse event-related cancer and infection.

## MSCs AND CANCER

### MSCs might form tumors

Like any other cell, when MSCs are manipulated in the long-term they might have chromosomal aberrations and produce tumors in healthy animals<sup>[14]</sup>; this has mainly been reported regarding mouse cells, which require extensive cultures for producing a significant number of hematopoietic-free MSCs<sup>[14]</sup>. For instance, it has been shown that intravenously administered NOC/SCID bone marrow-derived MSCs embolize within the lung capillaries, expand and invade the lung parenchyma and form tumor nodules<sup>[15]</sup>. These lesions rarely contain lung epithelial cells but they have the characteristics of cartilage and immature bone resembling well-differentiated osteosarcoma.

No transformation has been proven so far for human MSCs when expanded properly *ex vivo* (*i.e.*, non-exhausted and not forced to cell crisis)<sup>[14]</sup>. The Canadian Critical Care Trials Group has recently published a meta-analysis of randomized, non-randomized, controlled and uncontrolled, phase I and phase II clinical trials<sup>[16]</sup>; no association between autologous or allogeneic MSCs administration and tumor formation was reported in the 36 studies reviewed by them. Nonetheless, longer follow-up is required to draw a final conclusion regarding human

MSCs' tumorigenic potential.

### MSCs may promote tumor growth

Human bone marrow-derived MSCs have increased the growth of ERA $\alpha$  positive breast cancer cell lines (T47D, BT474 and ZR-75-1) in an *in vitro* three-dimensional tumor environment, but have had no effect on an ERA $\alpha$  negative cell line (MDA-MB-231)<sup>[17]</sup>; however, the growth rate of another ERA $\alpha$  negative cell line (MDA-MB-468) was high in the presence of human MSCs. Another study has shown that both human fetal MSCs and human adipose-derived MSCs transplanted subcutaneously into BALB/c-nu/nu mice alone or together with tumor cell lines F6 or SW480 (ratio 1:1 or 1:10), favored the growth of these tumor cell lines<sup>[18]</sup>.

Tumor cells obtained from primary breast cancer grown in the presence of human bone marrow-derived MSCs (ratio 1:1) and tested in secondary mice have been seen to have greater tumor-producing ability than cells obtained from primary tumors and grown in the absence of MSCs<sup>[19]</sup>. Besides, tumor incidence and/or size<sup>[18,20,21]</sup> as well as tumor vascularity<sup>[22]</sup> have all increased when breast, lung, colon or prostate tumor cells have been co-injected with human adipose-derived or bone marrow-derived MSCs. The same has been proven for osteosarcoma, melanoma and glioma tumor cells<sup>[23]</sup>. Another interesting observation concerned adipose tissue implant adjacent to lung cancer or Kaposi sarcoma xenografts resulting in a substantial increase in tumor size along with the appearance of stromal cells from the implant; adipose-derived MSCs can thus promote tumor growth<sup>[24]</sup>.

MSCs' innate tropism for established tumors has been widely reported<sup>[24]</sup>, yet the mechanism behind it still remains to be fully elucidated<sup>[25]</sup>. The explanation advanced to date is that tumors behave as unresolved wounds as their stroma closely resemble healing granulation tissue and they produce cytokines, chemokines and other chemoattractants<sup>[26]</sup> and MSCs chemotactic properties are similar to those of leukocytes<sup>[27,28]</sup>. MSCs tropism for tumors has been successfully exploited for the delivery of antitumor agents in animal models of lung and breast cancer and melanoma and glioma<sup>[25]</sup>.

### MSCs might promote metastasis

Breast cancer cells co-cultured with human bone marrow-derived MSCs (ratio 1:1) up-regulate the expression of oncogenes and proto-oncogenes associated with tissue invasion, angiogenesis and apoptosis (*i.e.*, N-cadherin, vimentin, Twist, Snail and E-cadherin)<sup>[29]</sup>. Such molecular changes have been accompanied by morphological and growth alterations, these being features of a more metastatic phenotype. It has been seen that  $0.5 \times 10^5$  breast cancer cells co-injected subcutaneously with  $1.3 \times 10^6$  human bone marrow-derived MSCs have significantly increased lung metastasis rate in NOD/SCID mice. This effect was lost when bone marrow-derived MSCs were injected



separately from tumor cells<sup>[20]</sup>. On the other hand, it has been shown that bone marrow-derived MSCs facilitate cancer cells [MCF-7, T47D low invasive cell lines and stromal cell-derived factor 1 (SDF-1)<sup>null</sup> MDA-MB-231 highly aggressive ones] homing into bone marrow and have modified the metastatic niche through trophic factor secretion (SDF-1 and CXCR4) and improved neovasculation in a xenogeneic mouse model<sup>[30]</sup>.

### **MSCs might inhibit tumor growth**

It has been shown that human bone marrow-derived MSCs interfere *in vitro* with small cell lung cancer (A549), esophageal cancer (Eca-109), Kaposi's sarcoma and leukemic cell line proliferation kinetics<sup>[31]</sup>. The foregoing was observed when  $0.5 \times 10^5$  tumor cells were co-cultivated with  $0.5 \times 10^5$  human bone marrow-derived MSCs but also when they were exposed to MSCs-conditioned medium; cells were arrested during the cell cycle G<sub>1</sub> phase in both cases by the downregulation of cyclin D2 and induction of apoptosis<sup>[32,33]</sup>. MSCs from other sources, including human fetal skin-derived MSCs and adipose-derived MSCs, have also inhibited the growth of human liver cancer cell lines<sup>[34]</sup>, breast cancer (MCF-7)<sup>[35]</sup> and primary leukemia cells by reducing their proliferation, colony formation and oncogene expression<sup>[22]</sup>. The intravenous injection of  $4 \times 10^6$  human bone marrow-derived MSCs into Kaposi's sarcoma-bearing nude mice has inhibited tumor cell growth<sup>[36]</sup>. A similar effect has been observed in an animal model of hepatocellular carcinoma and pancreatic tumors as altering cell cycle progression has led to decreased cell proliferation<sup>[22,37]</sup>; the same has happened with melanoma due to increased apoptosis of capillaries<sup>[38]</sup> and rat colon carcinoma growth has been inhibited when rat MSCs (the MPC1cE cell line) were co-implanted with tumor cells in a 1:1 or 1:10 ratio<sup>[39]</sup>.

Human fetal skin-derived MSCs (Z3 cell line) have also delayed liver tumor growth and decreased tumor size when injected with the same number of cells from the H7402 cell line in SCID mice<sup>[34]</sup>. Injecting human adipose-derived MSCs ( $1 \times 10^3$  cells/mm<sup>3</sup>) into established pancreatic cancer xenografts has led to apoptosis and the abrogation of tumor growth in female Swiss nude (athymic) mice<sup>[37]</sup>.

The role of MSCs in cancer thus remains paradoxical. Evidence to date has suggested that they are pro- as well as anti-tumorigenic<sup>[40-42]</sup> such discrepancy seems to depend on isolation and expansion conditions, cell source and dose, the administration route and the tumor model used.

## **MSCs AND INFECTION**

### **MSCs might increase infection**

MSCs can be recruited into inflamed sites secondary to microbial infection where they promote potent immune-suppressive activity<sup>[43,44]</sup>. For instance, it has

been shown that administering MSCs ( $1.25 \times 10^5$  cells/kg) to animals infected by *Trypanosoma cruzi* (*T. cruzi*, protozoa) or *Mycobacterium tuberculosis* (*Mtb*, bacteria) has worsened the natural course of infection. Activated macrophages play an essential role in host defense against *T. cruzi* as they can destroy intracellular parasites *via* interferon (INF)- $\gamma$ - and tumor necrosis factor (TNF)- $\alpha$ -stimulated nitric oxide (NO) production<sup>[45]</sup>. It has been shown that mice bone marrow-derived MSCs switch macrophages to an anti-inflammatory profile, thereby suppressing inflammatory cytokine production and enhancing interleukin (IL)-10 production<sup>[46]</sup>. An immune response to *Mtb* depends on IFN- $\gamma$ -producing T-lymphocytes activating macrophages to produce NO<sup>[47,48]</sup>. Bone marrow-derived MSCs ( $2.5 \times 10^5$ /kg) infusion into animals, which are normally resistant to this infection [transforming growth factor  $\beta$  (TGF- $\beta$ ) RIIDN transgenic mice], has resulted in making them susceptible to disease. Furthermore, it has been observed that donor MSCs have been recruited to the periphery of live bacteria-containing granuloma and have induced regulatory T-cell differentiation, thus resulting in immunosuppression.

A recent study aimed to prove the safety and feasibility of autologous bone marrow-derived MSCs infusion ( $1 \times 10^6$  cells/kg, two doses) into kidney allograft recipients, showing that three out of six enrolled patients developed an opportunistic viral infection<sup>[49]</sup>.

### **MSCs might decrease infection**

Regarding fungal infection, the intravenous administration of an IL-17-producing sub-population of bone marrow derived-MSCs ( $1 \times 10^6$  cells) significantly reduced the fungal burden of kidneys in immunocompetent mice, which had suffered invasive candidiasis<sup>[50]</sup>.

Both un-stimulated and IFN- $\gamma$  stimulated human MSCs can inhibit the growth of Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, as well as the growth of Gram-positive pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, group B *Streptococci* and *Enterococcus faecium*<sup>[46,51]</sup>. MSCs' antimicrobial effect depends on whether they have been stimulated<sup>[51]</sup>; while cathelicidin LL-37 antimicrobial peptide is critical to un-stimulated human MSCs, tryptophan-catabolizing enzyme heme oxygenase-1 and indoleamine-2,3-dioxygenase (IDO) are needed in IFN- $\gamma$ -stimulated human MSCs. Tryptophan depletion and toxic kynurenine accumulation leads to the inhibition of bacterial growth in the latter case. Differences between human and murine MSCs antibacterial activity have also been reported; murine MSCs do not produce cathelicidin LL-37 and cannot express IDO, even after stimulation with a combination of cytokines, but they do produce lipocalin 2 (an antimicrobial molecule)<sup>[52]</sup>.

Little data has been published concerning the



impact of MSCs on viral pathogens. One study has reported that IFN- $\gamma$ -stimulated human MSCs have reduced intracellular replication of cytomegalovirus and herpes simplex virus type 1 *in vitro*<sup>[46]</sup>, such effect being attributed to IDO activity<sup>[46]</sup>.

Together with MSCs' direct antimicrobial effect, it has been shown that they play an important role in the complex network of host immune response against pathogens, particularly regarding the dynamic coordination of the immune system's pro- and anti-inflammatory components<sup>[53]</sup>.

MSCs' antimicrobial activity observed *in vitro* has been clearly supported by animal models of experimental infection, such as polymicrobial sepsis<sup>[42]</sup>, lipopolysaccharide (LPS) administration<sup>[54]</sup> and pulmonary respiratory distress syndrome<sup>[55]</sup>. Regardless of MSCs source, administration route (intravenously *cf* intraperitoneally) or strategy (prophylaxis *cf* therapy), their administration leads to reduced pathogen burden and significantly improved survival rate<sup>[41,42,53,56]</sup>.

It has been shown that administering  $2.5 \times 10^5$  mouse bone marrow-derived MSCs led to decreased mortality, controlled multi-organ dysfunction/injury and reduced pulmonary and systemic inflammation in a clinically relevant model of polymicrobial sepsis where infection was settled after the inoculation of Gram-negative and Gram-positive organisms<sup>[42]</sup>. An endotoxemic rat model (involving intravenous LPS injection) has been used to demonstrate that administering  $2.5 \times 10^5$  human adipose-derived MSCs decreased inflammatory cytokine level in serum and the lungs, reduced inflammatory changes in the lungs, prevented apoptosis in the kidneys and reduced multi-organ injury<sup>[54]</sup>. A pulmonary respiratory distress syndrome model (induced by intratracheal endotoxin administration) has been used to show that the intrapulmonary delivery of mouse bone marrow-derived MSCs has down-regulated an LPS-induced inflammatory response and reduced lung injury, while direct lung injury by toxins or pneumonitis led to severe pulmonary edema and inflammation<sup>[57,58]</sup>.

MSCs' antimicrobial effect has also been demonstrated in the blood, peritoneum, liver and spleen, using a Gram-negative pneumonia model involving immunocompetent mice<sup>[42,44,56]</sup>.

As MSCs might lessen the development of infection, they have been used recently in a clinical study aimed at treating patients suffering acute respiratory distress syndrome (NCT01902082); one intravenous dose of  $1 \times 10^6$  cells/kg allogeneic adipose-derived MSCs proved to represent a safe and feasible therapeutic tool for this infection<sup>[59]</sup>.

MSCs have been seen as an innovative therapeutic tool for preventing or treating graft-versus-host disease (GvHD) following allogeneic hematopoietic stem cell transplant (HSCT)<sup>[60,61]</sup> owing to their immunosuppressive properties, such as not eliciting immunological responses from alloreactive T-lymphocytes and/or other immunological effector cells. However, it is not

known whether using immunosuppressive MSCs may inadvertently inhibit antimicrobial immune responses and ultimately result in an increased risk of infection in allogeneic HSCT recipients<sup>[62]</sup>, considering that infection is one of the major complications following an HSCT contributing to high morbidity and mortality indexes<sup>[63,64]</sup>. One open randomized clinical trial has demonstrated that acute grade II-IV and chronic GVHD incidence in 10 patients receiving a median  $3.4 \times 10^5$ /kg MSCs dose from a human leukocyte antigen-identical sibling donor was lower than in 15 patients who did not receive MSCs (11% *cf* 53% and 14% *cf* 29%, respectively)<sup>[65]</sup>. Unfortunately, this did not mean a lower risk for infectious complications. Early and mid-phase severe infection incidence was even higher in patients who had received a co-transplant of hematopoietic stem cells and MSCs compared to a control group, which did not receive MSCs, although differences were not statistically significant [4/10 (40%) *cf* 5/15 (33%)]. Patients receiving MSCs suffered from cytomegalovirus (CMV) interstitial pneumonia and bacterial and/or fungal infection whereas this was only seen in two of the patients who did not receive MSCs<sup>[65]</sup>; no patient treated with MSCs died because of infectious complications, whereas this happened in two control group patients who did not receive MSCs. This raises the question of whether infection severity is lower when MSCs are co-transplanted with a graft.

By contrast, another two non-randomized clinical trials, involving 20 patients<sup>[66]</sup> and 14 pediatric patients<sup>[67]</sup>, showed that co-transplanting MSCs did not result in higher infection incidence and severity when compared to historical controls.

On the other hand, multivariate analysis regarding a retrospective cohort study of 691 HSCT patients showed that GVHD grade II-IV, CMV infection and having received human bone marrow-derived MSCs were factors which were associated with overall pneumonia-related deaths<sup>[68]</sup>.

Thus, the role of MSCs in infection is paradoxical. Evidence reported to date suggests that there may be pro- as well as anti-microbial effects<sup>[40-42]</sup> and this seems to depend on isolation and expansion conditions, cell origin and dose and administration route and timing.

## MECHANISMS BEHIND MSCs CANCER-INDUCING EFFECT

### MSCs modify cancer cells

Although the cancer stem cell (CSC) concept was first introduced in hematological malignancies (chronic and acute leukemia)<sup>[69]</sup>, it has been identified during recent years in a variety of solid tumors such as glioblastomas, medulloblastomas and carcinomas<sup>[70]</sup>. It has been demonstrated that MSCs interact with CSC in human cancer and regulate their own self-renewal through cytokine networks involving IL-6 and CXCL7<sup>[19]</sup>. CSC-produced IL-6 interacts with IL6R/gp130 expressed on MSCs to produce CXCL7; this molecule



interacts with CSCs through the CXCR2 receptor where it induces the synthesis of others cytokines (*i.e.*, IL-8, IL-6, CXCL6, and CXCL5)<sup>[19]</sup>. These cytokines trigger CSC self-renewal and enhance their invasive properties while IL-6 mediates chemotaxis, which may facilitate MSCs homing to primary tumor growth sites. It has been shown that MSCs administered subcutaneously in mice having had a breast tumor xenograft became recruited to the tumors and produced IL-6 and IL-8, which accelerated their growth by regulating the CSC population<sup>[20]</sup>.

### ***MSCs might induce epithelial-to-mesenchymal transition***

Most malignancies have an epithelial origin, and cancer progression is often associated with epithelial-to-mesenchymal transition (EMT)<sup>[71]</sup>; this is a physiological process, which is recognized as being crucial for embryogenesis and wound healing. It involves epithelial cell conversion to mesenchymal cells through the disruption of cell-cell junctions and the reorganization of the actin cytoskeleton; EMT has gained much attention recently due to its role in converting benign lesions into invasive and metastatic tumors<sup>[72]</sup>. It is governed by complex networks, which are influenced by signals from the neoplastic microenvironment, such as collagen, cytokines and TGF $\beta$ , epidermal growth factor, fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and platelet-derived growth factor<sup>[71-73]</sup>. Interestingly, all the aforementioned factors are secreted by MSCs<sup>[9]</sup>.

### ***MSCs modify vasculogenesis***

Vasculogenesis plays a critical role in tumor growth<sup>[74]</sup>; MSCs could contribute towards tumor vasculogenesis because they act as pericytes but may also differentiate into endothelial cells and secrete provasculogenic factors<sup>[75-77]</sup>, thereby allowing blood vessel formation<sup>[75]</sup>. Vascular endothelial growth factor (VEGF) and FGF-2 are the two main MSCs-secreted vasculogenic factors involved in tumor neovascularization<sup>[76]</sup>. VEGF is known to regulate MSCs mobilization and recruitment to neovascularization sites and directs MSCs differentiation to vascular cell<sup>[78,79]</sup>. VEGF expression in MSCs can be enhanced by hypoxia, a common phenomenon in tumor tissue<sup>[80]</sup> whilst FGF-2 is a potent mitogen which is produced and secreted by endothelial cells and MSCs<sup>[81]</sup>. This factor has been implicated in cell proliferation and endothelial cell migration during tumor growth<sup>[81]</sup>; conversely, MSCs appear to reduce vascular density due to endothelial cell cytotoxicity in certain conditions<sup>[38]</sup>.

### ***MSCs modify anti-cancer immune response***

MSCs suppress both innate and adaptive immune responses<sup>[82,83]</sup>; they inhibit CD4<sup>+</sup> and CD8<sup>+</sup> T-cell proliferation<sup>[84]</sup> by producing a wide range of mediators, including TGF $\beta$ 1, HGF, insulin-like growth factor, prostaglandin E<sub>2</sub>, NO, heme oxygenase-1 and IDO<sup>[85-89]</sup>. MSCs also inhibit monocyte and hematopoietic progenitor proliferation and differentiation into mature dendritic

cells<sup>[32,90]</sup>. Other MSCs-induced effects regarding dendritic cells would be a loss of their ability to stimulate allo-responses<sup>[91]</sup>, acquiring a regulatory phenotype due to the production of large amounts of IL-10<sup>[91]</sup> and changing dendritic cells' cytokine secretion profile by MSCs-derived PGE<sub>2</sub><sup>[91]</sup>. MSCs alter the natural killer (NK) cell phenotype besides suppressing their proliferation and cytokine secretion<sup>[92]</sup>; this requires cell-to-cell contact and soluble factors (TGF $\beta$ 1 and PGE<sub>2</sub>). Hence, MSCs could promote an anti-inflammatory response within a tumor, thereby allowing its enlargement<sup>[93]</sup>. Systemically administered MSCs have promoted immune-tolerance in damaged organs, irrespective of whether donor cells home into them<sup>[7,8,94]</sup>. It is expected that MSCs would worsen the immune-destruction of tumor cells and thus facilitate tumor growth and metastasis. Conversely, increased macrophage and granulocyte infiltration in MSCs-injected tumors has been shown, suggesting that allogeneic MSCs immunogenicity might contribute towards their antitumor effect<sup>[32,39]</sup>.

Changes in MSCs microenvironment, together with changes in transformed cells, would also seem to contribute towards carcinogenesis<sup>[95]</sup>.

## **MECHANISMS BEHIND MSCs INFECTION ADVERSE EFFECTS**

### ***MSCs modify bacterial growth inhibition and clearance***

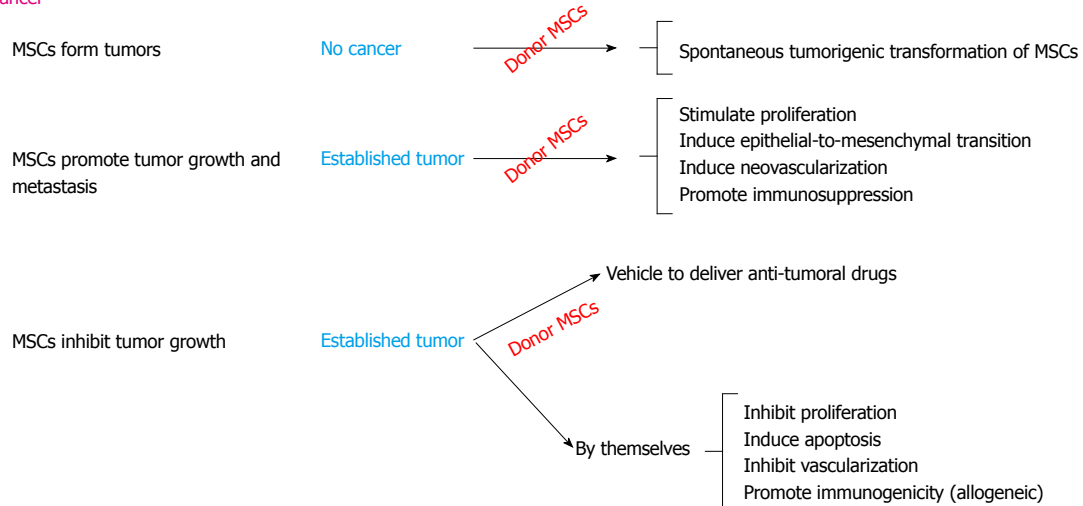
MSCs can participate in host defense through the secretion of antimicrobial peptides (cathelicidin LL-37<sup>[51]</sup> and lipocalin 2<sup>[52]</sup>), which can directly inhibit bacterial growth or kill the pathogens. The secretion of these soluble peptides improves resident phagocyte ability to clear bacteria through the up-regulation of pathways associated with monocyte/macrophage, phagocytosis, NK cell activity and antigen presentation<sup>[42]</sup> while MSCs antifungal activity means an increased amount of TH17 cells in the blood, thereby promoting TH1-type immune responses and restraining TH2-type ones<sup>[50]</sup>.

### ***MSCs modify anti-microorganism immune response***

MSCs induce a marked decrease in Toll-like receptor 2 expression, which plays a fundamental role in pathogen recognition and activation of innate immunity<sup>[96]</sup>. MSCs induce a marked increase in macrophage susceptibility to infection by parasites and bacteria. The mechanisms so involved appear to be linked to the production of inflammatory cytokines TNF- $\alpha$ , IL-12p70 and IFN- $\gamma$  which drive NO production<sup>[43]</sup>. MSCs switch activated macrophages into regulatory ones producing low levels of pro-inflammatory cytokines. MSCs could modify an immune response against microorganisms by inducing apoptosis and cell-cycle arrest of T-cells by producing NO, TGF $\beta$  or IDO<sup>[91]</sup>. MSCs can inhibit cellular immune responses and promote regulatory T-lymphocyte production, thereby establishing T-cell tolerance for microorganisms<sup>[97-100]</sup>.



### MSCs and cancer



### MSCs and infections

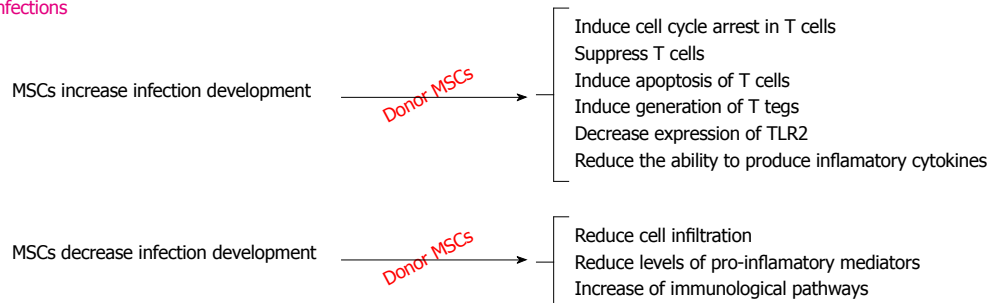


Figure 1 Paradoxical effect of donor mesenchymal stem cells in cancer and infection development. MSCs: Mesenchymal stem cells; TLR2: Toll-like receptor.

## ARE CANCER AND INFECTION ADVERSE EFFECTS ARISING FROM USING MSCs IN THERAPY?

*In vitro* and *in vivo* studies have demonstrated MSCs' pro- and anti-cancer and pro- and anti-infection effects nevertheless, most clinical trials have reported that MSCs-based therapy appears safe and has not been associated with severe adverse events. Together, due to MSCs' context-dependent potential to produce immune-modulatory factors they seem to be an ideal therapeutic tool for both cancer and infections.

## CONCLUSION

The pertinent literature reveals incongruity regarding the impact of MSCs on the development of cancer and infection (Figure 1); such paradoxical effect might be attributed to differences in isolation and expansion conditions, the source and dose of the cells being used, the administration route and its timing and host characteristics. MSCs immunomodulatory potential seems to be the leading mechanism responsible for such effects. Until conclusive data becomes available, cancer and infection will still be seen as adverse effects and therapeutic targets for using MSCs-based therapy.

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## REFERENCES

1. **Friedenstein AJ**, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* 1968; **6**: 230-247 [PMID: 5654088 DOI: 10.1097/00007890-196803000-00009]
2. **Pittenger MF**, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-147 [PMID: 10102814 DOI: 10.1126/science.284.5411.143]
3. **Phinney DG**, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells* 2007; **25**: 2896-2902 [PMID: 17901396 DOI: 10.1634/stemcells.2007-0637]
4. **Hoogduijn MJ**, Betjes MG, Baan CC. Mesenchymal stromal cells for organ transplantation: different sources and unique characteristics? *Curr Opin Organ Transplant* 2014; **19**: 41-46 [PMID: 24275893 DOI: 10.1097/MOT.0000000000000036]
5. **Minguell JJ**, Erices A, Conget P. Mesenchymal stem cells. *Exp Biol Med* (Maywood) 2001; **226**: 507-520 [PMID: 11395921]
6. **Rasmusson I**. Immune modulation by mesenchymal stem cells. *Exp Cell Res* 2006; **312**: 2169-2179 [PMID: 16631737 DOI: 10.1016/j.yexcr.2006.03.019]
7. **Ezquer F**, Ezquer M, Simon V, Conget P. The antidiabetic effect of



- MSCs is not impaired by insulin prophylaxis and is not improved by a second dose of cells. *PLoS One* 2011; **6**: e16566 [PMID: 21304603 DOI: 10.1371/journal.pone.0016566]
- 8 **Ezquer F**, Ezquer M, Simon V, Pardo F, Yañez A, Carpio D, Conget P. Endovenous administration of bone-marrow-derived multipotent mesenchymal stromal cells prevents renal failure in diabetic mice. *Biol Blood Marrow Transplant* 2009; **15**: 1354-1365 [PMID: 19822294 DOI: 10.1016/j.bbmt.2009.07.022]
- 9 **Caplan AI**, Dennis JE. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 2006; **98**: 1076-1084 [PMID: 16619257]
- 10 **Ishikane S**, Hosoda H, Yamahara K, Akitake Y, Kyoungsook J, Mishima K, Iwasaki K, Fujiwara M, Miyazato M, Kangawa K, Ikeda T. Allogeneic transplantation of fetal membrane-derived mesenchymal stem cell sheets increases neovascularization and improves cardiac function after myocardial infarction in rats. *Transplantation* 2013; **96**: 697-706 [PMID: 23912174 DOI: 10.1097/TP.0b013e31829f753d]
- 11 **Valle-Prieto A**, Conget PA. Human mesenchymal stem cells efficiently manage oxidative stress. *Stem Cells Dev* 2010; **19**: 1885-1893 [PMID: 20380515 DOI: 10.1089/scd.2010.0093]
- 12 **Lazarus HM**, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI. Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. *Bone Marrow Transplant* 1995; **16**: 557-564 [PMID: 8528172]
- 13 **Horwitz EM**, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, Muul L, Hofmann T. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proc Natl Acad Sci USA* 2002; **99**: 8932-8937 [PMID: 12084934 DOI: 10.1073/pnas.132252399]
- 14 **Rubio D**, Garcia S, De la Cueva T, Paz MF, Lloyd AC, Bernad A, Garcia-Castro J. Human mesenchymal stem cell transformation is associated with a mesenchymal-epithelial transition. *Exp Cell Res* 2008; **314**: 691-698 [PMID: 18201695 DOI: 10.1016/j.yexcr.2007.11.017]
- 15 **Aguilar S**, Nye E, Chan J, Loebinger M, Spencer-Dene B, Fisk N, Stamp G, Bonnet D, Janes SM. Murine but not human mesenchymal stem cells generate osteosarcoma-like lesions in the lung. *Stem Cells* 2007; **25**: 1586-1594 [PMID: 17363552 DOI: 10.1634/stemcells.2006-0762]
- 16 **Lalu MM**, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC, Granton J, Stewart DJ. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One* 2012; **7**: e47559 [PMID: 23133515 DOI: 10.1371/journal.pone.0047559]
- 17 **Sasser AK**, Mundy BL, Smith KM, Studebaker AW, Axel AE, Haidet AM, Fernandez SA, Hall BM. Human bone marrow stromal cells enhance breast cancer cell growth rates in a cell line-dependent manner when evaluated in 3D tumor environments. *Cancer Lett* 2007; **254**: 255-264 [PMID: 17467167]
- 18 **Zhu W**, Xu W, Jiang R, Qian H, Chen M, Hu J, Cao W, Han C, Chen Y. Mesenchymal stem cells derived from bone marrow favor tumor cell growth in vivo. *Exp Mol Pathol* 2006; **80**: 267-274 [PMID: 16214129 DOI: 10.1016/j.yexmp.2005.07.004]
- 19 **Liu S**, Ginestier C, Ou SJ, Clouthier SG, Patel SH, Monville F, Korkaya H, Heath A, Dutcher J, Kleer CG, Jung Y, Dontu G, Taichman R, Wicha MS. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res* 2011; **71**: 614-624 [PMID: 21224357 DOI: 10.1158/0008-5472.CAN-10-0538]
- 20 **Karnoub AE**, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007; **449**: 557-563 [PMID: 17914389]
- 21 **Prantl L**, Muehlberg F, Navone NM, Song YH, Vykoukal J, Logothetis CJ, Alt EU. Adipose tissue-derived stem cells promote prostate tumor growth. *Prostate* 2010; **70**: 1709-1715 [PMID: 20564322 DOI: 10.1002/pros.21206]
- 22 **Klopp AH**, Gupta A, Spaeth E, Andreeff M, Marini F. Concise review: Dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? *Stem Cells* 2011; **29**: 11-19 [PMID: 21280155 DOI: 10.1002/stem.559]
- 23 **Bian ZY**, Fan QM, Li G, Xu WT, Tang TT. Human mesenchymal stem cells promote growth of osteosarcoma: involvement of interleukin-6 in the interaction between human mesenchymal stem cells and Saos-2. *Cancer Sci* 2010; **101**: 2554-2560 [PMID: 20874851 DOI: 10.1111/j.1349-7006.2010.01731.x]
- 24 **Zhang Y**, Daquinag A, Traktuev DO, Amaya-Manzanares F, Simmons PJ, March KL, Pasqualini R, Arap W, Kolonin MG. White adipose tissue cells are recruited by experimental tumors and promote cancer progression in mouse models. *Cancer Res* 2009; **69**: 5259-5266 [PMID: 19491274 DOI: 10.1158/0008-5472.CAN-08-3444]
- 25 **Spaeth E**, Klopp A, Dembinski J, Andreeff M, Marini F. Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells. *Gene Ther* 2008; **15**: 730-738 [PMID: 18401438 DOI: 10.1038/gt.2008.39]
- 26 **Dvorak HF**. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986; **315**: 1650-1659 [PMID: 3537791]
- 27 **Ringe J**, Strassburg S, Neumann K, Endres M, Notter M, Burmester GR, Kaps C, Sittlinger M. Towards in situ tissue repair: human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2. *J Cell Biochem* 2007; **101**: 135-146 [PMID: 17295203 DOI: 10.1002/jcb.21172]
- 28 **Chamberlain G**, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007; **25**: 2739-2749 [PMID: 17656645 DOI: 10.1634/stemcells.2007-0197]
- 29 **Martin FT**, Dwyer RM, Kelly J, Khan S, Murphy JM, Curran C, Miller N, Hennessy E, Dockery P, Barry FP, O'Brien T, Kerin MJ. Potential role of mesenchymal stem cells (MSCs) in the breast tumour microenvironment: stimulation of epithelial to mesenchymal transition (EMT). *Breast Cancer Res Treat* 2010; **124**: 317-326 [PMID: 20087650 DOI: 10.1007/s10549-010-0734-1]
- 30 **Corcoran KE**, Trzaska KA, Fernandes H, Bryan M, Taborga M, Srinivas V, Packman K, Patel PS, Rameshwar P. Mesenchymal stem cells in early entry of breast cancer into bone marrow. *PLoS One* 2008; **3**: e2563 [PMID: 18575622 DOI: 10.1371/journal.pone.0002563]
- 31 **Tian LL**, Yue W, Zhu F, Li S, Li W. Human mesenchymal stem cells play a dual role on tumor cell growth in vitro and in vivo. *J Cell Physiol* 2011; **226**: 1860-1867 [PMID: 21442622 DOI: 10.1002/jcp.22511]
- 32 **Ramasamy R**, Fazekasova H, Lam EW, Soeiro I, Lombardi G, Dazzi F. Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. *Transplantation* 2007; **83**: 71-76 [PMID: 17220794 DOI: 10.1097/01.tp.0000244572.24780.54]
- 33 **Ramasamy R**, Lam EW, Soeiro I, Tisato V, Bonnet D, Dazzi F. Mesenchymal stem cells inhibit proliferation and apoptosis of tumor cells: impact on in vivo tumor growth. *Leukemia* 2007; **21**: 304-310 [PMID: 17170725 DOI: 10.1038/sj.leu.2404489]
- 34 **Qiao L**, Xu Z, Zhao T, Zhao Z, Shi M, Zhao RC, Ye L, Zhang X. Suppression of tumorigenesis by human mesenchymal stem cells in a hepatoma model. *Cell Res* 2008; **18**: 500-507 [PMID: 18364678 DOI: 10.1038/cr.2008.40]
- 35 **Qiao L**, Xu ZL, Zhao TJ, Ye LH, Zhang XD. Dkk-1 secreted by mesenchymal stem cells inhibits growth of breast cancer cells via depression of Wnt signalling. *Cancer Lett* 2008; **269**: 67-77 [PMID: 18571836 DOI: 10.1016/j.canlet.2008.04.032]
- 36 **Khakoo AY**, Pati S, Anderson SA, Reid W, Elshal MF, Rovira II, Nguyen AT, Malide D, Combs CA, Hall G, Zhang J, Raffeld M, Rogers TB, Stetler-Stevenson W, Frank JA, Reitz M, Finkel T. Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *J Exp Med* 2006; **203**: 1235-1247 [PMID: 16636132]



- 37 **Cousin B**, Ravet E, Poglio S, De Toni F, Bertuzzi M, Lulka H, Touil I, André M, Grolleau JL, Péron JM, Chavoin JP, Bourin P, Pénicaud L, Casteilla L, Buscail L, Cordelier P. Adult stromal cells derived from human adipose tissue provoke pancreatic cancer cell death both in vitro and in vivo. *PLoS One* 2009; **4**: e6278 [PMID: 19609435 DOI: 10.1371/journal.pone.0006278]
- 38 **Otsu K**, Das S, Houser SD, Quadri SK, Bhattacharya S, Bhattacharya J. Concentration-dependent inhibition of angiogenesis by mesenchymal stem cells. *Blood* 2009; **113**: 4197-4205 [PMID: 19036701 DOI: 10.1182/blood-2008-09-176198]
- 39 **Ohlsson LB**, Varas L, Kjellman C, Edvardsen K, Lindvall M. Mesenchymal progenitor cell-mediated inhibition of tumor growth in vivo and in vitro in gelatin matrix. *Exp Mol Pathol* 2003; **75**: 248-255 [PMID: 14611816]
- 40 **Alagesan S**, Griffin MD. Autologous and allogeneic mesenchymal stem cells in organ transplantation: what do we know about their safety and efficacy? *Curr Opin Organ Transplant* 2014; **19**: 65-72 [PMID: 24370985 DOI: 10.1097/MOT.0000000000000043]
- 41 **Hall SR**, Tsoyi K, Ith B, Padera RF, Lederer JA, Wang Z, Liu X, Perrella MA. Mesenchymal stromal cells improve survival during sepsis in the absence of heme oxygenase-1: the importance of neutrophils. *Stem Cells* 2013; **31**: 397-407 [PMID: 23132816 DOI: 10.1002/stem.1270]
- 42 **Mei SH**, Haitsma JJ, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, Liles WC, Stewart DJ. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 2010; **182**: 1047-1057 [PMID: 20558630 DOI: 10.1164/rccm.201001-0010OC]
- 43 **Maggini J**, Mirkin G, Bognanni I, Holmberg J, Piazzón IM, Nepomnaschy I, Costa H, Cañones C, Raiden S, Vermeulen M, Geffner JR. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS One* 2010; **5**: e9252 [PMID: 20169081 DOI: 10.1371/journal.pone.0009252]
- 44 **Raghuvanshi S**, Sharma P, Singh S, Van Kaer L, Das G. Mycobacterium tuberculosis evades host immunity by recruiting mesenchymal stem cells. *Proc Natl Acad Sci USA* 2010; **107**: 21653-21658 [PMID: 21135221 DOI: 10.1073/pnas.1007967107]
- 45 **Romano PS**, Cueto JA, Casassa AF, Vanrell MC, Gottlieb RA, Colombo MI. Molecular and cellular mechanisms involved in the Trypanosoma cruzi/host cell interplay. *IUBMB Life* 2012; **64**: 387-396 [PMID: 22454195 DOI: 10.1002/iub.1019]
- 46 **Meisel R**, Brockers S, Heseler K, Degistirici O, Bülle H, Woite C, Stuhlsatz S, Schwippert W, Jäger M, Sorg R, Henschler R, Seissler J, Dilloo D, Däubener W. Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. *Leukemia* 2011; **25**: 648-654 [PMID: 21242993]
- 47 **MacMicking J**, Xie QW, Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol* 1997; **15**: 323-350 [PMID: 9143691 DOI: 10.1146/annurev.immunol.15.1.323]
- 48 **Shiloh MU**, Nathan CF. Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria. *Curr Opin Microbiol* 2000; **3**: 35-42 [PMID: 10679417 DOI: 10.1016/S1369-5274(99)00048-X]
- 49 **Reinders ME**, de Fijter JW, Roelofs H, Bajema IM, de Vries DK, Schaapherder AF, Claas FH, van Miert PP, Roelen DL, van Kooten C, Fibbe WE, Rabelink TJ. Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. *Stem Cells Transl Med* 2013; **2**: 107-111 [PMID: 23349326 DOI: 10.5966/sctm.2012-0114]
- 50 **Yang R**, Liu Y, Kelk P, Qu C, Akiyama K, Chen C, Atsuta I, Chen W, Zhou Y, Shi S. A subset of IL-17(+) mesenchymal stem cells possesses anti-Candida albicans effect. *Cell Res* 2013; **23**: 107-121 [PMID: 23266891 DOI: 10.1038/cr.2012.179]
- 51 **Krasnodembkaya A**, Song Y, Fang X, Gupta N, Serikov V, Lee JW, Matthay MA. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 2010; **28**: 2229-2238 [PMID: 20945332 DOI: 10.1002/stem.544]
- 52 **Gupta N**, Krasnodembkaya A, Kapetanaki M, Mouded M, Tan X, Serikov V, Matthay MA. Mesenchymal stem cells enhance survival and bacterial clearance in murine Escherichia coli pneumonia. *Thorax* 2012; **67**: 533-539 [PMID: 22250097 DOI: 10.1136/thoraxjnl-2011-201176]
- 53 **Balan A**, Lucchini G, Schmidt S, Schneider A, Tramsen L, Kuci S, Meisel R, Bader P, Lehrnbecher T. Mesenchymal stromal cells in the antimicrobial host response of hematopoietic stem cell recipients with graft-versus-host disease-friends or foes? *Leukemia* 2014; **28**: 1941-1948 [PMID: 24762460]
- 54 **Shin S**, Kim Y, Jeong S, Hong S, Kim I, Lee W, Choi S. The therapeutic effect of human adult stem cells derived from adipose tissue in endotoxemic rat model. *Int J Med Sci* 2013; **10**: 8-18 [PMID: 23289000 DOI: 10.7150/ijms.5385]
- 55 **Curley GF**, Scott JA, Laffey JG. Therapeutic potential and mechanisms of action of mesenchymal stromal cells for acute respiratory distress syndrome. *Curr Stem Cell Res Ther* 2014; **9**: 319-329 [PMID: 24588087]
- 56 **Gonzalez-Rey E**, Anderson P, González MA, Rico L, Büscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009; **58**: 929-939 [PMID: 19136511 DOI: 10.1136/gut.2008.168534]
- 57 **Xu J**, Qu J, Cao L, Sai Y, Chen C, He L, Yu L. Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *J Pathol* 2008; **214**: 472-481 [PMID: 18213733 DOI: 10.1002/path.2302]
- 58 **Mei SH**, McCarter SD, Deng Y, Parker CH, Liles WC, Stewart DJ. Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. *PLoS Med* 2007; **4**: e269 [PMID: 17803352]
- 59 **Zheng G**, Huang L, Tong H, Shu Q, Hu Y, Ge M, Deng K, Zhang L, Zou B, Cheng B, Xu J. Treatment of acute respiratory distress syndrome with allogeneic adipose-derived mesenchymal stem cells: a randomized, placebo-controlled pilot study. *Respir Res* 2014; **15**: 39 [PMID: 24708472 DOI: 10.1186/1465-9921-15-39]
- 60 **Baron F**, Storb R. Mesenchymal stromal cells: a new tool against graft-versus-host disease? *Biol Blood Marrow Transplant* 2012; **18**: 822-840 [PMID: 21963621 DOI: 10.1016/j.bbmt.2011.09.003]
- 61 **Kim EJ**, Kim N, Cho SG. The potential use of mesenchymal stem cells in hematopoietic stem cell transplantation. *Exp Mol Med* 2013; **45**: e2 [PMID: 23306700 DOI: 10.1038/emm.2013.2]
- 62 **Nauta AJ**, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* 2007; **110**: 3499-3506 [PMID: 17664353 DOI: 10.1182/blood-2007-02-069716]
- 63 **Appelbaum FR**. The current status of hematopoietic cell transplantation. *Annu Rev Med* 2003; **54**: 491-512 [PMID: 12414918 DOI: 10.1146/annurev.med.54.101601.152456]
- 64 **Sparrelid E**, Hägglund H, Remberger M, Ringdén O, Lönnqvist B, Ljungman P, Andersson J. Bacteraemia during the aplastic phase after allogeneic bone marrow transplantation is associated with early death from invasive fungal infection. *Bone Marrow Transplant* 1998; **22**: 795-800 [PMID: 9827978 DOI: 10.1038/sj.bmt.1701404]
- 65 **Ning H**, Yang F, Jiang M, Hu L, Feng K, Zhang J, Yu Z, Li B, Xu C, Li Y, Wang J, Hu J, Lou X, Chen H. The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. *Leukemia* 2008; **22**: 593-599 [PMID: 18185520 DOI: 10.1038/sj.leu.2405090]
- 66 **Baron F**, Lechanteur C, Willems E, Bruck F, Baudoux E, Seidel L, Vanbellinghen JF, Hafroui K, Lejeune M, Gothot A, Fillet G, Beguin Y. Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. *Biol Blood Marrow Transplant* 2010; **16**: 838-847 [PMID: 20109568 DOI: 10.1016/j.bbmt.2010.01.011]
- 67 **Ball LM**, Bernardo ME, Roelofs H, Lankester A, Cometa A, Egeler RM, Locatelli F, Fibbe WE. Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and



- may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. *Blood* 2007; **110**: 2764-2767 [PMID: 17638847]
- 68 **Forsl w U**, Blennow O, LeBlanc K, Ringd n O, Gustafsson B, Mattsson J, Remberger M. Treatment with mesenchymal stromal cells is a risk factor for pneumonia-related death after allogeneic hematopoietic stem cell transplantation. *Eur J Haematol* 2012; **89**: 220-227 [PMID: 22765507 DOI: 10.1111/j.1600-0609.2012.01824.x]
  - 69 **Bonnet D**, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730-737 [PMID: 9212098 DOI: 10.1038/nm0797-730]
  - 70 **Ricci-Vitiani L**, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111-115 [PMID: 17122771]
  - 71 **Cannito S**, Novo E, di Bonzo LV, Busletta C, Colombatto S, Parola M. Epithelial-mesenchymal transition: from molecular mechanisms, redox regulation to implications in human health and disease. *Antioxid Redox Signal* 2010; **12**: 1383-1430 [PMID: 19903090 DOI: 10.1089/ars.2009.2737]
  - 72 **Thompson EW**, Newgreen DF, Tarin D. Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? *Cancer Res* 2005; **65**: 5991-5995; discussion 5995 [PMID: 16024595]
  - 73 **Kong D**, Wang Z, Sarkar SH, Li Y, Banerjee S, Saliganan A, Kim HR, Cher ML, Sarkar FH. Platelet-derived growth factor-D overexpression contributes to epithelial-mesenchymal transition of PC3 prostate cancer cells. *Stem Cells* 2008; **26**: 1425-1435 [PMID: 18403754 DOI: 10.1634/stemcells.2007-1076]
  - 74 **Hiratsuka S**. Vasculogenesis, angiogenesis and special features of tumor blood vessels. *Front Biosci* (Landmark Ed) 2011; **16**: 1413-1427 [PMID: 21196239]
  - 75 **Rajantie I**, Imonen M, Almainaite A, Ozerdem U, Alitalo K, Salven P. Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. *Blood* 2004; **104**: 2084-2086 [PMID: 15191949 DOI: 10.1182/blood-2004-01-0336]
  - 76 **Spaeth EL**, Dembinski JL, Sasser AK, Watson K, Klopp A, Hall B, Andreeff M, Marini F. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PLoS One* 2009; **4**: e4992 [PMID: 19352430 DOI: 10.1371/journal.pone.0004992]
  - 77 **Bexell D**, Gunnarsson S, Tormin A, Darabi A, Gisselsson D, Roybon L, Scheding S, Bengzon J. Bone marrow multipotent mesenchymal stroma cells act as pericyte-like migratory vehicles in experimental gliomas. *Mol Ther* 2009; **17**: 183-190 [PMID: 18985030 DOI: 10.1038/mt.2008.229]
  - 78 **Beckermann BM**, Kallifatidis G, Groth A, Frommhold D, Apel A, Mattern J, Salnikov AV, Moldenhauer G, Wagner W, Diehlmann A, Saffrich R, Schubert M, Ho AD, Giese N, B chler MW, Friess H, B chler P, Herr I. VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma. *Br J Cancer* 2008; **99**: 622-631 [PMID: 18665180 DOI: 10.1038/sj.bjc.6604508]
  - 79 **Gy ngy si M**, Posa A, Pavo N, Hemetsberger R, Kvakan H, Steiner-B ker S, Petr si Z, Manczur F, Pavo IJ, Edes IF, Wojta J, Glogar D, Huber K. Differential effect of ischaemic preconditioning on mobilisation and recruitment of haematopoietic and mesenchymal stem cells in porcine myocardial ischaemia-reperfusion. *Thromb Haemost* 2010; **104**: 376-384 [PMID: 20352158 DOI: 10.1160/TH09-08-0558]
  - 80 **Potier E**, Ferreira E, Andriamanalijaona R, Pujol JP, Oudina K, Logeart-Avramoglou D, Petite H. Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. *Bone* 2007; **40**: 1078-1087 [PMID: 17276151 DOI: 10.1016/j.bone.2006.11.024]
  - 81 **Delli-Bovi P**, Curatola AM, Newman KM, Sato Y, Moscatelli D, Hewick RM, Rifkin DB, Basilico C. Processing, secretion, and biological properties of a novel growth factor of the fibroblast growth factor family with oncogenic potential. *Mol Cell Biol* 1988; **8**: 2933-2941 [PMID: 3043199]
  - 82 **Law S**, Chaudhuri S. Mesenchymal stem cell and regenerative medicine: regeneration versus immunomodulatory challenges. *Am J Stem Cells* 2013; **2**: 22-38 [PMID: 23671814]
  - 83 **Shi M**, Liu ZW, Wang FS. Immunomodulatory properties and therapeutic application of mesenchymal stem cells. *Clin Exp Immunol* 2011; **164**: 1-8 [PMID: 21352202 DOI: 10.1111/j.1365-2249.2011.04327.x]
  - 84 **Glennie S**, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 2005; **105**: 2821-2827 [PMID: 15591115 DOI: 10.1182/blood-2004-09-3696]
  - 85 **Bartholomew A**, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002; **30**: 42-48 [PMID: 11823036 DOI: 10.1016/S0301-472X(01)00769-X]
  - 86 **Di Nicola M**, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; **99**: 3838-3843 [PMID: 11986244 DOI: 10.1182/blood.V99.10.3838]
  - 87 **Meisel R**, Zibert A, Laryea M, G bel U, D ubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004; **103**: 4619-4621 [PMID: 15001472 DOI: 10.1182/blood-2003-11-3909]
  - 88 **Chabannes D**, Hill M, Merieau E, Rossignol J, Brion R, Souillou JP, Anegon I, Cuturi MC. A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood* 2007; **110**: 3691-3694 [PMID: 17684157 DOI: 10.1182/blood-2007-02-075481]
  - 89 **Gieseke F**, Sch tt B, Viebahn S, Koscielniak E, Friedrich W, Handgretinger R, M ller I. Human multipotent mesenchymal stromal cells inhibit proliferation of PBMCs independently of IFN gammaR1 signaling and IDO expression. *Blood* 2007; **110**: 2197-2200 [PMID: 17522338 DOI: 10.1182/blood-2007-04-083162]
  - 90 **Nauta AJ**, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. *J Immunol* 2006; **177**: 2080-2087 [PMID: 16887966 DOI: 10.4049/jimmunol.177.4.2080]
  - 91 **Aggarwal S**, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005; **105**: 1815-1822 [PMID: 15494428 DOI: 10.1182/blood-2004-04-1559]
  - 92 **Sotiropoulou PA**, Perez SA, Gritzapis AD, Baxevas CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006; **24**: 74-85 [PMID: 16099998 DOI: 10.1634/stemcells.2004-0359]
  - 93 **Razmkhah M**, Jaberipour M, Erfani N, Habibagahi M, Talei AR, Ghaderi A. Adipose derived stem cells (ASCs) isolated from breast cancer tissue express IL-4, IL-10 and TGF- 1 and upregulate expression of regulatory molecules on T cells: do they protect breast cancer cells from the immune response? *Cell Immunol* 2011; **266**: 116-122 [PMID: 20970781 DOI: 10.1016/j.cellimm.2010.09.005]
  - 94 **Ezquer FE**, Ezquer ME, Parrau DB, Carpio D, Y  ez AJ, Conget PA. Systemic administration of multipotent mesenchymal stromal cells reverts hyperglycemia and prevents nephropathy in type 1 diabetic mice. *Biol Blood Marrow Transplant* 2008; **14**: 631-640 [PMID: 18489988 DOI: 10.1016/j.bbmt.2008.01.006]
  - 95 **Dvorak HF**, Weaver VM, Tlsty TD, Bergers G. Tumor microenvironment and progression. *J Surg Oncol* 2011; **103**: 468-474 [PMID: 21480238 DOI: 10.1002/jso.21709]
  - 96 **Mo IF**, Yip KH, Chan WK, Law HK, Lau YL, Chan GC. Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors. *BMC Cell Biol* 2008; **9**: 52 [PMID: 18799018 DOI: 10.1186/1471-2121-9-52]



- 97 **Niedbala W**, Besnard AG, Jiang HR, Alves-Filho JC, Fukada SY, Nascimento D, Mitani A, Pushparaj P, Alqahtani MH, Liew FY. Nitric oxide-induced regulatory T cells inhibit Th17 but not Th1 cell differentiation and function. *J Immunol* 2013; **191**: 164-170 [PMID: 23720815 DOI: 10.4049/jimmunol.1202580]
- 98 **Yuan J**, Zhang G, Yang X, Liu K, Wang F. Transplantation of allograft transforming growth factor- $\beta$ 1 transfected CD103<sup>+</sup> lamina propria dendritic cells could effectively induce antigen-specific regulatory T cells in vivo. *Transplant Proc* 2013; **45**: 3408-3413 [PMID: 24182825 DOI: 10.1016/j.transproceed.2013.07.056]
- 99 **Curran TA**, Jalili RB, Farrokhi A, Ghahary A. IDO expressing fibroblasts promote the expansion of antigen specific regulatory T cells. *Immunobiology* 2014; **219**: 17-24 [PMID: 23891282 DOI: 10.1016/j.imbio.2013.06.008]
- 100 **Yan Z**, Zhuansun Y, Chen R, Li J, Ran P. Immunomodulation of mesenchymal stromal cells on regulatory T cells and its possible mechanism. *Exp Cell Res* 2014; **324**: 65-74 [PMID: 24681107 DOI: 10.1016/j.yexcr.2014.03.013]

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## Mitochondria as therapeutic targets for cancer stem cells

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somatic stem cells and are responsible for tumor initiation, chemoresistance, and metastasis. Evidence for the CSCs existence has been reported for a number of human cancers. The CSC mitochondria have been shown recently to be an important target for cancer treatment, but clinical significance of CSCs and their mitochondria properties remain unclear. Mitochondria-targeted agents are considerably more effective compared to other agents in triggering apoptosis of CSCs, as well as general cancer cells, *via* mitochondrial dysfunction. Mitochondrial metabolism is altered in cancer cells because of their reliance on glycolytic intermediates, which are normally destined for oxidative phosphorylation. Therefore, inhibiting cancer-specific modifications in mitochondrial metabolism, increasing reactive oxygen species production, or stimulating mitochondrial permeabilization transition could be promising new therapeutic strategies to activate cell death in CSCs as well, as in general cancer cells. This review analyzed mitochondrial function and its potential as a therapeutic target to induce cell death in CSCs. Furthermore, combined treatment with mitochondria-targeted drugs will be a promising strategy for the treatment of relapsed and refractory cancer.

**Key words:** Cancer stem cells; Mitochondria; Relapsed and refractory cancer; Therapeutic target; Mitochondrial energy metabolism

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**Core tip:** This review is devoted to the analysis of mitochondrial function as a therapeutic target to induce cell death in cancer stem cells (CSCs). In particular, we focused on the differences in energy metabolism and features between CSC and non-CSC mitochondria, and between CSCs and normal stem cells. We described the roles of mitochondria that may make CSCs more susceptible to anti-cancer treatment and apoptosis, and how these may be useful to develop novel strategies for cancer treatment, such as through combined therapy

### Abstract

Cancer stem cells (CSCs) are maintained by their



with specific mitochondrial-targeting drugs.

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## INTRODUCTION

Over the last decade, cancer therapies have improved the quality of life of cancer patients. However, although almost all developed anti-cancer drugs are apparently successful following initial therapy, secondary tumors development and disease relapse is common. The limitation of classical anti-cancer therapies has been attributed recently to the existence of cancer stem cells (CSCs), which are quiescent, have relatively small population, and highly drug-resistant cells. CSCs act like stem cells (SCs) and are responsible for cancer growth and metastasis<sup>[1]</sup>. Through the continued effort of many researchers, CSCs features have been revealed, such as anti-cancer drug resistance, metastasis, proliferation, hypoxic tolerance, and the capacity for neovessel induction<sup>[2,3]</sup>.

Mitochondria-targeted drugs may overcome potentially the drug-resistance mechanisms that have progressed toward conventional chemo-therapeutics in cancer<sup>[4-7]</sup>. Mitochondria produce ATP, but they also mediate cell death and produce reactive oxygen species (ROS). Although ROS are affected in the regulation of various cellular responses, excessive production may be harmful to the cell<sup>[8]</sup>. Cancer cells also exhibit extensive metabolic rearrangement that makes them more susceptible to alteration of mitochondria than normal cells<sup>[9,10]</sup>. However, mitochondrial properties of CSCs in tumors remain unknown.

This review analyzed the potential role of mitochondria as a therapeutic target for inducing cell death in CSCs. In particular, we focused on the differences in energy metabolism and mitochondrial features between CSCs and non-CSCs, as well as between CSCs and normal SCs, and how these unique features of CSCs may increase the susceptibility of CSCs to anti-cancer treatment and apoptosis induction. We described how CSC mitochondria may be useful targets for the development of novel cancer treatment strategies, such as targeting CSCs *via* combination therapy with specific mitochondrial-targeting drugs.

## CURRENT STATUS OF CSCs

### History

The concept of CSCs is many decades old<sup>[11]</sup>. In the middle of 1800s, the embryonal rest theory of cancer introduced the idea that cancer arises from SCs, but

the existence of CSCs in tumors could not be verified due to a lack of techniques. Furth *et al.*<sup>[12]</sup> first alluded to CSCs in 1937 when they showed that a single cell within a tumor initiates the generation of new tumor in a recipient mouse<sup>[12]</sup>. This finding was defined in the 1960s and 1970s by the development of quantitative methods to measure the tumorigenic ability able to sustain tumor growth *in vivo*. In the middle of 1900s, Radiolabeling permitted the measurements of cellular phenotype such as cell proliferation, lifespan, and hierarchical organizations within normal tissues<sup>[13]</sup>. Al-Hajj *et al.*<sup>[14]</sup> and Singh *et al.*<sup>[15]</sup> represented that a small subset of cells within breast and brain tumors can be isolated prospectively and can generate phenotypically heterogeneous tumor *in vivo*. Thus, these various evidences represent that diverse solid tumors are organized hierarchically and sustained by a distinct subpopulation of CSCs.

### Identification of CSCs

CSCs are classified according to several properties such as the presence of cell surface markers and their occupancy in the Fluorescence Activated Cell Sorting (FACS) analysis. Flow cytometry with antibodies against cell surface antigens has been the preferred method for characterizing and sorting normal stem cells. However, differences between CSC and normal SC markers are not well defined, and CSCs and normal SCs share some surface markers.

Most of CSCs studies isolate CSCs marker or a combination of markers, which is expressed heterogeneously in a certain tumor type. Based on this marker heterogeneity, subpopulations including CSCs are isolated from original tumors and injected into immunodeficient mice, after which tumor growth is assessed several weeks or months later. Table 1 shows current CSC markers according to cancer types, as FACS markers allow for consistent sorting according to marker expression. For example, Al-Hajj *et al.*<sup>[14]</sup> used a marker combination of the CD24 and CD44 as an indicator of breast CSC, and the CD133 marker has been shown to be both normal SC and CSC marker<sup>[16-20]</sup>.

### Stem cells and CSCs

The first embryonic SC lines were developed from the inner cell mass of early embryos in 1998<sup>[21]</sup>. In 1999 and 2000, it was discovered that it could produce different cell types through manipulating adult mouse tissues, indicating that stem cell differentiation and proliferation could be controlled externally. Both somatic SCs and CSCs generate numerous daughter cells, differentiate into a variety of cell types, actively express telomerase, activate anti-apoptotic pathways, increase active membrane transports, and metastasize<sup>[22]</sup>. Moreover, SCs are induced to differentiate by niche signaling and outer environmental stimuli. Niche signaling keeps the undifferentiation of SCs until they are stimulated to



**Table 1** Markers used to identify stem cells and cancer stem cells

Marker	Cancer origin	Marker properties	Ref.
ALDH1	Breast	Catalyzes the oxidation of aliphatic and aromatic aldehydes Converts retinol to retinoic acid	[81]
ABC135	Melanomas	AdSC ATP binding cassette family Involved in transport of sterol and other lipids	[82]
Bmi-1	Breast, prostate, leukemias, neuroblastomas	HSC, NSC, and AdSC marker	[83,84]
CD20	Metastatic melanomas	Hematopoietic marker	[85]
CD29	Breast, colon	AdSC marker	[86,87]
CD34	Leukemias, sarcomas	HSC, MSC marker	[88-91]
CD44	Breast, pancreas, colon, head and neck, prostate	Adhesion molecule related to metastasis HSC and pluripotent stem cell marker Normal prostate epithelial stem cell marker	[91-96]
CD49f	Prostate	Adhesion to extracellular matrix	[97]
CD90	Liver, breast, glioblastomas	Glycoprotein, role in stem cell differentiation MSC marker	[98-100]
CD113	Lung, pancreas, colon, glioblastoma, melanomas, etc.	HSC, NSC AdSC (colon) marker	[16-18,101-104]
CD117	Breast, ovarian, lung, glioblastoma	Progenitor cell marker	[105,106]
Oct4	Many carcinomas	Embryonic stem cell and induced pluripotent stem cell marker	[107,108]
Sca-1	Lung	Skin epithelial stem cell and HSC marker	[109]

AdSC: Adult stem cell marker; HSC: Hematopoietic stem cell; NSC: Neuronal stem cell; MSC: Mesenchymal stem cell.

generate new cells, suggesting a similarity with signaling pathways that govern normal SC proliferation. Local environment signaling can initiate CSC proliferation, and thus, trigger tumor initiation and growth<sup>[23]</sup>. Therefore, SC markers and features may not be effective therapeutic targets for inhibiting CSC growth.

## MITOCHONDRIA AND CANCER

### Roles of mitochondria

As the main energy producers, mitochondria produce ATP using the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). However, they also generate ROS during this process, which are harmful to the cell if produced excessively. In addition, mitochondria play a crucial role for the regulation of cell death pathways and intracellular Ca<sup>2+</sup> homeostasis. Mitochondria activate apoptosis by regulating the releasement of proapoptotic proteins space to the cytosol from the mitochondrial intermembrane<sup>[7]</sup>, and they also play a crucial role in non-apoptotic cell death<sup>[24]</sup>.

Key regulators related to cell death and other cellular processes in the mitochondria are frequently altered in cancer cells<sup>[8]</sup>, as cancer cell mitochondria differ functionally and structurally compare with that of normal cells<sup>[25]</sup>. Fast growing tumors result in hypoxia because of an inadequate amount of oxygen from the local vasculature. In addition, cancer cells include the DNA mutation of mitochondria and nucleus, which affect the OXPHOS components and result in ROS overproduction, wasteful ATP production, and mitochondrial oxidative damage<sup>[25]</sup>. Warburg<sup>[26]</sup> pioneered research on the cancer-related alterations in mitochondrial respiration and suggested a mechanism to explain how they progress during the tumorigenesis.

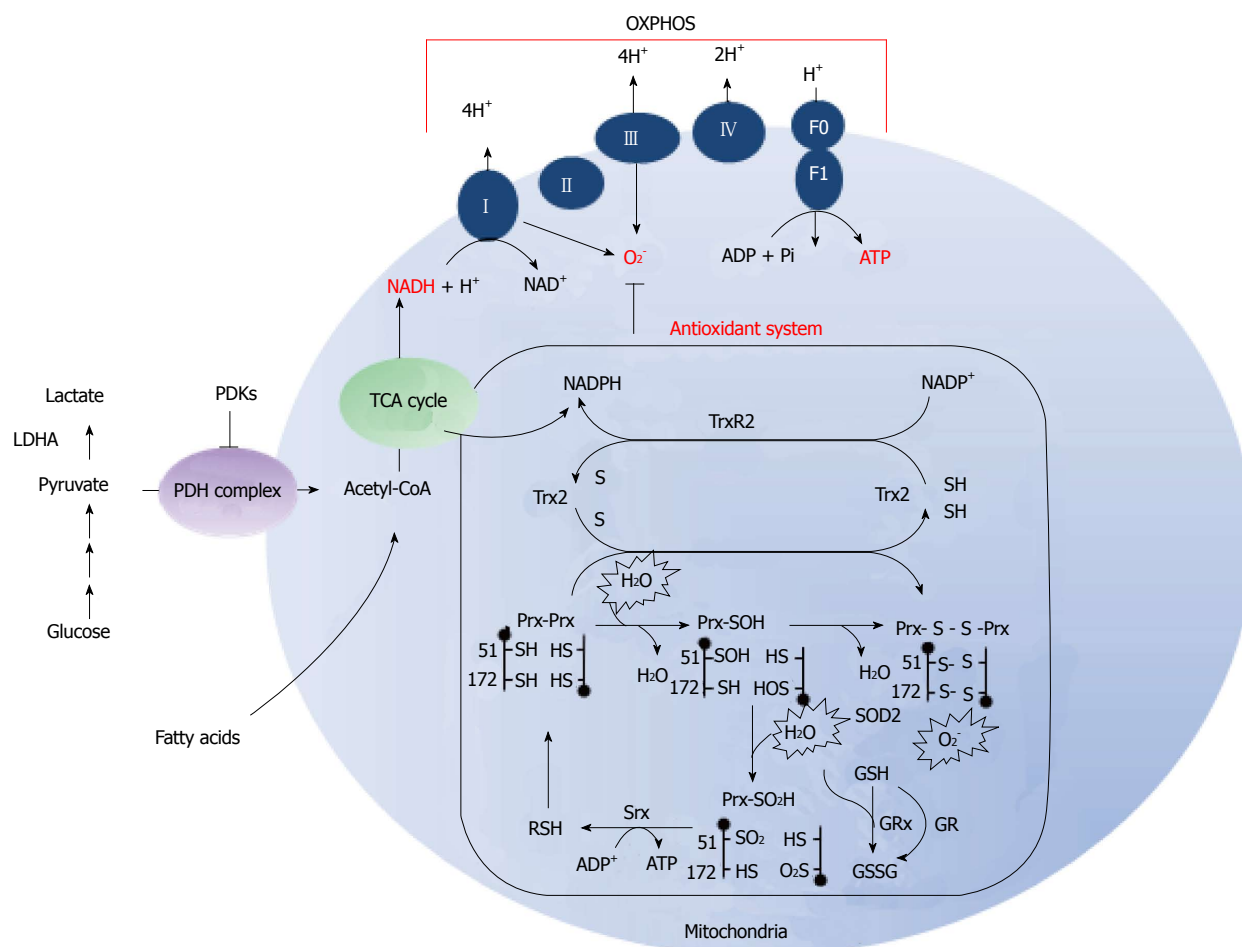
The proposed mechanism differs from that in non-malignant cells utilizing OXPHOS. Although aerobic glycolysis has been corroborated in cancer cells, the function of mitochondria has been controversial<sup>[27]</sup>. In cancer cells, the aerobic glycolysis generate glycolytic intermediates to the pentose phosphate pathway. Moreover, the glycolytic ATP generation is important for survival in hypoxic conditions<sup>[28]</sup>. In OXPHOS, the ATP synthesis requires much oxygen, which leads to continuous the ROS production such as superoxide anion, organic peroxide, and hydrogen peroxide<sup>[29]</sup>. If the generated ROS are not eliminated by redox regulating system, they may cause cellular damage.

### Mitochondrial antioxidant system

Mitochondria have a multi-level network of redox-defense systems for the elimination of hydrogen peroxide (Figure 1). Glutathione and glutathione peroxidases require nicotinamide adenine dinucleotide phosphate (NADPH) for the reduction of H<sub>2</sub>O<sub>2</sub> and other peroxides generated in the mitochondria. Mitochondrial redox balances are also regulated by the mitochondrial inner membrane electrochemical gradient, which mitochondrial Complex V (ATP synthase) uses to produce ATP from ADP and inorganic phosphate (Pi).

Moreover, the physiological significance of mitochondrial redox balance has been highlighted by the antioxidant genes-deletion and over-expression. As antioxidant defense system, Peroxiredoxin (Prx) 3, Prx5, superoxide dismutase 2, and thioredoxin 2 eliminates ROS produced in mitochondria<sup>[30,31]</sup>. Knockout (KO) of Prx3 mice result in induction of oxidative damage<sup>[32]</sup>, KO of thioredoxin 2 mice showed an embryonic lethal phenotype<sup>[33]</sup> and KO of superoxide dismutase 2 mice die within 3 wk of birth because of mitochondrial





**Figure 1 Antioxidant and oxidative phosphorylation systems in mitochondria.** Under normal conditions, normal cells rely primarily on oxidative phosphorylation for ATP synthesis, whereas cancer cells rely more on glycolysis. Pyruvate from glycolysis is converted to acetyl-CoA, CO<sub>2</sub>, and NADH by pyruvate dehydrogenase (PDH). Acetyl-CoA enters the TCA cycle by the citrate synthase-mediated reaction with oxaloacetate to generate citrate. NADH is oxidized first by Complex I in the electron transport chain (OXPHOS). Electrons from Complex I and II are transferred to coenzyme Q10, then passed on to Complex III, cytochrome c, Complex IV, and finally to O<sub>2</sub> to generate H<sub>2</sub>O. O<sub>2</sub> is converted to H<sub>2</sub>O<sub>2</sub> through the action of SOD2 and/or spontaneous dismutation. H<sub>2</sub>O<sub>2</sub> is eliminated by three mechanisms: (1) glutathione (GSH) peroxidase (GPx) coupled to GSH and GSH reductase (GR); (2) Prx3 coupled to Trx2 and Trx reductase (TrxR 2); and (3) non-enzymatic eliminating by redox compounds. The H<sub>2</sub>O<sub>2</sub> selectively oxidized cysteine Cys-SH to Cys-SOH, which then reacts with the resolving cysteine Cys-SH of the other subunit in the homodimer to form an intermolecular disulfide bond. The disulfide bond is reduced by Trx2. Moreover, the generated Cys-SOH is oxidized to Cys-SO<sub>2</sub>H. Reactivation of the enzyme is achieved by reduction of the Cys-SO<sub>2</sub>H moiety and is catalyzed by sulfiredoxin (Srx). Nicotinamide adenine dinucleotide phosphate (NADPH) is utilized by the reductases in the peroxidase system (GR and TrxR) to reduce disulfide bonds formed in proteins during the elimination of H<sub>2</sub>O<sub>2</sub>. TCA: Tricarboxylic acid; ATP: Adenosine triphosphatase; ADP: Adenosine diphosphate.

oxidative damage and severe neurodegeneration<sup>[34,35]</sup>. Therefore, the inhibition of antioxidant systems may provide a targeted therapy that leads to mitochondrial dysfunction and cell death.

### Mitochondrial membrane potential

Mitochondria harbor a robust mitochondrial trans-membrane potential ( $\Delta\Psi_m$ ), and the exchange of small metabolites between the mitochondrial matrix and the cytosol is induced by the low conductance of permeability transition pore complex (PTPC)<sup>[36]</sup>. The rupture of mitochondrial membranes leading to functional impairment result in the release of toxic mitochondrial intermembrane space proteins, such as apoptosis-inducing factor and cytochrome *c*, into the cytosol<sup>[37]</sup>. Under apoptotic conditions, including ROS and Ca<sup>2+</sup> overload, the PTPC presumes a high conductance state allowing uncontrolled influx of small solutes

into the matrix of mitochondria. This mitochondrial permeability transition (MPT) leads to osmotic swelling of the mitochondrial matrix and dissipation of the  $\Delta\Psi_m$ <sup>[38,39]</sup>, and eventually cell death occurs due to mitochondrial outer membrane permeabilization<sup>[40]</sup>. The MPT is triggered by reagents increasing ROS generation, cytosolic Ca<sup>2+</sup> concentrations, or acting on the PTPC. Therefore, the induction of mitochondrial membrane permeabilization are attractive targets to develop drug for cancer therapy.

### Mitochondria-targeted cancer therapy

As mentioned above, mitochondria play important role in apoptosis, but also trigger cell death through various mechanisms<sup>[41-43]</sup>. Various mitochondria-targeted strategies for cancer treatment have been developed over the last decade<sup>[6,44]</sup> that focused on the development of agents that regulate the MPT,



Bcl-2 family proteins, and ROS production in cancer<sup>[6]</sup>. Numerous molecules, acting on mitochondria, are currently used or being tested in clinical trials<sup>[45]</sup>. Several experimental anti-cancer drugs, such as ceramide<sup>[46]</sup>, CD437<sup>[47]</sup>, and MKT077<sup>[48]</sup>, and clinically approved anti-cancer drugs, such as etoposide<sup>[49]</sup>, paclitaxel<sup>[50]</sup>, and vinorelbine<sup>[51]</sup>, induce apoptosis *via* mitochondria dysfunction. Furthermore, determining of pathophysiological differences of mitochondria between cancer cells and normal cells, will improve the selectivity of mitochondria-targeted anti-cancer agents.

## MITOCHONDRIA OF CSCs

Because mitochondria play a key role in the alteration of oxidative stress, energy status, and apoptotic stimuli, scientists have assumed that they are also involved in the regulation of stemness and differentiation in SCs. Researchers have attempted to employ mitochondrial properties in the selection of SCs<sup>[52]</sup>. Lonergan *et al.*<sup>[53]</sup> and Bavister<sup>[54]</sup> suggested that functional mitochondrial characteristics, such as subcellular localization and metabolic activity could verify stemness, SC stability, and pluripotency. Mitochondria are localized in perinuclear sites in embryonic stem cells (ESCs) and have a more scattered distribution throughout the cytoplasm after differentiation and senescence<sup>[55]</sup>.

Mitochondrial metabolic activity is also related to cell differentiation, as early passages of an adult primate stromal cell line have a higher oxygen consumption rate (OCR) and a low ATP/ mitochondrial DNA content compared with long-term cultured cells<sup>[53]</sup>. In CD34<sup>+</sup> hematopoietic SCs, a low mitochondrial OCR and mitochondrial mass result in a predominantly perinuclear mitochondrial arrangement<sup>[56]</sup>.

Antioxidant enzyme expression also shows a dramatic change during differentiation<sup>[57]</sup>. Moreover, ROS play an agonistic role in the differentiation of ESCs. Enhanced intracellular ROS as the differentiation stimulus may act on transplanted SCs into the cardiovascular lineage<sup>[58]</sup>, indicating that mitochondrial redox metabolism act as a crucial regulator in cardiac differentiation of SCs. Furthermore, Plotnikov *et al.*<sup>[59]</sup> suggest a correlation of the mitochondrial function and the status of neural SCs.

SC mitochondria play important roles in maintaining stemness and differentiation. However, whether the roles of CSC mitochondria are similar to SC mitochondria or cancer cells in general is uncertain. Two hypotheses on the origin of CSCs, both of which contribute to acute myeloid leukemia<sup>[1,60]</sup>, have been proposed. One hypothesis of the origin of CSCs is that they are derivatives of SCs residing in various organs. Genetic mutations and epigenetic changes, which are crucial for initiation and progression of tumor growth, accumulate in long-lived stem cells, and the transformation of SCs into CSCs initiates carcinogenesis.

CSCs may also have a greater differentiation potential than other SCs. (SCs can be divided into the following groups based on differentiation potential: the totipotent, pluripotent, multipotent, and unipotent group). Another hypothesis assumes the existence of ESC-like cells that convert into CSCs when they are exposed to damaging environmental factors. Additional differentiation and mutation of these cells may also contribute to development of CSCs<sup>[61]</sup>. Based on these reports, the CSCs may be more differentiated than normal SCs and likewise, the mitochondrial properties of CSCs are different from those of SCs or general cancer cells.

Recently, Ye *et al.*<sup>[62]</sup> determined the mitochondrial features between lung CSCs and non-CSCs. As a results, it is showed a lower mtDNA contents, lower OCR, glucose consumption, intracellular ATP and ROS level in the lung CSCs compared to non-CSCs. Leukemia CSCs showed a low ROS level and reduced OXPHOS compared with that of non-CSCs<sup>[63]</sup>. However, Pastò *et al.*<sup>[64]</sup> reported that CSCs exhibited over-expressed genes related to glucose uptake, oxidative phosphorylation, and fatty acid  $\beta$ -oxidation, indicating higher ability to direct pyruvate towards the TCA cycle. As reported, ovarian CSCs showed higher mitochondrial ROS production and  $\Delta\Psi_m$  than non-CSCs. In addition, targeting mitochondrial biogenetics induced caspase-independent cell death in ovarian CSCs<sup>[65]</sup>. In glioma CSCs, a higher mitochondrial reserve capacity was measured as compared to the differentiated cells<sup>[66]</sup>. Glioblastoma CSCs also depend on OXPHOS for their energy production and survival<sup>[67]</sup>. Besides, breast CSCs have higher ATP content compared to their differentiated progeny<sup>[68]</sup>. Based on these studies, CSCs mitochondria showed the different roles and features according to the cancer type. A summary of the mitochondrial features between CSCs and non-CSCs according to cancer origin is highlighted in Table 2. Although the mitochondrial features of CSCs in several cancers are not identical, CSCs mitochondria obviously differ from those of non-CSCs. Moreover, mitochondrial features of CSCs have not been clearly defined in other cancer types. Most importantly, little has been known about the mitochondrial features related to energy metabolism and the ROS/antioxidant enzyme system of CSCs in colon, stomach, liver, bone, and prostate cancer. Therefore, defining these features will be essential for developing a mitochondria-targeted therapeutic drug that induces death of CSCs, and therefore, reduces the risk of relapsed or refractory cancer.

## CLINICAL IMPLICATION AND THERAPEUTIC TARGETS OF CSCs

Despite the recent surge of published studies on CSCs, the clinical significance of this population remains unclear and has been slow in progression of the development of clinical agents to eliminate CSCs. However, most experts agree that effective



**Table 2 Mitochondrial features of cancer stem cells according to cancer origin**

Cancer origin	Mitochondria features			Energy metabolism of CSC	Target/drug for CSCs	Ref.
	Feature	CSC	Non-CSC			
Breast	Glucose uptake	High	Low	OXPHOS		[68]
	ATP contents	High	Low			
	OCR	High	Low			
	Lactate production	Low	High			
	Membrane potential	High	Low			
Glioma	Glucose consumption	Low	High	OXPHOS		[66]
	ATP contents	High	Low			
	Lactate production	Low	High			
	OCR	High	Low		IMP-2	[67]
	ATP contents	High	Low			
Leukemia	ROS	Low	High	Low glycolysis Low OXPHOS	Bcl-2/ ABT263	[63]
	Proliferation rate	Slow	Fast			
	OCR	Low	High			
	Lactate production	Low	High			
	ATP contents	Low	High			
Lung	Glucose consumption	Low	High			[62]
	OCR	Low	High			
	ROS level	Low	High			
	ATP contents	Low	High			
	Membrane potential	High	Low			
Ovarian	Mitochondrial DNA	Low	High	OXPHOS	NV-128	[65] [64]
	ROS	High	Low			
	Membrane potential	High	Low			
	ATP contents	High	Low			
	Glucose deprivation	Resist	Sensitive			

CSC: Cancer stem cell; OCR: Oxygen consumption rate; ROS: Reactive oxygen species; OXPHOS: Oxidative phosphorylation; ABT263: Bcl-2 inhibitor; NV-128: Isoflavone derivative (play a role as inhibitor of mitochondrial function); IMP-2: Insulin-like growth factor 2 mRNA-binding protein 2.

anti-cancer drugs should be targeted toward CSCs in addition to non-CSCs. Current cancer treatments such as conventional chemotherapy and radiotherapy target rapidly proliferating cells that make up the bulk of the tumor, but do not specifically target CSCs. Thus, the hypotheses on the origin of CSCs may explain the development of relapsed and metastatic cancer. In cancer therapy, the new paradigm requires development of novel anti-cancer drug molecules and drug targets to assess drug responses of CSCs.

Altered expression of genes involved in apoptosis, survival, and DNA repair machinery are among the multiple mechanisms responsible for the chemoresistance of leukemia<sup>[69]</sup>, brain<sup>[70]</sup>, pancreatic<sup>[71]</sup>, breast<sup>[72]</sup>, melanoma<sup>[73,74]</sup>, and colon cancer<sup>[75]</sup> CSCs. Liu *et al.*<sup>[23]</sup> reports that CD133<sup>+</sup> glioblastoma cells isolated from patients have a high expression of genes in the Bcl-2 and inhibitor of apoptosis (IAP) families. Moreover, several types of CSCs have upregulated ATP binding cassette (ABC) pumps that make them resistant to various chemotherapeutics<sup>[73,74]</sup>. Therefore, finding targets that efficiently promote CSC cell death is important and a focus of intensive research. Dong and colleagues demonstrate that loss of fructose-1,6-bisphosphatase in breast CSCs induces glycolysis, as well as inhibiting oxygen consumption and ROS generation, through the suppression of mitochondrial Complex I activity<sup>[76]</sup>. The report implies

that overproduction of ROS and reduction in glucose metabolism may be effective against breast CSCs. Hirsch *et al.*<sup>[77]</sup> showed that metformin, an AMPK activator and Complex I inhibitor often used as the first-line drug for treating diabetes, and selectively kills CSCs in breast cancer cell lines. The novel isoflavone derivative NV-128 significantly decreased mitochondrial function, as shown by a decreases in ATP, Complex I, and Complex IV levels, and induced cell death in ovarian CSCs<sup>[65]</sup>. These results demonstrate that specific mitochondrial targeted compounds can induce cell death in chemoresistant CSCs and may be a new venue for treating ovarian cancer patients with relapsed or metastatic cancer. The new-generation taxoid SB-T-1214 significantly inhibited stemness gene expression profiles and induced cell death in both CSCs and general cancer cells, indicating its promise in overcoming relapsed and refractory cancer due to CSCs<sup>[78]</sup>. Finally, mitochondria-targeted vitamin E succinate (MitoVES), which includes the positively charged triphenylphosphonium group, may be the most well-characterized toxic agent in its ability to induce apoptosis in breast CSCs<sup>[79]</sup>. Meanwhile, it was reported that a drug which inhibits the self-renewal of CSCs by targeting of Notch and Hedgehog pathway has been developed<sup>[80]</sup>. It was also reported that has been developed a drugs, which can eliminate CSCs by targeting cell surface markers such as CD133 and EpCAM. However, the use of these drugs increases



the exposure to side effects due to the sharing of signaling pathway and cell surface marker with normal SCs. Thus, it is important to understand how CSCs differ from normal SCs and differentiated cells. Moreover, a full understanding of the mitochondrial function and energy metabolism in CSCs contributes to the development of the agents targeting mitochondrial functions (such as ROS overproduction, energy metabolism inhibition, and antioxidant protein inhibition), and presents a need to develop new strategies to target CSCs in the clinical field<sup>[80]</sup>.

## CONCLUSION

In summary, the mitochondria are an important tool to investigate CSCs properties and to develop anti-cancer drugs. However, the properties and clinical significance of mitochondria in CSCs have not been verified. Because mitochondria-targeted therapy may open new strategies for the treatment of relapsed and refractory cancer, mitochondrial properties unique to CSCs need to be defined. Furthermore, combined treatment with mitochondrial-targeted and anti-cancer drugs may specifically induce the death of both CSCs and general cancer cells and promises to be a novel cancer therapy.

## REFERENCES

1. **Reya T**, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105-111 [PMID: 11689955 DOI: 10.1038/35102167]
2. **Visvader JE**, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008; **8**: 755-768 [PMID: 18784658 DOI: 10.1038/nrc2499]
3. **Li Z**, Rich JN. Hypoxia and hypoxia inducible factors in cancer stem cell maintenance. *Curr Top Microbiol Immunol* 2010; **345**: 21-30 [PMID: 20582533 DOI: 10.1007/82\_2010\_75]
4. **Song IS**, Kim HK, Lee SR, Jeong SH, Kim N, Ko KS, Rhee BD, Han J. Mitochondrial modulation decreases the bortezomib-resistance in multiple myeloma cells. *Int J Cancer* 2013; **133**: 1357-1367 [PMID: 23463417 DOI: 10.1002/ijc.28149]
5. **Song IS**, Jeong YJ, Jeong SH, Heo HJ, Kim HK, Lee SR, Ko TH, Youm JB, Kim N, Ko KS, Rhee BD, Han J. Combination treatment with 2-methoxyestradiol overcomes bortezomib resistance of multiple myeloma cells. *Exp Mol Med* 2013; **45**: e50 [PMID: 24158003 DOI: 10.1038/emmm.2013.104]
6. **Fulda S**, Galluzzi L, Kroemer G. Targeting mitochondria for cancer therapy. *Nat Rev Drug Discov* 2010; **9**: 447-464 [PMID: 20467424 DOI: 10.1038/nrd3137]
7. **Song IS**, Kim HK, Jeong SH, Lee SR, Kim N, Rhee BD, Ko KS, Han J. Mitochondrial peroxiredoxin III is a potential target for cancer therapy. *Int J Mol Sci* 2011; **12**: 7163-7185 [PMID: 22072940 DOI: 10.3390/ijms12107163]
8. **Gogvadze V**, Orrenius S, Zhivotovsky B. Mitochondria in cancer cells: what is so special about them? *Trends Cell Biol* 2008; **18**: 165-173 [PMID: 18296052 DOI: 10.1016/j.tcb.2008.01.006]
9. **Bellance N**, Lestienne P, Rossignol R. Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis. *Front Biosci (Landmark Ed)* 2009; **14**: 4015-4034 [PMID: 19273331]
10. **Kroemer G**, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. *Cancer Cell* 2008; **13**: 472-482 [PMID: 18538731 DOI: 10.1016/j.ccr.2008.05.005]
11. **Dick JE**. Stem cell concepts renew cancer research. *Blood* 2008; **112**: 4793-4807 [PMID: 19064739 DOI: 10.1182/blood-2008-08-077941]
12. **Furth J**, Kahn M C, Breedis C. The transmission of leukaemia of mice with a single cell. *Am J Cancer* 1937; **31**: 276-282 [DOI: 10.1158/ajc.1937.276]
13. **Clermont Y**, Leblond CP. Renewal of spermatogonia in the rat. *Am J Anat* 1953; **93**: 475-501 [DOI: 10.1002/aja.1000930308]
14. **Al-Hajj M**, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003; **100**: 3983-3988 [PMID: 12629218 DOI: 10.1073/pnas.0530291100]
15. **Singh SK**, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB. Identification of human brain tumour initiating cells. *Nature* 2004; **432**: 396-401 [PMID: 15549107 DOI: 10.1038/nature03128]
16. **O'Brien CA**, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007; **445**: 106-110 [PMID: 17122772 DOI: 10.1038/nature05372]
17. **Ricci-Vitiani L**, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111-115 [PMID: 17122771 DOI: 10.1038/nature05384]
18. **Singh SK**, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003; **63**: 5821-5828 [PMID: 14522905]
19. **Florek M**, Haase M, Marzesco AM, Freund D, Ehninger G, Huttner WB, Corbeil D. Prominin-1/CD133, a neural and hematopoietic stem cell marker, is expressed in adult human differentiated cells and certain types of kidney cancer. *Cell Tissue Res* 2005; **319**: 15-26 [PMID: 15558321 DOI: 10.1007/s00441-004-1018-z]
20. **Mehra N**, Penning M, Maas J, Beerepoot LV, van Daal N, van Gils CH, Giles RH, Voest EE. Progenitor marker CD133 mRNA is elevated in peripheral blood of cancer patients with bone metastases. *Clin Cancer Res* 2006; **12**: 4859-4866 [PMID: 16914572 DOI: 10.1158/1078-0432.CCR-06-0422]
21. **Thomson JA**, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: 9804556]
22. **Wicha MS**, Liu S, Dontu G. Cancer stem cells: an old idea-a paradigm shift. *Cancer Res* 2006; **66**: 1883-1890; discussion 1895-1896 [PMID: 16488983 DOI: 10.1158/0008-5472.CAN-05-3153]
23. **Clarke MF**, Fuller M. Stem cells and cancer: two faces of eve. *Cell* 2006; **124**: 1111-1115 [PMID: 16564000 DOI: 10.1016/j.cell.2006.03.011S0092-8674(06)00312-6]
24. **Galluzzi L**, Kroemer G. Necroptosis: a specialized pathway of programmed necrosis. *Cell* 2008; **135**: 1161-1163 [PMID: 19109884 DOI: 10.1016/j.cell.2008.12.004]
25. **Modica-Napolitano JS**, Singh KK. Mitochondrial dysfunction in cancer. *Mitochondrion* 2004; **4**: 755-762 [PMID: 16120430 DOI: 10.1016/j.mito.2004.07.027]
26. **Warburg O**. On the origin of cancer cells. *Science* 1956; **123**: 309-314 [PMID: 13298683]
27. **Weinhouse S**. On respiratory impairment in cancer cells. *Science* 1956; **124**: 267-269 [PMID: 13351638]
28. **Weinberg F**, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, Lopez M, Kalyanaraman B, Mutlu GM, Budinger GR, Chandel NS. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc Natl Acad Sci USA* 2010; **107**: 8788-8793 [PMID: 20421486 DOI: 10.1073/pnas.1003428107]
29. **Reed DJ**. Glutathione: toxicological implications. *Annu Rev Pharmacol Toxicol* 1990; **30**: 603-631 [PMID: 2188580 DOI: 10.1146/annurev.pa.30.040190.003131]
30. **Rabilloud T**, Heller M, Rigobello MP, Bindoli A, Aebersold R, Lunardi J. The mitochondrial antioxidant defence system and its response to oxidative stress. *Proteomics* 2001; **1**: 1105-1110 [PMID: 11990504 DOI: 10.1002/1615-9861(200109)1: 9<1105::AID-PROT1105>3.0.CO;2-M]



- 31 **Banmeyer I**, Marchand C, Clippe A, Knoops B. Human mitochondrial peroxiredoxin 5 protects from mitochondrial DNA damages induced by hydrogen peroxide. *FEBS Lett* 2005; **579**: 2327-2333 [PMID: 15848167 DOI: 10.1016/j.febslet.2005.03.027]
- 32 **Huh JY**, Kim Y, Jeong J, Park J, Kim I, Huh KH, Kim YS, Woo HA, Rhee SG, Lee KJ, Ha H. Peroxiredoxin 3 is a key molecule regulating adipocyte oxidative stress, mitochondrial biogenesis, and adipokine expression. *Antioxid Redox Signal* 2012; **16**: 229-243 [PMID: 21902452 DOI: 10.1089/ars.2011.3952]
- 33 **Nonn L**, Williams RR, Erickson RP, Powis G. The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice. *Mol Cell Biol* 2003; **23**: 916-922 [PMID: 12529397]
- 34 **Lebovitz RM**, Zhang H, Vogel H, Cartwright J, Dionne L, Lu N, Huang S, Matzuk MM. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc Natl Acad Sci USA* 1996; **93**: 9782-9787 [PMID: 8790408]
- 35 **Hinerfeld D**, Traini MD, Weinberger RP, Cochran B, Doctrow SR, Harry J, Melov S. Endogenous mitochondrial oxidative stress: neurodegeneration, proteomic analysis, specific respiratory chain defects, and efficacious antioxidant therapy in superoxide dismutase 2 null mice. *J Neurochem* 2004; **88**: 657-667 [PMID: 14720215 DOI: 10.1046/j.1471-4159.2003.02195.x]
- 36 **Bouchier-Hayes L**, Muñoz-Pinedo C, Connell S, Green DR. Measuring apoptosis at the single cell level. *Methods* 2008; **44**: 222-228 [PMID: 18314052 DOI: 10.1016/j.ymeth.2007.11.007]
- 37 **Nakagawa T**, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 2005; **434**: 652-658 [PMID: 15800626 DOI: 10.1038/nature03317]
- 38 **Baines CP**, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* 2007; **9**: 550-555 [PMID: 17417626 DOI: 10.1038/ncb1575]
- 39 **Marzo I**, Brenner C, Zamzami N, Jürgensmeier JM, Susin SA, Vieira HL, Prévost MC, Xie Z, Matsuyama S, Reed JC, Kroemer G. Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science* 1998; **281**: 2027-2031 [PMID: 9748162]
- 40 **Kroemer G**, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 2007; **87**: 99-163 [PMID: 17237344 DOI: 10.1152/physrev.00013.2006]
- 41 **Gulbins E**, Dreschers S, Bock J. Role of mitochondria in apoptosis. *Exp Physiol* 2003; **88**: 85-90 [PMID: 12525857 DOI: 10.1113/eph8802503]
- 42 **Hiendleder S**, Schmutz SM, Erhardt G, Green RD, Plante Y. Trans-mitochondrial differences and varying levels of heteroplasmy in nuclear transfer cloned cattle. *Mol Reprod Dev* 1999; **54**: 24-31 [PMID: 10423294 DOI: 10.1002/(SICI)1098-2795(199909)54:1<24::AID-MRD4>3.0.CO;2-S]
- 43 **Waterhouse NJ**, Goldstein JC, Kluck RM, Newmeyer DD, Green DR. The (Hole) study of mitochondria in apoptosis. *Methods Cell Biol* 2001; **66**: 365-391 [PMID: 11396012]
- 44 **Fantini VR**, Leder P. Mitochondriotoxic compounds for cancer therapy. *Oncogene* 2006; **25**: 4787-4797 [PMID: 16892091 DOI: 10.1038/sj.onc.12095991209599]
- 45 **Pathania D**, Millard M, Neamati N. Opportunities in discovery and delivery of anticancer drugs targeting mitochondria and cancer cell metabolism. *Adv Drug Deliv Rev* 2009; **61**: 1250-1275 [PMID: 19716393 DOI: 10.1016/j.addr.2009.05.010]
- 46 **Stover TC**, Sharma A, Robertson GP, Kester M. Systemic delivery of liposomal short-chain ceramide limits solid tumor growth in murine models of breast adenocarcinoma. *Clin Cancer Res* 2005; **11**: 3465-3474 [PMID: 15867249 DOI: 10.1158/1078-0432.CCR-04-1770]
- 47 **Holmes WF**, Soprano DR, Soprano KJ. Elucidation of molecular events mediating induction of apoptosis by synthetic retinoids using a CD437-resistant ovarian carcinoma cell line. *J Biol Chem* 2002; **277**: 45408-45419 [PMID: 12237293 DOI: 10.1074/jbc.M204600200]
- 48 **Propper DJ**, Braybrooke JP, Taylor DJ, Lodi R, Styles P, Cramer JA, Collins WC, Levitt NC, Talbot DC, Ganesan TS, Harris AL. Phase I trial of the selective mitochondrial toxin MKT077 in chemo-resistant solid tumours. *Ann Oncol* 1999; **10**: 923-927 [PMID: 10509153]
- 49 **Robertson JD**, Gogvadze V, Zhivotovsky B, Orrenius S. Distinct pathways for stimulation of cytochrome c release by etoposide. *J Biol Chem* 2000; **275**: 32438-32443 [PMID: 10961984 DOI: 10.1074/jbc.C000518200]
- 50 **Kidd JF**, Pilkington MF, Schell MJ, Fogarty KE, Skepper JN, Taylor CW, Thorn P. Paclitaxel affects cytosolic calcium signals by opening the mitochondrial permeability transition pore. *J Biol Chem* 2002; **277**: 6504-6510 [PMID: 11724773 DOI: 10.1074/jbc.M106802200]
- 51 **Chinnery PF**, Taylor GA, Howell N, Andrews RM, Morris CM, Taylor RW, McKeith IG, Perry RH, Edwardson JA, Turnbull DM. Mitochondrial DNA haplogroups and susceptibility to AD and dementia with Lewy bodies. *Neurology* 2000; **55**: 302-304 [PMID: 10908912]
- 52 **Bertoncello I**, Hodgson GS, Bradley TR. Multiparameter analysis of transplantable hemopoietic stem cells: I. The separation and enrichment of stem cells homing to marrow and spleen on the basis of rhodamine-123 fluorescence. *Exp Hematol* 1985; **13**: 999-1006 [PMID: 2865163]
- 53 **Lonergan T**, Brenner C, Bavister B. Differentiation-related changes in mitochondrial properties as indicators of stem cell competence. *J Cell Physiol* 2006; **208**: 149-153 [PMID: 16575916 DOI: 10.1002/jcp.20641]
- 54 **Bavister BD**. The mitochondrial contribution to stem cell biology. *Reprod Fertil Dev* 2006; **18**: 829-838 [PMID: 17147931]
- 55 **Barnett DK**, Kimura J, Bavister BD. Translocation of active mitochondria during hamster preimplantation embryo development studied by confocal laser scanning microscopy. *Dev Dyn* 1996; **205**: 64-72 [PMID: 8770552 DOI: 10.1002/(SICI)1097-0177(199601)205:1<64::AID-AJA6>3.0.CO;2-3]
- 56 **Piccoli C**, Ria R, Scrima R, Cela O, D'Aprile A, Boffoli D, Falzetti F, Tabilio A, Capitanio N. Characterization of mitochondrial and extra-mitochondrial oxygen consuming reactions in human hematopoietic stem cells. Novel evidence of the occurrence of NAD(P)H oxidase activity. *J Biol Chem* 2005; **280**: 26467-26476 [PMID: 15883163 DOI: 10.1074/jbc.M500047200]
- 57 **Rhee SG**, Kang SW, Chang TS, Jeong W, Kim K. Peroxiredoxin, a novel family of peroxidases. *IUBMB Life* 2001; **52**: 35-41 [PMID: 11795591 DOI: 10.1080/15216540252774748]
- 58 **Sauer H**, Wartenberg M. Reactive oxygen species as signaling molecules in cardiovascular differentiation of embryonic stem cells and tumor-induced angiogenesis. *Antioxid Redox Signal* 2005; **7**: 1423-1434 [PMID: 16356105 DOI: 10.1089/ars.2005.7.1423]
- 59 **Plotnikov EY**, Marei MV, Podgornyi OV, Aleksandrova MA, Zorov DB, Sukhikh GT. Functional activity of mitochondria in cultured neural precursor cells. *Bull Exp Biol Med* 2006; **141**: 142-146 [PMID: 16929986]
- 60 **Miyamoto T**, Weissman IL, Akashi K. AML1/ETO-expressing nonleukemic stem cells in acute myelogenous leukemia with 8; 21 chromosomal translocation. *Proc Natl Acad Sci USA* 2000; **97**: 7521-7526 [PMID: 10861016]
- 61 **Kucia M**, Ratajczak MZ. Stem cells as a two edged sword--from regeneration to tumor formation. *J Physiol Pharmacol* 2006; **57** Suppl 7: 5-16 [PMID: 17228093]
- 62 **Ye XQ**, Li Q, Wang GH, Sun FF, Huang GJ, Bian XW, Yu SC, Qian GS. Mitochondrial and energy metabolism-related properties as novel indicators of lung cancer stem cells. *Int J Cancer* 2011; **129**: 820-831 [PMID: 21520032 DOI: 10.1002/ijc.25944]
- 63 **Lagadinou ED**, Sach A, Callahan K, Rossi RM, Neering SJ, Minhajuddin M, Ashton JM, Pei S, Grose V, O'Dwyer KM, Liesveld JL, Brookes PS, Becker MW, Jordan CT. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell* 2013; **12**:



- 329-341 [PMID: 23333149 DOI: 10.1016/j.stem.2012.12.013]
- 64 **Pastò A**, Bellio C, Pilotto G, Ciminale V, Silic-Benussi M, Guzzo G, Rasola A, Frasson C, Nardo G, Zulato E, Nicoletto MO, Manicone M, Indraccolo S, Amadori A. Cancer stem cells from epithelial ovarian cancer patients privilege oxidative phosphorylation, and resist glucose deprivation. *Oncotarget* 2014; **5**: 4305-4319 [PMID: 24946808 DOI: 2010]
  - 65 **Alvero AB**, Montagna MK, Holmberg JC, Craveiro V, Brown D, Mor G. Targeting the mitochondria activates two independent cell death pathways in ovarian cancer stem cells. *Mol Cancer Ther* 2011; **10**: 1385-1393 [PMID: 21677151 DOI: 10.1158/1535-7163.MCT-11-0023]
  - 66 **Vlasi E**, Lagadec C, Vergnes L, Matsutani T, Masui K, Poulou M, Popescu R, Della Donna L, Evers P, Dekmezian C, Reue K, Christofk H, Mischel PS, Pajonk F. Metabolic state of glioma stem cells and nontumorigenic cells. *Proc Natl Acad Sci USA* 2011; **108**: 16062-16067 [PMID: 21900605 DOI: 10.1073/pnas.1106704108]
  - 67 **Janiszewska M**, Suvà ML, Riggi N, Houtkooper RH, Auwerx J, Clément-Schatlo V, Radovanovic I, Rheinbay E, Provero P, Stamenkovic I. Imp2 controls oxidative phosphorylation and is crucial for preserving glioblastoma cancer stem cells. *Genes Dev* 2012; **26**: 1926-1944 [PMID: 22899010 DOI: 10.1101/gad.188292.112]
  - 68 **Vlasi E**, Lagadec C, Vergnes L, Reue K, Frohnen P, Chan M, Alhiyari Y, Dratver MB, Pajonk F. Metabolic differences in breast cancer stem cells and differentiated progeny. *Breast Cancer Res Treat* 2014; **146**: 525-534 [PMID: 25007966 DOI: 10.1007/s10549-014-3051-2]
  - 69 **Essers MA**, Trumpp A. Targeting leukemic stem cells by breaking their dormancy. *Mol Oncol* 2010; **4**: 443-450 [PMID: 20599449 DOI: 10.1016/j.molonc.2010.06.001]
  - 70 **Bao S**, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006; **444**: 756-760 [PMID: 17051156 DOI: 10.1038/nature05236]
  - 71 **Lonardo E**, Hermann PC, Heeschen C. Pancreatic cancer stem cells - update and future perspectives. *Mol Oncol* 2010; **4**: 431-442 [PMID: 20580623 DOI: 10.1016/j.molonc.2010.06.002]
  - 72 **McDermott SP**, Wicha MS. Targeting breast cancer stem cells. *Mol Oncol* 2010; **4**: 404-419 [PMID: 20599450 DOI: 10.1016/j.molonc.2010.06.005]
  - 73 **Frank NY**, Pendse SS, Lapchak PH, Margaryan A, Shlain D, Doeing C, Sayegh MH, Frank MH. Regulation of progenitor cell fusion by ABCB5 P-glycoprotein, a novel human ATP-binding cassette transporter. *J Biol Chem* 2003; **278**: 47156-47165 [PMID: 12960149 DOI: 10.1074/jbc.M308700200]
  - 74 **Frank NY**, Margaryan A, Huang Y, Schatton T, Waaga-Gasser AM, Gasser M, Sayegh MH, Sadee W, Frank MH. ABCB5-mediated doxorubicin transport and chemoresistance in human malignant melanoma. *Cancer Res* 2005; **65**: 4320-4333 [PMID: 15899824 DOI: 10.1158/0008-5472.CAN-04-3327]
  - 75 **Boman BM**, Huang E. Human colon cancer stem cells: a new paradigm in gastrointestinal oncology. *J Clin Oncol* 2008; **26**: 2828-2838 [PMID: 18539961 DOI: 10.1200/JCO.2008.17.6941]
  - 76 **Dong C**, Yuan T, Wu Y, Wang Y, Fan TW, Miriyala S, Lin Y, Yao J, Shi J, Kang T, Lorkiewicz P, St Clair D, Hung MC, Evers BM, Zhou BP. Loss of FBPI by Snail-mediated repression provides metabolic advantages in basal-like breast cancer. *Cancer Cell* 2013; **23**: 316-331 [PMID: 23453623 DOI: 10.1016/j.ccr.2013.01.022]
  - 77 **Hirsch HA**, Iliopoulos D, Struhl K. Metformin inhibits the inflammatory response associated with cellular transformation and cancer stem cell growth. *Proc Natl Acad Sci USA* 2013; **110**: 972-977 [PMID: 23277563 DOI: 10.1073/pnas.1221055110]
  - 78 **Botchkina GI**, Zuniga ES, Das M, Wang Y, Wang H, Zhu S, Savitt AG, Rowe RA, Leyfman Y, Ju J, Shroyer K, Ojima I. New-generation taxoid SB-T-1214 inhibits stem cell-related gene expression in 3D cancer spheroids induced by purified colon tumor-initiating cells. *Mol Cancer* 2010; **9**: 192 [PMID: 20630067 DOI: 10.1186/1476-4598-9-192]
  - 79 **Biasutto L**, Dong LF, Zoratti M, Neuzil J. Mitochondrially targeted anti-cancer agents. *Mitochondrion* 2010; **10**: 670-681 [PMID: 20601192 DOI: 10.1016/j.mito.2010.06.004]
  - 80 **Loureiro R**, Mesquita KA, Oliveira PJ, Vega-Naredo I. Mitochondria in cancer stem cells: a target for therapy. *Recent Pat Endocr Metab Immune Drug Discov* 2013; **7**: 102-114 [PMID: 23360288 DOI: 10.2174/18722148113079990006]
  - 81 **Ginestier C**, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, Jacquemier J, Viens P, Kleer CG, Liu S, Schott A, Hayes D, Birnbaum D, Wicha MS, Dontu G. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007; **1**: 555-567 [PMID: 18371393 DOI: 10.1016/j.stem.2007.08.014]
  - 82 **Schatton T**, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M, Zhan Q, Jordan S, Duncan LM, Weishaupt C, Fuhlbrigge RC, Kupper TS, Sayegh MH, Frank MH. Identification of cells initiating human melanomas. *Nature* 2008; **451**: 345-349 [PMID: 18202660 DOI: 10.1038/nature06489]
  - 83 **Sangiorgi E**, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* 2008; **40**: 915-920 [PMID: 18536716 DOI: 10.1038/ng.165]
  - 84 **Lukacs RU**, Memarzadeh S, Wu H, Witte ON. Bmi-1 is a crucial regulator of prostate stem cell self-renewal and malignant transformation. *Cell Stem Cell* 2010; **7**: 682-693 [PMID: 21112563 DOI: 10.1016/j.stem.2010.11.013]
  - 85 **Fang D**, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S, Van Belle PA, Xu X, Elder DE, Herlyn M. A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res* 2005; **65**: 9328-9337 [PMID: 16230395 DOI: 10.1158/0008-5472.CAN-05-1343]
  - 86 **Shackleton M**, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. Generation of a functional mammary gland from a single stem cell. *Nature* 2006; **439**: 84-88 [PMID: 16397499 DOI: 10.1038/nature04372]
  - 87 **Pontier SM**, Muller WJ. Integrins in mammary-stem-cell biology and breast-cancer progression--a role in cancer stem cells? *J Cell Sci* 2009; **122**: 207-214 [PMID: 19118213 DOI: 10.1242/jcs.040394]
  - 88 **Krause DS**, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. *Blood* 1996; **87**: 1-13 [PMID: 8547630]
  - 89 **Furness SG**, McNagny K. Beyond mere markers: functions for CD34 family of sialomucins in hematopoiesis. *Immunol Res* 2006; **34**: 13-32 [PMID: 16720896 DOI: 10.1385/IR.34:1:13]
  - 90 **Rongioletti F**, Donati P, Amantea A, Ferrara G, Montinari M, Santoro F, Parodi A. Obesity-associated lymphoedematous mucinosis. *J Cutan Pathol* 2009; **36**: 1089-1094 [PMID: 19222694 DOI: 10.1111/j.1600-0560.2008.01239.x]
  - 91 **Prince ME**, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF, Ailles LE. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci USA* 2007; **104**: 973-978 [PMID: 17210912 DOI: 10.1073/pnas.0610117104]
  - 92 **Dalerba P**, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, Shelton AA, Parmiani G, Castelli C, Clarke MF. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci USA* 2007; **104**: 10158-10163 [PMID: 17548814 DOI: 10.1073/pnas.0703478104]
  - 93 **Li C**, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. *Cancer Res* 2007; **67**: 1030-1037 [PMID: 17283135 DOI: 10.1158/0008-5472.CAN-06-2030]
  - 94 **Collins AT**, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005; **65**: 10946-10951 [PMID: 16322242 DOI: 10.1158/0008-5472.CAN-05-2018]
  - 95 **Günthert U**, Hofmann M, Rudy W, Reber S, Zöller M, Haussmann I, Matzku S, Wenzel A, Ponta H, Herrlich P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991; **65**: 13-24 [PMID: 1707342 DOI: 10.1016/0092-8674(91)90403-L]
  - 96 **Zöller M**. CD44: can a cancer-initiating cell profit from an



- abundantly expressed molecule? *Nat Rev Cancer* 2011; **11**: 254-267 [PMID: 21390059 DOI: 10.1038/nrc3023]
- 97 **Burger PE**, Xiong X, Coetzee S, Salm SN, Moscatelli D, Goto K, Wilson EL. Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with high capacity to reconstitute prostatic tissue. *Proc Natl Acad Sci USA* 2005; **102**: 7180-7185 [PMID: 15899981 DOI: 10.1073/pnas.0502761102]
  - 98 **Yang ZF**, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P, Chu PW, Lam CT, Poon RT, Fan ST. Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell* 2008; **13**: 153-166 [PMID: 18242515 DOI: 10.1016/j.ccr.2008.01.013]
  - 99 **Augello A**, Kurth TB, De Bari C. Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. *Eur Cell Mater* 2010; **20**: 121-133 [PMID: 21249629]
  - 100 **Salcido CD**, Larochele A, Taylor BJ, Dunbar CE, Varticovski L. Molecular characterisation of side population cells with cancer stem cell-like characteristics in small-cell lung cancer. *Br J Cancer* 2010; **102**: 1636-1644 [PMID: 20424609 DOI: 10.1038/sj.bjc.6605668]
  - 101 **Eramo A**, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C, De Maria R. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ* 2008; **15**: 504-514 [PMID: 18049477 DOI: 10.1038/sj.cdd.4402283]
  - 102 **Hermann PC**, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 2007; **1**: 313-323 [PMID: 18371365 DOI: 10.1016/j.stem.2007.06.002]
  - 103 **Liu G**, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir IR, Lu L, Irvin D, Black KL, Yu JS. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. *Mol Cancer* 2006; **5**: 67 [PMID: 17140455 DOI: 10.1186/1476-4598-5-67]
  - 104 **Mizrak D**, Brittan M, Alison M. CD133: molecule of the moment. *J Pathol* 2008; **214**: 3-9 [PMID: 18067118 DOI: 10.1002/path.2283]
  - 105 **Zhang S**, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH, Nephew KP. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 2008; **68**: 4311-4320 [PMID: 18519691 DOI: 10.1158/0008-5472.CAN-08-0364]
  - 106 **Ponnusamy MP**, Batra SK. Ovarian cancer: emerging concept on cancer stem cells. *J Ovarian Res* 2008; **1**: 4 [PMID: 19014671 DOI: 10.1186/1757-2215-1-4]
  - 107 **Monk M**, Holding C. Human embryonic genes re-expressed in cancer cells. *Oncogene* 2001; **20**: 8085-8091 [PMID: 11781821 DOI: 10.1038/sj.onc.1205088]
  - 108 **Carpenter MK**, Rosler E, Rao MS. Characterization and differentiation of human embryonic stem cells. *Cloning Stem Cells* 2003; **5**: 79-88 [PMID: 12713704 DOI: 10.1089/153623003321512193]
  - 109 **Kim CF**, Jackson EL, Woelfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005; **121**: 823-835 [PMID: 15960971 DOI: 10.1016/j.cell.2005.03.032]

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## Vital roles of stem cells and biomaterials in skin tissue engineering

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### Abstract

Tissue engineering essentially refers to technology for growing new human tissue and is distinct from regenerative medicine. Currently, pieces of skin are already being fabricated for clinical use and many other tissue types may be fabricated in the future.

Tissue engineering was first defined in 1987 by the United States National Science Foundation which critically discussed the future targets of bioengineering research and its consequences. The principles of tissue engineering are to initiate cell cultures *in vitro*, grow them on scaffolds *in situ* and transplant the composite into a recipient *in vivo*. From the beginning, scaffolds have been necessary in tissue engineering applications. Regardless, the latest technology has redirected established approaches by omitting scaffolds. Currently, scientists from diverse research institutes are engineering skin without scaffolds. Due to their advantageous properties, stem cells have robustly transformed the tissue engineering field as part of an engineered bilayered skin substitute that will later be discussed in detail. Additionally, utilizing biomaterials or skin replacement products in skin tissue engineering as strategy to successfully direct cell proliferation and differentiation as well as to optimize the safety of handling during grafting is beneficial. This approach has also led to the cells' application in developing the novel skin substitute that will be briefly explained in this review.

**Key words:** Hair follicle stem cells; Skin repair; Tissue engineering; Chitosan; Collagen

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**Core tip:** Biomaterials and epithelial stem cells, and especially hair follicle stem cells are vital components for successful skin tissue engineering. Ignoring one of these components will decrease the opportunity for skin tissue engineering to foster complete healing through skin repair and will increase the failure of skin grafting in the clinical setting. The latest technology, new raw biomaterials and information on the significant contribution of stem cells are likely to be of great benefit to skin tissue engineering.



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## INTRODUCTION

In classical bioengineered skin, the engineered epidermis is reconstructed from a skin autograft or allograft serving as a cellular dressing. The limitations addressed by this technique are pain and scar formation at the donor site, impaired wound healing and non-healing wounds, insufficient material to cover a large defect area and autoimmune rejection in the case of an allograft. The emergence of skin tissue engineering has led to robust innovation in skin substitutes and skin replacement products for burn and wound management. Diseased or injured skin may rule out a particular treatment because of wound depth. Superficial, partial and full-thickness wounds require diverse skin substitutes that simultaneously function as a primary dressing. Although various commercial skin substitutes are available, novel findings on fabrication techniques for biomaterials and on regulators of wound healing have highly encouraged scientists to develop new engineered skin substitutes that offer an effective remedy for wound care and wound management. The combination of stem cells or other cells with a specifically designed novel biomaterial has resulted in different impacts on engineered skin after wounding. An ideal biomaterial with multiple combinations of cultured cells and a collectively established broad knowledge of the healing process are the main criteria for the future development of skin substitutes. Eventually, skin substitutes that can be kept frozen, that are ready for use, inexpensive in cost, less labor intensive, and permanently adherent to the wound bed, that yield an impressive cosmetic outcome and that do not contain animal or human serum will definitely be in increased demand for skin tissue engineering purposes.

## DEFINITION OF STEM CELLS

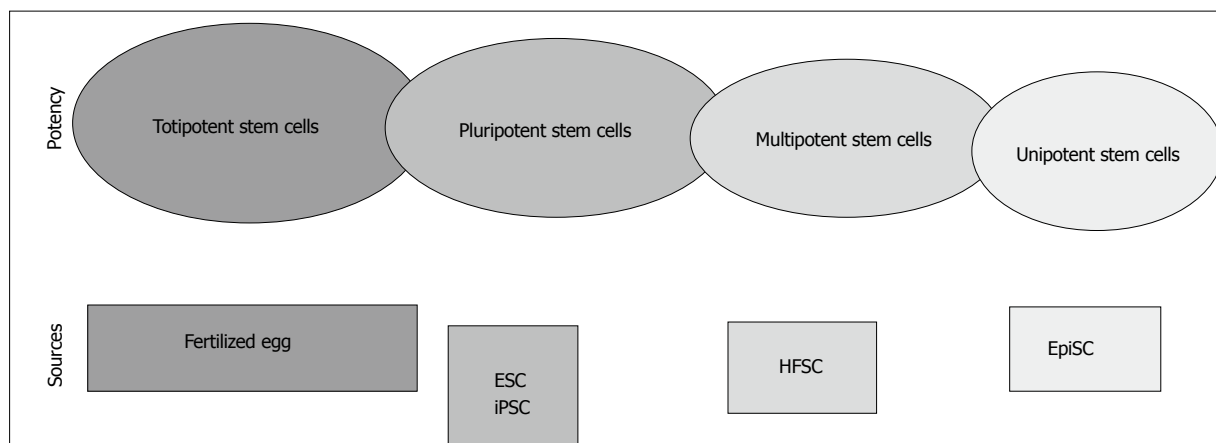
Stem cells have at least two advantageous properties. First, these cells are capable of dividing or renewing themselves for long periods of time with the identical morphology and phenotypic characteristics as their parent cells. This self-renewal mechanism is crucial for recovering degenerated and damaged cells, tissues or organs. Second, the differentiation of stem cells is a mechanism that can maintain the cellular and molecular integrity of adjacent or other cells. Stem cells differentiate into specialized cells to perform a specific function<sup>[1]</sup> after receiving internal or external signals, which are signs of signalling pathway changes in the

cells' microenvironment. Differentiation starts when the cells discontinue renewal. Differentiation abilities are used to rank stem cells' potency (Figure 1). Stem cells that are able to differentiate into one cell type are known as unipotent stem cells, such as epidermal stem cells which regenerate differentiated epidermis<sup>[2]</sup>. Hair follicle stem cells (HFSC) are able to differentiate into multiple types of structures with specialized functions in the body including hair follicles<sup>[3]</sup>, epidermis<sup>[4]</sup>, sebaceous glands<sup>[5]</sup> and neurons<sup>[6]</sup>. These stem cells are known as multipotent stem cells. Pluripotent stem cells are stem cells that have the ability to differentiate into ectoderm, mesoderm and endoderm germ layers as demonstrated by embryonic stem cells and induced pluripotent stem cells<sup>[7]</sup>. Totipotent stem cells have the excellent ability to differentiate into whole tissues. This total potency can be observed in the fertilized eggs of humans or animals.

## SKIN STEM CELLS

The skin is known as cutaneous or integumentary and its appendages constitute the integumentary system. The skin is the largest organ, contributing 15%-20% of body mass to form the external covering of the body. The skin has two main components: the epidermis and the dermis. The epidermis is derived from ectoderm, composed of a keratinized stratified squamous epithelium that grows continuously and simultaneously maintains its normal thickness by the process of desquamation<sup>[8]</sup>. The dermis is derived from mesoderm, composed of a connective tissue that provides mechanical support, strength and thickness to the skin. The hypodermis is not a part of the skin but lies beneath the dermis and is equivalent to the subcutaneous fascia. This layer contains adipose tissue arranged into lobules that are separated by connective tissue septa. The epithelial skin appendages are composed of the hair follicles and hairs, sweat glands, sebaceous glands, nails and mammary glands. The skin forms an effective barrier against pathogen invasion, chemicals and ultraviolet light<sup>[9]</sup>; participates in homeostasis<sup>[10]</sup> by regulating the body temperature and water loss; conveys sensory information; and function in pigmentation<sup>[11]</sup>, physical appearance, wound repair and regeneration<sup>[12]</sup>. The skin is considered as a stem cell zoo, as it accommodates a variety of stem cell niches. A niche is the microenvironment in which stem cells remain quiescent. The dermal papilla is a stem cell niche for mesenchymal stem cells (MSC) which initiate hair follicle growth<sup>[13]</sup>. The superior bulge is a stem cell niche for HFSC<sup>[14]</sup> and melanocyte stem cells (MeSC)<sup>[15]</sup>. Multipotent HFSC not only function to maintain the hair growth cycle<sup>[16]</sup>, but also give rise to sebaceous glands<sup>[17]</sup> and contribute to skin re-epithelialization<sup>[18]</sup>, which form neo-epidermis by differentiating into keratinocytes within the wound. MeSC supply melanin for hair and skin pigmentation<sup>[19]</sup> and are concurrently involved in skin re-epithelialization<sup>[20]</sup>. The abundant fat





**Figure 1** The potency of stem cells. ESC: Embryonic stem cells; iPSC: Induced pluripotent stem cells; HFSC: Hair follicle stem cells; EpiSC: Epidermal stem cells.

in the subcutaneous layer contains adipose stem cells (ASC)<sup>[21]</sup> which are efficient in promoting skin repair<sup>[22]</sup>. Collectively, the evidence suggests that skin's distinct resident stem cell pools have a crucial function in skin repair and regeneration. Therefore, it is very important for scientists to develop a good understanding of this idea before skin substitutes can be engineered in the future.

## SKIN TISSUE ENGINEERING

Skin tissue engineering is the first field within tissue engineering that successfully engineered tissue in the laboratory. As the largest organ, the skin has high potential risk of injuries and diseases. Skin damaged requires a skin replacement product from either a natural or an artificial source. Skin replacement products are in high demand for the treatment of burns and wounds, leading the industrial sector to highly invest in skin tissue engineering. Skin tissue engineering utilizes biomaterials, stem cells, connective tissues and an established broad knowledge of the mechanism of the acute and chronic healing processes. The main target of skin tissue engineering is to produce an excellent skin replacement product for application in wound repair, especially in the case of full-thickness skin loss, with the goal of less or no scar formation after wounding. However, skin replacement products are not able to replace damaged skin as completely as native skin can, leaving a scar. Consequently, the field of skin tissue engineering is challenging.

## BIOMATERIALS

Collagen gel is the first biomaterial or skin replacement product that has been used in skin tissue engineering to replace the use of allografts and autografts. Essentially, to produce an excellent skin substitute, a biomaterial must be able to support cell growth and differentiation in a similar manner as in the

cells' original microenvironment. Most importantly, biomaterials with multiple graded pore sizes allow the acceleration of tissue reconstruction<sup>[23]</sup>. For example, microscopic pores improve cell attachment, proliferation and responses<sup>[24]</sup> and macroscopic pores that are at least 100  $\mu\text{m}$  in diameter play a role in enhancing the ingrowth of cells and blood capillaries<sup>[25]</sup>. To allow three-dimensional (3-D) tissue reconstruction *in vitro*, a biomaterial should support uniform cell spreading into interconnected pores for the engineering specific tissue<sup>[26]</sup>. Biomaterials with a single pore size have certain limitations, only allowing one cell type to grow according to the pore size whereas, biomaterials with multiple graded pore sizes can reconstruct various types of tissues simultaneously<sup>[27]</sup>. A previous study has suggested that a pore size 20-120  $\mu\text{m}$  in diameter is suitable for skin tissue engineering<sup>[28]</sup>. Biomaterials must have the ability to absorb the nutrients involved in wound healing and the exudate of the wound bed which are important factors in skin tissue engineering. The absorption ability is known as the water uptake ratio (WUR). Biomaterials with a high WUR are suitable for use in full-thickness wounds that contain excess exudates and inflammation that lead to impaired wound healing. Meanwhile, a lower WUR is suitable for partial thickness wounds<sup>[29]</sup>. An appropriate WUR enhances the biological activity of skin equivalents and contributes to hydrophilicity and the maintenance of 3-D structure. A moist wound bed is required to enhance dermal regeneration and wound closure which can be achieved if a scaffold has suitable water vapor permeability (WVP). The WVP depends on the scaffold thickness and the ratio of the scaffold area to the water surface area<sup>[30]</sup>. A suitable WVP is an important factor for wound dressing. If the WVP is too high, the wound bed will be dry and it may increase metabolic activity. In contrast, if the WVP is too low, the accumulation of exudates will trigger the onset of bacterial growth. Following the criteria mentioned above, a fabricated skin replacement product will retain the cell-cell and cell-biomaterial signaling, allowing the complete layer



**Table 1** Current skin replacement products

Brand	Cell and biomaterial	Wound type
Apligraf®, United States	Human keratinocytes and fibroblasts cultured on collagen	Full thickness
Biobrane®, United Kingdom	Silicone membrane attached to a nylon mesh	Partial thickness
CellerateRX®, United States	Gel containing 65% collagen	Superficial
Cryoskin®, United Kingdom	Keratinocytes cultured on silicone	Superficial
Dermagraft®, United States	Cryopreserved human fibroblasts from foreskin cultured on a polyglactin mesh scaffold	Full thickness
EpiDex®, Switzerland	HFSC cultured on silicone	Full thickness
EpiFix®, United States	Human amniotic membrane	Full thickness
Integra®, United States	Semi-permeable silicone membrane composed of bovine tendon collagen	Full thickness
MyDerm™, Malaysia	Human fibroblasts and keratinocytes cultured on fibrin-silk	Full thickness
OASIS™, United States	Porcine small intestinal submucosa	Full thickness
OrCel™, United States	Human keratinocytes and fibroblasts cultured on bovine collagen	Full thickness
PriMatrix, United States	Collagen from fetal bovine dermis	Full thickness
Transcyte™, United States	Human newborn fibroblasts cultured on nylon mesh	Full thickness

HFSC: Hair follicle stem cells.

of skin to be engineered. Subsequently, the engineered skin substitute can be used for grafting purposes, this is how skin tissue engineering works. Most importantly, the engineered skin must be tolerated by the host, be retained permanently and later be able to degrade slowly over time. A permanent skin replacement product is a primary dressing that is ideal for application in great skin loss because it is used only once during treatment, until healing is completed. In contrast, a temporary skin replacement product must be changed many times during treatment which may increase the cost of wound management and wound care. Commercial skin replacement products are collagen, chitosan or fibrin-based and these products have been broadly used in clinical applications as shown in Table 1.

Apligraf is a permanent cellular based skin replacement product for the treatment of non-healing wounds including diabetic foot ulcer and venous leg ulcer. It was approved by the United States Food and Drug Administration's guidelines (FDA) for use in non-infected, partial and full-thickness skin ulcers.

Biobrane is a temporary acellular based skin replacement product. It originated from porcine dermal collagen which is bonded to a nylon mesh and nylon membrane to form a biosynthetic skin dressing for use in superficial wound, partial-thickness wound and donor site wound dressing.

CellerateRX is a patented form of Type I bovine collagen for the treatment of diabetic or other impaired healing wounds. It is provided in a powder and a gel form which can be used as alone or a powder-gel combination.

Cryoskin is a cell spray-based skin replacement product and is prepared upon request by clinicians. It is made from allogeneic donor cells which are originally isolated from a newborn foreskin biopsy. Cryoskin spray is suitable for the treatment of chronic wounds and burns.

Dermagraft is a permanent cryopreserved cellular based skin replacement product. It composed of collagen which is isolated from neonatal foreskin biopsy and cultured on biodegradable mesh. Dermagraft was

approved by the FDA for use in diabetic foot ulcers.

EpiDex is a permanent autologous epidermal skin replacement product that is isolated directly from epithelial stem cells of patients. The patient's own hair was plucking using non-surgical procedure and then grafted onto a chronic wound.

EpiFix is a dehydrated amniotic membrane allograft. It is provided in sheets form, does not require FDA approval as human amniotic membrane is considered not significantly changed from its original structure. EpiFix is used in the treatment of acute and chronic wounds.

Integra is a bilayer engineered skin replacement product. It composed of porous matrix derived from bovine tendon collagen, glycosaminoglycan and a semi-permeable polysiloxane. Integra is used in the treatment of chronic and traumatic wounds.

MyDerm is an autologous bilayer engineered skin replacement product. It contained the mixture of fibrin-keratinocytes that seeded onto a piece of medical grade of silk. The mixture of fibrin-fibroblasts is then seeded onto fibrin-keratinocyte skin equivalent. MyDerm is used for the treatment of wounds and burns.

OASIS is a xenogeneic porous collagen matrix which derived from porcine small intestinal submucosa. It was approved by the FDA for the management of pressure ulcers, venous ulcers, diabetic ulcers and chronic vascular ulcers.

OrCel is an allogeneic bilayered cellular based matrix. It is biodegradable, made of composite bovine collagen which contained skin cells. OrCel was approved by the FDA for use in patients with dystrophic epidermolysis bullosa.

PriMatrix is a xenogeneic acellular based dermal matrix. It is derived from fetal bovine dermis. PriMatrix is provided in meshed, fenestrated and solid form. It was approved by the FDA for pressure and venous stasis ulcers.

Transcyte is an allogeneic bilayer skin replacement product for the treatment of burn. It made of human fibroblasts grown on nylon mesh and then combined with a synthetic epidermal layer. Transcyte has been



approved by the FDA for temporary covering over burn wounds.

### Collagen

Collagen is the main protein component in connective tissue and its most abundant sources are found in mammals and marine animals. Collagen is easily obtained from porcine and bovine sources which provide bone, skin, tendon and many other parts of the body as raw materials. Unfortunately, prion disease that may transfer to humans after these materials' use<sup>[31]</sup> has led scientists to have seek an alternative source. Currently, fish skin, seaweed, jellyfish and other marine sources are in high demand for isolating collagen and are the ideal sources for skin tissue engineering because prion disease transmission to humans is eliminated if marine-based sources are used compared with mammalian-based sources. Collagen-based biomaterials encode antimicrobial activity<sup>[32]</sup> and do not support colonization by bacteria in a full-thickness wound. The prevention of infection in wounds leads to minimized scar formation and promote wound healing<sup>[33]</sup>. Due to their biocompatibility and biodegradability, collagen biomaterials have been established for use in skin repair in the clinical setting since last decade with various forms such as a gel<sup>[34]</sup>, sponge<sup>[35]</sup>, film<sup>[36]</sup> and paste<sup>[37]</sup>.

### Chitosan

Chitosan is a biopolymer obtained from deacetylated chitin and the second most abundant biopolymer sources after collagen. Crude chitin can be found in shellfish. Chitosan is naturally insoluble unless in acidic solutions, and possesses a positive charge and low cytotoxicity which broadly imply excellence as a DNA carrier<sup>[38]</sup>, protein nanocarrier<sup>[39]</sup>, drug delivery system<sup>[40]</sup>, siRNA nanovector<sup>[41]</sup> and growth factor carrier<sup>[42]</sup>. Based on its composition, the structure of chitosan is similar to that of glycosaminoglycans. Glycosaminoglycans are a component of the extracellular matrix and are an important substrate for tissue constructs<sup>[43]</sup>. Chitosan arrests bleeding in major hepatic injuries and is effective for use in patients who suffer major injury with clotting dysfunction<sup>[44]</sup>. Therefore, chitosan is preferable for tissue engineering and especially for engineered skin applications. The used of a novel technique to develop a chitosan sponge by electrospinning has produced a chitosan nanofiber that provides excellent substrate-cell adhesion, proliferation and differentiation<sup>[45]</sup>. During skin repair and regeneration, nanofibers contribute to increased vascularization, re-epithelialization and enhanced granulation tissue formation. The major skin cells, particularly epithelial and epidermal cells and also fibroblasts grow well on this type of biomaterial.

### Fibrin

Fibrin-based biomaterials are well-established biological

sealants for skin tissue engineering. However, the use of raw blood to isolate the fibrin has limited fibrin's use as a novel skin substitute. Commercial fibrin in kit form can successfully heal a wound but is relatively expensive for the majority of patients. Fibrin gel is continuously in demand in plastics and reconstructive surgery as it minimizes subcutaneous seroma formation and decreases wound morbidity<sup>[46]</sup>. The combination of fibrin with bone marrow mesenchymal stem cells can improve the condition of scalded skin, providing strong self-repair capability and promising an acceptable cosmetic appearance with hair follicle formation. This application is more suitable for the treatment of patients with burns in emergency cases<sup>[47]</sup>. Long-lasting fibrin biomaterials ensure stable and functional angiogenesis by highly tunable and sustained delivery of growth factors<sup>[48]</sup>.

## MESENCHYMAL STEM CELLS

Mesenchymal stem cells also known as mesenchymal stromal cells, have broadly contributed to cellular therapy and skin tissue engineering. MSC are spindle-shaped, or similar to morphology to but phenotypically different from fibroblasts. Bone marrow derived-MSC were first isolated in 1981<sup>[49]</sup> before MSC from other tissues were explored. Skin derived-MSC were first isolated from mice in 2001, and were initially named skin-derived precursors<sup>[50]</sup>. In the skin, MSC are predominantly found in the dermal papilla, functioning in secreting diverse growth factors after wounding to promote fibroblast proliferation and collagen formation and to elicit intrinsic stem cell differentiation<sup>[51]</sup>, serving as a modulator to activate macrophages<sup>[52]</sup>, and directly affecting hair follicle morphogenesis<sup>[53]</sup> and neo-dermis reconstruction<sup>[54]</sup>. MSC elicit leukocyte migration for skin homeostasis and produce hepatocyte growth factor and basic fibroblast growth factor to inhibit scar formation at the wound site<sup>[55]</sup>. The MSC-based skin substitute constructs have increased paracrine factor levels in promoting skin repair<sup>[56]</sup>.

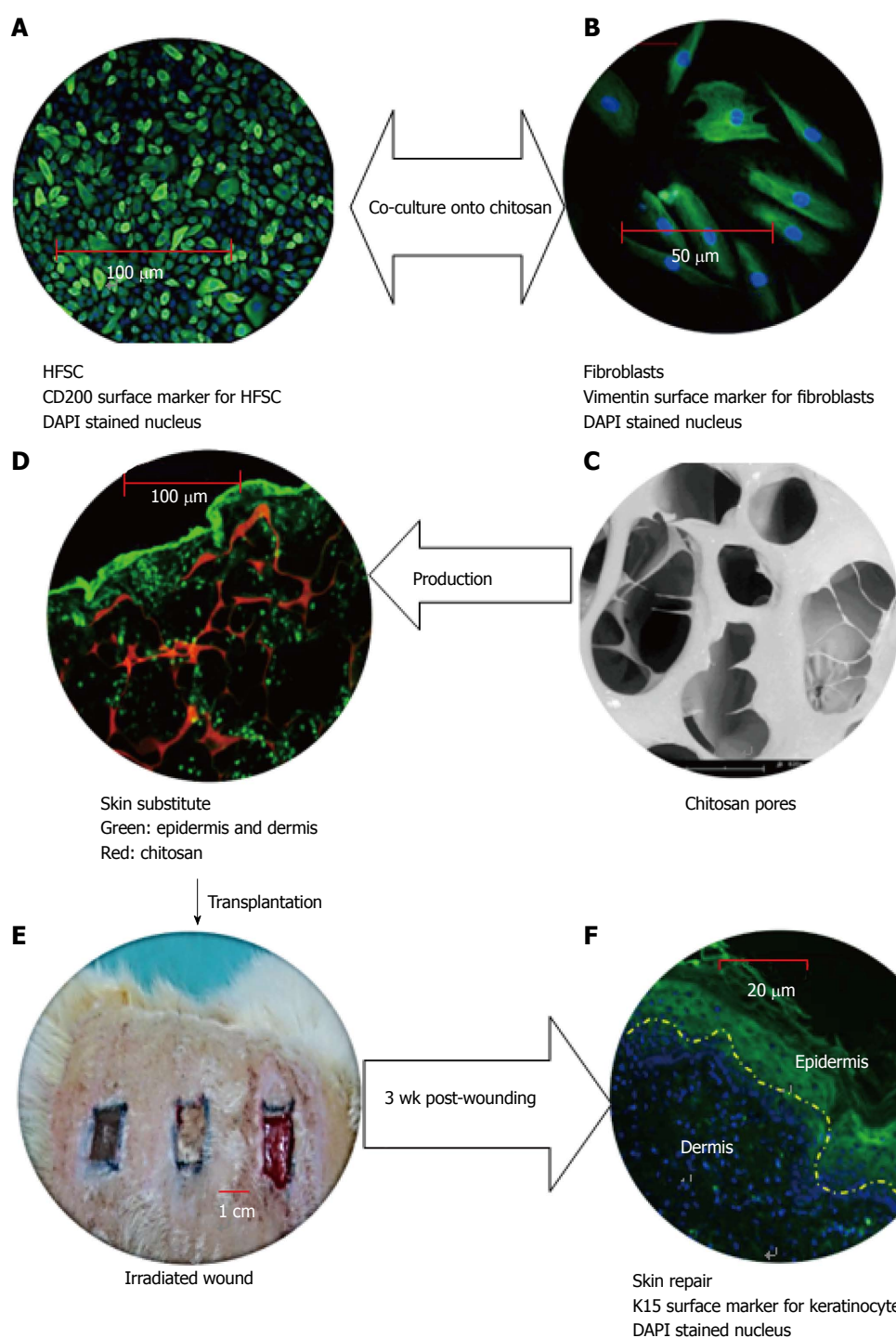
## FIBROBLASTS

Apart from stem cells, other components of the dermis such as fibroblasts are important in skin tissue engineering<sup>[57]</sup>. Fibroblasts synthesize and deposit extracellular protein after wounding, producing growth and angiogenic factors that accelerate cell proliferation and angiogenesis<sup>[58]</sup>. Fibroblasts are also transformed into myofibroblasts that express contractile fibers. As a myofibroblast contracts, forces are applied to the collagen fibers, thus reducing the size of the wound<sup>[59]</sup>. Cultured fibroblasts in biomaterials have been reported to improve regeneration of the dermis<sup>[60]</sup> and epidermis<sup>[61]</sup> especially by re-epithelialization<sup>[57]</sup>. This specific role of fibroblasts, especially in improving scarring and the contraction of skin wounds has encouraged researchers





**Figure 2** The rapid proliferating of hair follicle stem cells. At hour 0, cells were separated (A) and then closely adhered to a plate (B) until one of the cells doubled (arrow head) at 12.59 h post-culture (C). Magnification 400  $\times$ .



**Figure 3** The vital role of hair follicle stem cells in skin tissue engineering. HFSC: Hair follicle stem cells; DAPI: 4', 6-diamidino-2-phenylindole.



to highly prioritize facilitating these cells' use in skin replacement therapies<sup>[62]</sup>. Additionally, a previous study has demonstrated that fibroblasts from the dermis are the best source for skin tissue engineering applications compared with fibroblasts from other sources based on yields and reduced contraction of wounds<sup>[63]</sup>.

## ADIPOSE STEM CELLS

Fat accommodates adipose tissue-derived stem cells usually known as adipose stem cells<sup>[21]</sup>. ASC are actually MSC therefore, ASC express the same surface markers and highly contribute to skin repair as well. ASC improve neo epidermis formation and skin aging<sup>[64]</sup> by accelerating angiogenesis<sup>[65]</sup> and fibroblast proliferation<sup>[66]</sup>. The use of ASC in skin repair increases the deposition of extra cellular matrix components such as collagen type-VI which has the potential to improve the protein deficiency<sup>[67]</sup>. A mixture of fibroblasts and ASC can improve the epidermal morphogenesis of tissue-engineered skin<sup>[68]</sup>. The ability of ASC to grow on porous biomaterial and to differentiate into fibrovascular, endothelial and epithelial tissues for skin repair<sup>[69]</sup> has proven these cells' excellent capability as a cellular source for skin tissue engineering.

## HFSCs

HFSC and their progenitors are directly involved in hair and skin regeneration during skin homeostasis<sup>[70]</sup>. Based on their anatomical classification and function, HFSC have mainly been called epithelial stem cells<sup>[71,72]</sup> and have been broadly known as epidermal stem cells<sup>[73,74]</sup> as well as bulge stem cells<sup>[75,76]</sup> in diverse studies. The critical role of HFSC in epidermal homeostasis<sup>[77]</sup> has led to their application in fabricating neo epidermis in skin tissue engineering. Due to their vital characteristic, HFSC are capable of proliferating rapidly and this phenomenon is shown in Figure 2.

The use of HFSC in skin tissue engineering was successfully performed in chronic wounding in 2002, when Limat and Hunziker demonstrated that their HFSC-silicone constructs contributed to accelerated the healing of patients' leg ulcers<sup>[78]</sup>. To date, the contribution of HFSC to ameliorating impaired healing due to radiation has stimulated the interest of tissue engineers in using epithelial stem cells for other tissue reconstruction. In Figure 3 shows that the chitosan based skin substitute which composed of HFSC-fibroblasts is an ideal source for skin tissue engineering. The chitosan composite was then used for transplanting into irradiated wound. Full-thickness wounds 1 cm by 1 cm in size were excised and treated using the novel skin substitute. As a result, a complete re-epithelialization occurred during skin repair after 3 wk post-wounding.

## CONCLUSION

This review has summarized the fundamentals of

stem cells and especially the terminology for stem cell potency which has occasionally created confusion in certain experiments. Stem cell potency provides the necessary guideline for skin engineering experiments. The importance of fibroblast, HFSC, MSC, ASC, chitosan, collagen and fibrin in skin tissue engineering highlights their critical role in the repair process. The use of stem cells and biomaterials is especially important in the case of impaired wound healing and in cases involving major excisional skin defects.

## REFERENCES

- 1 **Hilmi ABM**, Halim AS, Noor NM, Lim CK, Idris Z, Pohchi A, Asma H, Wahab SFA, Tiede S, and Paus R: A simple culture method for epithelial stem cells derived from human hair follicle. *Central European Journal of Biology* 2013; **8**: 432-439 [DOI: 10.2478/s11535-013-0149-6]
- 2 **Potten CS**, Booth C. Keratinocyte stem cells: a commentary. *J Invest Dermatol* 2002; **119**: 888-899 [PMID: 12406335 DOI: 10.1046/j.1523-1747.2002.00020.x]
- 3 **Lien WH**, Polak L, Lin M, Lay K, Zheng D, Fuchs E. In vivo transcriptional governance of hair follicle stem cells by canonical Wnt regulators. *Nat Cell Biol* 2014; **16**: 179-190 [PMID: 24463605 DOI: 10.1038/ncb2903]
- 4 **Liang X**, Bhattacharya S, Bajaj G, Guha G, Wang Z, Jang HS, Leid M, Indra AK, Ganguli-Indra G. Delayed cutaneous wound healing and aberrant expression of hair follicle stem cell markers in mice selectively lacking Ctip2 in epidermis. *PLoS One* 2012; **7**: e29999 [PMID: 22383956 DOI: 10.1371/journal.pone.0029999]
- 5 **Frances D**, Niemann C. Stem cell dynamics in sebaceous gland morphogenesis in mouse skin. *Dev Biol* 2012; **363**: 138-146 [PMID: 2227295 DOI: 10.1016/j.ydbio.2011.12.028]
- 6 **Amoh Y**, Kanoh M, Niiyama S, Hamada Y, Kawahara K, Sato Y, Hoffman RM, Katsuoka K. Human hair follicle pluripotent stem (hFPS) cells promote regeneration of peripheral-nerve injury: an advantageous alternative to ES and iPS cells. *J Cell Biochem* 2009; **107**: 1016-1020 [PMID: 19507228 DOI: 10.1002/jcb.22204]
- 7 **Halim AS**, Lim CK, Mohd Hilmi AB, Arman Zaharil MS. New Era of Regenerative Medicine: an Islamic Perspective. In: Bahari MS, editor. *Cell and Tissue Culture: Research and Technology from Islamic Perspective*. Gombak: IIUM Press, 2014: 110-142
- 8 **Ross MH**, Kaye GI, Pawlina W. *Histology: a text and atlas with cell and molecular biology*. Philadelphia: Lippincott Williams & Wilkins, 2003
- 9 **Proksch E**, Brandner JM, Jensen JM. The skin: an indispensable barrier. *Exp Dermatol* 2008; **17**: 1063-1072 [PMID: 19043850]
- 10 **Dean LG**, Breslin A, Ross EZ. Is it hot in here? Thermoregulation and homeostasis through an exercise activity. *Adv Physiol Educ* 2014; **38**: 99-100 [PMID: 24585478 DOI: 10.1152/advan.00101.2013]
- 11 **Yu M**, Finner A, Shapiro J, Lo B, Barekatin A, McElwee KJ. Hair follicles and their role in skin health. *Exp Rev Dermatol* 2006; **1**: 855-871 [DOI: 10.1586/17469872.1.6.855]
- 12 **Mohd Hilmi AB**, Halim AS, Jaafar H, Asiah AB, Hassan A. Chitosan dermal substitute and chitosan skin substitute contribute to accelerated full-thickness wound healing in irradiated rats. *Biomed Res Int* 2013; **2013**: 795458 [PMID: 24324974 DOI: 10.1155/2013/795458]
- 13 **Rompolas P**, Deschene ER, Zito G, Gonzalez DG, Saotome I, Haberman AM, Greco V. Live imaging of stem cell and progeny behaviour in physiological hair-follicle regeneration. *Nature* 2012; **487**: 496-499 [PMID: 22763436 DOI: 10.1038/nature11218]
- 14 **Ito M**, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ, Cotsarelis G. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nat Med* 2005; **11**: 1351-1354 [PMID: 16288281]



- 15 **Tanimura S**, Tadokoro Y, Inomata K, Binh NT, Nishie W, Yamazaki S, Nakauchi H, Tanaka Y, McMillan JR, Sawamura D, Yancey K, Shimizu H, Nishimura EK. Hair follicle stem cells provide a functional niche for melanocyte stem cells. *Cell Stem Cell* 2011; **8**: 177-187 [PMID: 21295274 DOI: 10.1016/j.stem.2010.11.029]
- 16 **Purba TS**, Haslam IS, Poblet E, Jiménez F, Gandarillas A, Izeta A, Paus R. Human epithelial hair follicle stem cells and their progeny: current state of knowledge, the widening gap in translational research and future challenges. *Bioessays* 2014; **36**: 513-525 [PMID: 24665045 DOI: 10.1002/bies.201300166]
- 17 **Niemann C**, Horsley V. Development and homeostasis of the sebaceous gland. *Semin Cell Dev Biol* 2012; **23**: 928-936 [PMID: 22960253 DOI: 10.1016/j.semdcb.2012.08.010]
- 18 **Takeda N**, Jain R, Leboeuf MR, Padmanabhan A, Wang Q, Li L, Lu MM, Millar SE, Epstein JA. Hopx expression defines a subset of multipotent hair follicle stem cells and a progenitor population primed to give rise to K6+ niche cells. *Development* 2013; **140**: 1655-1664 [PMID: 23487314 DOI: 10.1242/dev.093005]
- 19 **Nishimura EK**. Melanocyte stem cells: a melanocyte reservoir in hair follicles for hair and skin pigmentation. *Pigment Cell Melanoma Res* 2011; **24**: 401-410 [PMID: 21466661 DOI: 10.1111/j.1755-148X.2011.00855.x]
- 20 **Chou WC**, Takeo M, Rabbani P, Hu H, Lee W, Chung YR, Carucci J, Overbeek P, Ito M. Direct migration of follicular melanocyte stem cells to the epidermis after wounding or UVB irradiation is dependent on Mc1r signaling. *Nat Med* 2013; **19**: 924-929 [PMID: 23749232 DOI: 10.1038/nm.3194]
- 21 **Sotiropoulou PA**, Candi A, Maseré G, De Clercq S, Youssef KK, Lapouge G, Dahl E, Semeraro C, Denecker G, Marine JC, Blanpain C. Bcl-2 and accelerated DNA repair mediates resistance of hair follicle bulge stem cells to DNA-damage-induced cell death. *Nat Cell Biol* 2010; **12**: 572-582 [PMID: 20473297 DOI: 10.1038/ncb2059]
- 22 **Meruane MA**, Rojas M, Marcelain K. The use of adipose tissue-derived stem cells within a dermal substitute improves skin regeneration by increasing neoangiogenesis and collagen synthesis. *Plast Reconstr Surg* 2012; **130**: 53-63 [PMID: 22418720 DOI: 10.1097/PRS.0b013e3182547e04]
- 23 **Ma PX**. Biomimetic materials for tissue engineering. *Adv Drug Deliv Rev* 2008; **60**: 184-198 [PMID: 18045729]
- 24 **Ma PX**, Choi JW. Biodegradable polymer scaffolds with well-defined interconnected spherical pore network. *Tissue Eng* 2001; **7**: 23-33 [PMID: 11224921]
- 25 **Chen VJ**, Ma PX. Nano-fibrous poly(L-lactic acid) scaffolds with interconnected spherical macropores. *Biomaterials* 2004; **25**: 2065-2073 [PMID: 14741621]
- 26 **Zeltinger J**, Sherwood JK, Graham DA, Mueller R, Griffith LG. Effect of pore size and void fraction on cellular adhesion, proliferation, and matrix deposition. *Tissue Eng* 2001; **7**: 557-572 [PMID: 11694190]
- 27 **Oh SH**, Park IK, Kim JM, Lee JH. In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method. *Biomaterials* 2007; **28**: 1664-1671 [PMID: 17196648]
- 28 **Yannas IV**, Lee E, Orgill DP, Skrabut EM, Murphy GF. Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. *Proc Natl Acad Sci USA* 1989; **86**: 933-937 [PMID: 2915988]
- 29 **Guptaa B**. Textile-based smart wound dressings. *Indian J of Fibre & Textile Research* 2010; **35**: 174-187
- 30 **Hu Y**, Topolkaev V, Hiltner A, and Baer E. Measurement of water vapor transmission rate in highly permeable films. *J Appl Polym Sci* 2001; **81**: 1624-1633 [DOI: 10.1002/app.1593]
- 31 **Aguzzi A**, Zhu C. Five questions on prion diseases. *PLoS Pathog* 2012; **8**: e1002651 [PMID: 22570608 DOI: 10.1371/journal.ppat.1002651]
- 32 **Abdillahi SM**, Balvanović S, Baumgarten M, Mörgelin M. Collagen VI encodes antimicrobial activity: novel innate host defense properties of the extracellular matrix. *J Innate Immun* 2012; **4**: 371-376 [PMID: 22398575 DOI: 10.1159/000335239]
- 33 **Zhou Y**, Yang H, Liu X, Mao J, Gu S, Xu W. Potential of quaternization-functionalized chitosan fiber for wound dressing. *Int J Biol Macromol* 2013; **52**: 327-332 [PMID: 23089086 DOI: 10.1016/j.ijbiomac.2012.10.012]
- 34 **Grunert P**, Borde BH, Hudson KD, Macielak MR, Bonassar LJ, Härtl R. Annular repair using high-density collagen gel: a rat-tail in vivo model. *Spine (Phila Pa 1976)* 2014; **39**: 198-206 [PMID: 24253790 DOI: 10.1097/BRS.000000000000103]
- 35 **Shin YH**, Seo YK, Yoon HH, Yoo BY, Song KY, Park JK. Comparison of hair dermal cells and skin fibroblasts in a collagen sponge for use in wound repair. *Biotechnology and Bioengineering* 2011; **16**: 793-800 [DOI: 10.1007/s12257-010-0448-6]
- 36 **Wahl DA**, Czernuszka JT. Collagen-hydroxyapatite composites for hard tissue repair. *Eur Cell Mater* 2006; **11**: 43-56 [PMID: 16568401]
- 37 **Shevchenko RV**, Sibbons PD, Sharpe JR, James SE. Use of a novel porcine collagen paste as a dermal substitute in full-thickness wounds. *Wound Repair Regen* 2008; **16**: 198-207 [PMID: 18318805 DOI: 10.1111/j.1524-475X.2008.00360.x]
- 38 **Lu H**, Dai Y, Lv L, Zhao H. Chitosan-graft-polyethylenimine/DNA nanoparticles as novel non-viral gene delivery vectors targeting osteoarthritis. *PLoS One* 2014; **9**: e84703 [PMID: 24392152 DOI: 10.1371/journal.pone.0084703]
- 39 **Mattu C**, Li R, and Ciardelli G. Chitosan nanoparticles as therapeutic protein nanocarriers: The effect of pH on particle formation and encapsulation efficiency. *Polymer Composites* 2013; **34**: 1538-1545 [DOI: 10.1002/pc.22415]
- 40 **Meng L**, Huang W, Wang D, Huang X, Zhu X, Yan D. Chitosan-based nanocarriers with pH and light dual response for anticancer drug delivery. *Biomacromolecules* 2013; **14**: 2601-2610 [PMID: 23819825 DOI: 10.1021/bm400451v]
- 41 **Liu X**, Ma L, Mao Z, and Gao C. Chitosan-based biomaterials for tissue repair and regeneration. Chitosan for Biomaterials II: Springer, 2011: 81-127
- 42 **Tsuchiya N**, Sato S, Kigami R, Kawano E, Takane M, Arai Y, Ito K, Ogiso B. Effect of a chitosan sponge impregnated with platelet-derived growth factor on bone augmentation beyond the skeletal envelope in rat calvaria. *J Oral Sci* 2014; **56**: 23-28 [PMID: 24739704]
- 43 **Hilmi AB**, Halim AS, Hassan A, Lim CK, Noorsal K, Zainol I. In vitro characterization of a chitosan skin regenerating template as a scaffold for cells cultivation. *Springerplus* 2013; **2**: 79 [PMID: 23503998 DOI: 10.1186/2193-1801-2-79]
- 44 **Millner R**, Lockhart AS, Marr R. Chitosan arrests bleeding in major hepatic injuries with clotting dysfunction: an in vivo experimental study in a model of hepatic injury in the presence of moderate systemic heparinisation. *Ann R Coll Surg Engl* 2010; **92**: 559-561 [PMID: 20522310 DOI: 10.1308/003588410X12699663903593]
- 45 **Dhurai B**, Saraswathy N, Maheswaran R, Sethupathi P, Vanitha P, Vigneshwaran S, and Rameshbabu V. Electrospinning of curcumin loaded chitosan/poly (lactic acid) nanofilm and evaluation of its medicinal characteristics. *Frontiers of Materials Science* 2013; **7**: 350-361 [DOI: 10.1007/s11706-013-0222-8]
- 46 **Köhler G**, Koch O, Antoniou S, Lechner M, Mayer F, and Emmanuel K. Prevention of Subcutaneous Seroma Formation in Open Ventral Hernia Repair Using a New Low-Thrombin Fibrin Sealant. *World J Surg* 2014; **38**: 1-7 [DOI: 10.1007/s00268-014-2691-z]
- 47 **Yang Y**, Zhang W, Li Y, Fang G, Zhang K. Scalded skin of rat treated by using fibrin glue combined with allogeneic bone marrow mesenchymal stem cells. *Ann Dermatol* 2014; **26**: 289-295 [PMID: 24966626 DOI: 10.5021/ad.2014.26.3.289]
- 48 **Sacchi V**, Mittermayr R, Hartinger J, Martino MM, Lorentz KM, Wolbank S, Hofmann A, Largo RA, Marschall JS, Groppa E, Gianni-Barrera R, Ehrbar M, Hubbell JA, Redl H, Banfi A. Long-lasting fibrin matrices ensure stable and functional angiogenesis by highly tunable, sustained delivery of recombinant VEGF164. *Proc Natl Acad Sci USA* 2014; **111**: 6952-6957 [PMID: 24778233 DOI: 10.1073/pnas.1404605111]
- 49 **Fridenshtein Ala**. [Stromal bone marrow cells and the hematopoietic



- microenvironment]. *Arkiv Patol* 1982; **44**: 3-11 [PMID: 7181706]
- 50 **Toma JG**, Akhavan M, Fernandes KJ, Barnabé-Heider F, Sadikot A, Kaplan DR, Miller FD. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001; **3**: 778-784 [PMID: 11533656]
- 51 **Ma S**, Xie N, Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. *Cell Death Differ* 2014; **21**: 216-225 [PMID: 24185619 DOI: 10.1038/cdd.2013.158]
- 52 **Ulivi V**, Tasso R, Cancedda R, Descalzi F. Mesenchymal stem cell paracrine activity is modulated by platelet lysate: induction of an inflammatory response and secretion of factors maintaining macrophages in a proinflammatory phenotype. *Stem Cells Dev* 2014; **23**: 1858-1869 [PMID: 24720766 DOI: 10.1089/scd.2013.0567]
- 53 **Ramos R**, Guerrero-Juarez CF, Plikus MV. Hair follicle signaling networks: a dermal papilla-centric approach. *J Invest Dermatol* 2013; **133**: 2306-2308 [PMID: 24030645 DOI: 10.1038/jid.2013.262]
- 54 **Biernaskie J**, Paris M, Morozova O, Fagan BM, Marra M, Pevny L, Miller FD. SKPs derive from hair follicle precursors and exhibit properties of adult dermal stem cells. *Cell Stem Cell* 2009; **5**: 610-623 [PMID: 19951689 DOI: 10.1016/j.stem.2009.10.019]
- 55 **Ennis WJ**, Sui A, Bartholomew A. Stem Cells and Healing: Impact on Inflammation. *Adv Wound Care* (New Rochelle) 2013; **2**: 369-378 [PMID: 24587974 DOI: 10.1089/wound.2013.0449]
- 56 **Maxson S**, Lopez EA, Yoo D, Danilkovitch-Miagkova A, Leroux MA. Concise review: role of mesenchymal stem cells in wound repair. *Stem Cells Transl Med* 2012; **1**: 142-149 [PMID: 23197761 DOI: 10.5966/sctm.2011-0018]
- 57 **Driskell RR**, Lichtenberger BM, Hoste E, Kretschmar K, Simons BD, Charalambous M, Ferron SR, Herault Y, Pavlovic G, Ferguson-Smith AC, Watt FM. Distinct fibroblast lineages determine dermal architecture in skin development and repair. *Nature* 2013; **504**: 277-281 [PMID: 24336287 DOI: 10.1038/nature12783]
- 58 **Newman AC**, Nakatsu MN, Chou W, Gershon PD, Hughes CC. The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Mol Biol Cell* 2011; **22**: 3791-3800 [PMID: 21865599 DOI: 10.1091/mbc.E11-05-0393]
- 59 **Holloway S**, Harding K, Stechmiller JK, Schultz G. Acute and chronic wound healing. In: Baranoski S, Ayello EA, editors. *Wound care essential*. 3rd ed. Ambler: Lippincott Williams & Wilkins, 2012: 89
- 60 **Souto LR**, Rehder J, Vassallo J, Cintra ML, Kraemer MH, Puzzi MB. Model for human skin reconstructed in vitro composed of associated dermis and epidermis. *Sao Paulo Med J* 2006; **124**: 71-76 [PMID: 16878189]
- 61 **Wang HM**, Chou YT, Wen ZH, Wang CZ, Chen CH, Ho ML. Novel biodegradable porous scaffold applied to skin regeneration. *PLoS One* 2013; **8**: e56330 [PMID: 23762223 DOI: 10.1371/journal.pone.0056330]
- 62 **Levinson H**. A Paradigm of Fibroblast Activation and Dermal Wound Contraction to Guide the Development of Therapies for Chronic Wounds and Pathologic Scars. *Adv Wound Care* (New Rochelle) 2013; **2**: 149-159 [PMID: 24527338 DOI: 10.1089/wound.2012.0389]
- 63 **van den Bogaardt AJ**, van Zuijlen PP, van Galen M, Lamme EN, Middelkoop E. The suitability of cells from different tissues for use in tissue-engineered skin substitutes. *Arch Dermatol Res* 2002; **294**: 135-142 [PMID: 12029501]
- 64 **Metral E**, Santos MD, Amélie Thépotl WR, Mojallal A, Auxenfans C, and Damour O. Adipose-derived Stem Cells Promote Skin Homeostasis and Prevent its Senescence in an In vitro Skin Model. *J Stem Cell Res Ther* 2014; **4**: 194 [DOI: 10.4172/2157-7633.1000194]
- 65 **Matsuda K**, Falkenberg KJ, Woods AA, Choi YS, Morrison WA, Dilley RJ. Adipose-derived stem cells promote angiogenesis and tissue formation for in vivo tissue engineering. *Tissue Eng Part A* 2013; **19**: 1327-1335 [PMID: 23394225 DOI: 10.1089/ten.TEA.2012.0391]
- 66 **Zhao J**, Hu L, Liu J, Gong N, Chen L. The effects of cytokines in adipose stem cell-conditioned medium on the migration and proliferation of skin fibroblasts in vitro. *Biomed Res Int* 2013; **2013**: 578479 [PMID: 24416724 DOI: 10.1155/2013/578479]
- 67 **Alexeev V**, Arita M, Donahue A, Bonaldo P, Chu ML, Igoucheva O. Human adipose-derived stem cell transplantation as a potential therapy for collagen VI-related congenital muscular dystrophy. *Stem Cell Res Ther* 2014; **5**: 21 [PMID: 24522088 DOI: 10.1186/scrt411]
- 68 **Lu W**, Yu J, Zhang Y, Ji K, Zhou Y, Li Y, Deng Z, Jin Y. Mixture of fibroblasts and adipose tissue-derived stem cells can improve epidermal morphogenesis of tissue-engineered skin. *Cells Tissues Organs* 2012; **195**: 197-206 [PMID: 21494022 DOI: 10.1159/000324921]
- 69 **Altman AM**, Yan Y, Matthias N, Bai X, Rios C, Mathur AB, Song YH, Alt EU. IFATS collection: Human adipose-derived stem cells seeded on a silk fibroin-chitosan scaffold enhance wound repair in a murine soft tissue injury model. *Stem Cells* 2009; **27**: 250-258 [PMID: 18818439 DOI: 10.1634/stemcells.2008-0178]
- 70 **Hsu YC**, Fuchs E. A family business: stem cell progeny join the niche to regulate homeostasis. *Nat Rev Mol Cell Biol* 2012; **13**: 103-114 [PMID: 22266760 DOI: 10.1038/nrm3272]
- 71 **Yang R**, Zheng Y, Burrows M, Liu S, Wei Z, Nace A, Guo W, Kumar S, Cotsarelis G, Xu X. Generation of folliculogenic human epithelial stem cells from induced pluripotent stem cells. *Nat Commun* 2014; **5**: 3071 [PMID: 24468981 DOI: 10.1038/ncomms4071]
- 72 **Jaks V**, Kasper M, Toftgård R. The hair follicle-a stem cell zoo. *Exp Cell Res* 2010; **316**: 1422-1428 [PMID: 20338163 DOI: 10.1016/j.yexcr.2010.03.014]
- 73 **Snippert HJ**, Haegebarth A, Kasper M, Jaks V, van Es JH, Barker N, van de Wetering M, van den Born M, Begthel H, Vries RG, Stange DE, Toftgård R, Clevers H. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. *Science* 2010; **327**: 1385-1389 [PMID: 20223988 DOI: 10.1126/science.1184733]
- 74 **Blanco S**, Kurowski A, Nichols J, Watt FM, Benitah SA, Frye M. The RNA-methyltransferase Misu (NSun2) poises epidermal stem cells to differentiate. *PLoS Genet* 2011; **7**: e1002403 [PMID: 22144916 DOI: 10.1371/journal.pgen.1002403]
- 75 **Demehri S**, Kopan R. Notch signaling in bulge stem cells is not required for selection of hair follicle fate. *Development* 2009; **136**: 891-896 [PMID: 19211676 DOI: 10.1242/dev.030700]
- 76 **Zhang Y**, Yu J, Shi C, Huang Y, Wang Y, Yang T, Yang J. Lef1 contributes to the differentiation of bulge stem cells by nuclear translocation and cross-talk with the Notch signaling pathway. *Int J Med Sci* 2013; **10**: 738-746 [PMID: 23630438 DOI: 10.7150/ijms.5693]
- 77 **Blanpain C**, Fuchs E. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat Rev Mol Cell Biol* 2009; **10**: 207-217 [PMID: 19209183 DOI: 10.1038/nrm2636]
- 78 **Limat A**, Hunziker T. Use of epidermal equivalents generated from follicular outer root sheath cells in vitro and for autologous grafting of chronic wounds. *Cells Tissues Organs* 2002; **172**: 79-85 [PMID: 12426484 DOI: 10.1159/000065615]

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## Neural differentiation from embryonic stem cells *in vitro*: An overview of the signaling pathways

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particularly those associated with *in vitro* differentiation. The inducers and pathways explored include retinoic acid, Wnt/ $\beta$ -catenin, transforming growth factor/bone morphogenetic protein, Notch, fibroblast growth factor, cytokine, Hedgehog, c-Jun N-terminal kinase/mitogen-activated protein kinase and others. Some other miscellaneous molecular factors that have been reported in the literature are also summarized and discussed. These include calcium, calcium receptor, calcineurin, estrogen receptor, Hox protein, ceramide, glycosaminoglycan, ginsenoside Rg1, opioids, two pore channel 2, nitric oxide, chemically defined medium, cell-cell interactions, and physical stimuli. The interaction or crosstalk between these signaling pathways and factors will be explored. Elucidating these signals in detail should make a significant contribution to future progress in stem cell biology and allow, for example, better comparisons to be made between differentiation *in vivo* and *in vitro*. Of equal importance, a comprehensive understanding of the pathways that are involved in the development of neurons from ESCs *in vitro* will also accelerate their application as part of translational medicine.

**Key words:** Neurons; Differentiation; Embryonic stem cells; Signaling; Pathways

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**Core tip:** Neurons are derived from embryonic stem cells (ESCs) and have been a focus of research recently, particularly because of their application in regenerative medicine. Here we review and summarize the signaling pathways that have been reported to be involved in neuronal differentiation of ESCs, mainly *in vitro*. The inducers and pathways explored include retinoic acid, Wnt/ $\beta$ -catenin, transforming growth factor/bone morphogenetic protein, Notch, fibroblast growth factor, cytokine, Hedgehog, c-Jun N-terminal kinase/mitogen-activated protein kinase and others. Some miscellaneous factors are also explored. Elucidating these signals in

### Abstract

Neurons derived from embryonic stem cells (ESCs) have gained great merit in both basic research and regenerative medicine. Here we review and summarize the signaling pathways that have been reported to be involved in the neuronal differentiation of ESCs,



detail should make a significant contribution to future progress in stem cell biology and should also accelerate the application of stem cells in translational medicine.

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## INTRODUCTION

Having been established from the inner cell mass of the blastocyst, embryonic stem cells (ESCs) possess pluripotency and can theoretically differentiate into all kinds of embryonic tissue. During the last decade, a number of studies have reported the differentiation of ESCs into a range of embryonic tissues. These compelling results were done either by stimulating the cells with particular molecules or by simulating the environmental cues of the early embryo. Importantly, these differentiated cells can then be used in regenerative medicine and for drug discovery. Hence, it is important to elucidate the detailed involvement of signals and signaling pathways in these processes before these cells are used for therapeutic purposes. Compared to development *in vivo*, neurons that differentiate from ESCs *in vitro* seem to develop *via* a similar pattern and thus have become a promising field in terms of the medical applications in stem cell research.

Basically, a default model for development *in vivo* is hypothesized as that induction of neural differentiation reflects the earliest fate in determining neurons in the ectoderm. The neural inducers involved in the process have been found to be bone morphogenetic protein (BMP)-binding molecule called noggin in *Xenopus*<sup>[1]</sup> and FGF in chicken<sup>[2]</sup>. Utilizing a serum-free and patterning factor-free condition to cultivate mouse ESCs (mESCs), Tropepe *et al.*<sup>[3]</sup> found that neural progenitors differentiated specifically from these stem cells. Neural differentiation occurs spontaneously and does not require the presence of any extrinsic neural inducer in a special culture system named serum-free culture of embryoid body-like aggregates with quick reaggregation (SFEBq)<sup>[4]</sup>. ESCs were found to selectively differentiate into neural progenitors efficiently (> 95% of total cells). The endogenous signals can also be minimized by adding the inhibitors of Wnt and/or Nodal such as dickkopf-1 and/or lefty-1, respectively. Furthermore, utilizing transforming growth factor  $\beta$  (TGF $\beta$ ) antagonists to abolish the SMAD signaling is shown to enhance neural differentiation, especially in induced pluripotent stem cells and human ESCs (hESCs)<sup>[5]</sup>. All the results are in agreement with the "neural default

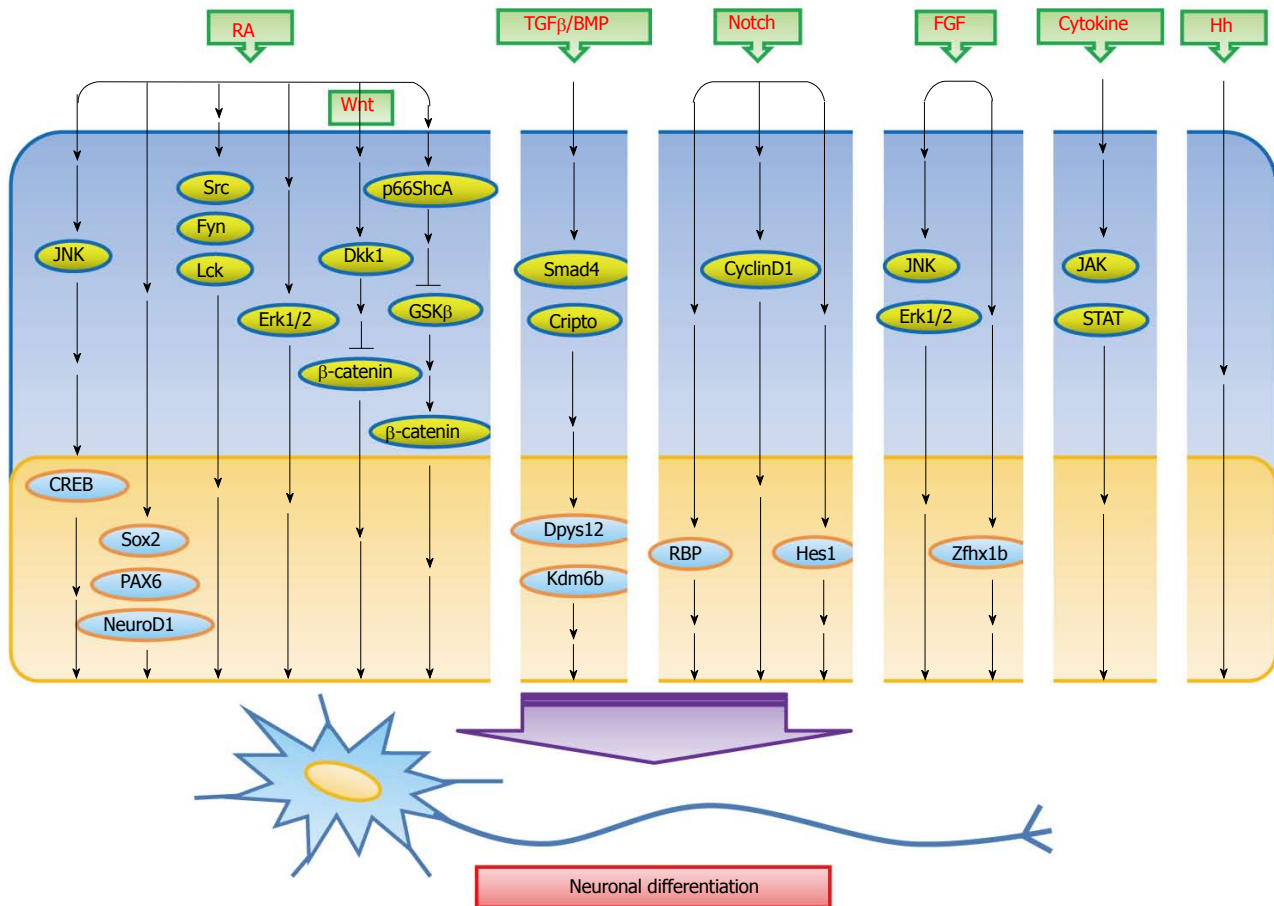
model" of embryonic development.

Neural cells were one of the first lineages to be developed from ESCs *in vitro*<sup>[6,7]</sup>. Many studies have effectuated subsequently to differentiate ESCs into the creation of different cellular subtypes such as neurons and glial cells. These ESCs can be surgically transferred back into vertebrate embryos and are then participated in brain development<sup>[8]</sup>. Work on ESCs over the last three decades has been concentrated on elucidating the characteristics of stem cells, particularly the differentiation approaches to obtain specific cell types such as neurons. Neural differentiation by ESCs is able to provide insights into the mechanisms involved in neural induction. In this review, we focus on obtaining an improving understanding of the overall signaling that underlies neural differentiation from ESCs *in vitro*. The inducers and pathways explored include retinoic acid (RA), Wnt/ $\beta$ -catenin, TGF/BMP, Notch, FGF, cytokine, Hedgehog, JNK/mitogen-activated protein kinase (MAPK) and others (Figure 1). This should help researchers with exploring the various areas of stem cell biology and also help to enhance the application of ESCs differentiation in regenerative medicine.

## THE RETINOIC ACID INDUCTION PATHWAY

RA typically is used at a range of concentrations from 5  $\mu$ mol/L to 5 mmol/L in order to facilitate the differentiation of ESCs into neural progenitor cells (NPCs). Shan *et al.*<sup>[9]</sup> found that the active cyclic AMP response element-binding protein (CREB) protein was obviously increased after treatment with 5  $\mu$ mol/L RA treatment during the differentiation/formation of embryoid body (EB). Inhibition of CREB activity was found to result in EBs switching the differentiation to other germ layers fate, while enhanced expression of CREB was found to augment NPCs differentiation. RA promoted the expression of active CREB by increasing the activity of c-Jun N-terminal kinase (JNK). These results show that JNK/CREB is likely to be a crucial factor in the RA-induction of NPCs differentiation<sup>[9]</sup>. In addition, Theus *et al.*<sup>[10]</sup> found that neurons derived from ESCs after RA induction led to significant neurite growth. This event is associated with an spread expression pattern of Src kinase from the cell body to the neurite processes and an up-regulated expression of Src, Fyn and Lck<sup>[10]</sup>. These neurons were characterized by the expression of neurofilament, synaptophysin and NMDA as well as the presence of kainate currents; they were found to have become vulnerable to excitotoxicity and went on to form functional excitatory synapses. Since these events were abolished when the cells were grown in the addition of the Src family kinase inhibitor PP2, it seems likely that the pathway induced by RA is





**Figure 1** A simplified scheme outlining the signaling pathways described in the text. The inducers that induce embryonic stem cells to differentiate into neurons reported thus far including RA, Wnt/ $\beta$ -catenin, TGF/ $\beta$ BMP, Notch, FGF, cytokine, Hedgehog, JNK/MAPK and others. The inducers in the middle part of figure mediate signaling molecules that bring about the differentiation into neurons.  $\rightarrow$  means stimulation. Some miscellaneous molecules/factors mentioned at the end of this text are not included in this figure. RA: Retinoic acid; TGF: Transforming growth factor; BMP: Bone morphogenetic protein; CREB: cAMP response element-binding protein; Dkk-1: Dickkopf-related protein 1; Erk: Extracellular signal-regulated kinase; FGF: Fibroblast growth factor; Hh: Hedgehog; JAK: Janus kinase; JNK: C-Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase.

mediated *via* Src<sup>[10]</sup>. Furthermore, Tonge *et al.*<sup>[11]</sup> demonstrated that neural differentiation of hESCs and embryonal carcinoma cells induced by RA needs both prolonged exposure of RA and cellular interaction that is done by the presence of a high cell density. These factors are required for the increase of the expression of various neural genes (*NeuroD1*, *PAX6* and *Sox2*) and the development of a neuronal appearance. They also found that inhibition of GSK3 $\beta$  activity was able to block the RA-induced differentiation of neural lineage derived from ESCs. This finding suggests a role for properly modulated Wnt signaling in this process<sup>[11]</sup>. After RA induction for 1-5 d, Li *et al.*<sup>[12]</sup> found that there is a dramatic increase in extracellular signal related kinase 1/2 (Erk 1/2) phosphorylation (p-Erk 1/2) and that this can be attenuated by treatment of U0126, a p-Erk 1/2 inhibitor. Furthermore, both the expression of associated cytoskeletal proteins and the number of NeuN-positive cells are dramatically reduced after the inhibition of p-Erk 1/2. As a result there was an increase in the differentiating ESCs of the nuclear translocation of STAT3, together with a

decrease in the expression of NGF, BDNF and GDNF molecules. These results imply that phosphorylation/activation of Erk 1/2 is a key signaling that is essential for survival of ESCs and early neural differentiation<sup>[12]</sup>. Recently, Glaser *et al.*<sup>[13]</sup> found that when mESCs were initiated to progress into neural differentiation with RA, the expression of Oct-4 and the P2X7 receptor, an ATP-gated cation channel, were reduced. Utilizing KN-62, a specific P2X7 receptor inhibitor, they found an increased number of SSEA-1 and type III  $\beta$ -tubulin expressing double-positive cells. This confirms the appearance of neuroectodermal differentiation and it would seem that the neural fate determination of mESCs is dependent on suppression of P2X7 receptor activity<sup>[13]</sup>.

RA could also mediate crosstalk among other signaling pathways such as the Wnt/ $\beta$ -catenin, FGF, and Erk pathways in order to induce neural differentiation. This is based on the finding that 4-d of RA treatment substantially increases the synthesis of the Dickkopf-related protein 1 (Dkk-1), a Wnt antagonist, and induces the expression of the Wnt/



Dkk-1 co-receptor LRP6<sup>[14]</sup>. When recombinant Dkk-1 was utilized, the EBs presented in a similar manner to treatment with RA, namely there was an induction of two neural markers, the distal-less homeobox gene (*Dlx-2*) and nestin gene. Dkk-1 overexpression was found to be able to block the Wnt pathway, as evidenced by a decrease of  $\beta$ -catenin protein in the nucleus. These findings show that the prevention of the canonical Wnt pathway is a prerequisite for neural differentiation of ESCs when this is induced by RA treatment<sup>[14]</sup>. Conversely, judging from the expression of neural marker *Hoxc4*, Otero *et al.*<sup>[15]</sup> found that neural differentiation can be initiated by overexpressing  $\beta$ -catenin alone or combination with RA. Nevertheless, RA treatment was found to inhibit the  $\beta$ -catenin-induced production of tyrosine hydroxylase positive neurons, which suggests that the effects of RA are only partially dependent on  $\beta$ -catenin signaling. These results also suggest that  $\beta$ -catenin signaling enhances determination of neural lineage in ESCs. Moreover,  $\beta$ -catenin signaling could play a role of required co-factor in RA-induced pathway so as to permit the neural differentiation<sup>[15]</sup>. Papadimou *et al.*<sup>[16]</sup> reported that p66ShcA is increased during neural induction of ESCs *in vitro*. Overexpression of p66ShcA in ESCs ablates GSK-3 $\beta$  kinase activation which in turn to stabilize  $\beta$ -catenin protein. In parallel, p66ShcA over-expression was found to result in both mESCs and hESCs undergoing neural induction as predicted and accelerated neural differentiation. Thus there seems to be a role for p66ShcA in the regulation of Wnt/ $\beta$ -catenin pathway as well as in ESCs neutralization. Based on the above, p66ShcA would seem to also participate in a part of the RA-induction pathway<sup>[16]</sup>. Furthermore, Engberg *et al.*<sup>[17]</sup> monitor ESCs containing reporter genes that allowed the detection of markers associated with the early neural plate and the primitive streak and its progeny. When RA signaling is inhibited, they found that the change from neural to mesodermal fate develops. In addition, neural induction in ESCs needs RA to block Nodal signaling. Thus, the mechanism by which Wnt signaling pathway inhibits neural development could be interpreted as *via* facilitation of Nodal signaling pathway<sup>[17]</sup>. Stavridis *et al.*<sup>[18]</sup> shows that retinoid repression of fibroblast growth factor (FGF) signaling is able to promote the onset of neural differentiation. Induction of FGF8 by RA and subsequent Erk activity under early differentiation conditions could function to ascertain the loss of self-renewal. Nevertheless, a progressing inhibition of FGF4 by RA would seem to be associated with an overall decrease in Erk activity at the later stage. The admission of a neural or a non-neural fate is therefore decided by an inhibition of FGF signaling. Hence, inhibition of FGF/Erk activity would enhance ESCs self-renewal, but a subsequent abolishment of FGF signaling seems to have the opposite effect and act as a driver for differentiation<sup>[18]</sup>.

## THE TGF $\beta$ /BMP PATHWAY

It has been speculated that a default mechanism for neural differentiation might be involved in regulating the property of neural stem cell identity directly from ESCs. As above-mentioned, Tropepe *et al.*<sup>[3]</sup> characterized that the neural lineage of differentiation from a nascent stem cell is modulated negatively by TGF $\beta$ -related signaling. Moreover, differentiated mESCs *in vitro* with *Smad4* or *Cripto* genes knockout have been found to produce increased numbers of neurons<sup>[19]</sup>. The profiles analysis of gene expression *in vitro* further demonstrates that cells bearing *Smad4* gene deletion were inclined to possess expressing patterns of mid-hindbrain and anterior hindbrain. However, the *Cripto* knockout cells tended to express gene markers of rostral central nervous system (CNS) in addition to other previous genes. Thus it would seem that *Smad4*<sup>-/-</sup> ESCs exhibit differentiation of mesoderm while *Cripto*<sup>-/-</sup> ESCs develop into epidermal/neuroectodermal cell types<sup>[19]</sup>. To investigate the role of BMP-4 in the determination of either epidermal or neural fate, Gambaro *et al.*<sup>[20]</sup> demonstrated that treatment of BMP-4 on murine ESCs results in the significant apoptosis of neural precursor cells which contain Sox-1 expression. Furthermore, counteraction of the SMAD pathway by overexpression of SMAD6, an inhibitor SMAD (I-SMAD), hinders the BMP4-induced apoptosis. Utilizing Noggin and SB431542, Chambers *et al.*<sup>[5]</sup> shown that these two inhibitors of SMAD signaling are sufficient to allow the induction of neural differentiation derived from hESCs.

Genome-wide mapping was used to obtain plausible downstream candidates within the TGF $\beta$ /BMP pathway that are involved in ESCs differentiation. Fei *et al.*<sup>[21]</sup> mapped the gene promoters on a genome-wide scale to search for the target sequences bound with SMAD1, SMAD4, and SMAD5. They found that these molecules were associated with many developmental regulators and these were abundant in terms of H3K4 and H3K27 trimethylation bivalent markers. These promoters were found to be repressed when cells were in the self-renewing state, whereas these promoters underwent rapid induction upon differentiation. In the same context, the results from SMAD loss-of-function experiments further supported the hypothesis that BMP mediating signaling *via* SMAD does not directly affect self-renewal, whereas is necessary for various processes relevant to differentiation. Within the various SMAD-associated genes, they were able to identify two regulators which have been known to participate in the early neural differentiation regulated by BMP. These genes are *Dpysl2* (also known as *Crmp2*) and the H3K27 demethylase *Kdm6b* (also known as *Jmjd3*). Bertacchi *et al.*<sup>[22]</sup> also adopted a global gene expression approach and were able to show that mESCs produce, secrete, and respond to BMPs during neural differentiation *in vitro*. Utilizing the analysis of



several markers of dorsoventral and anterior/posterior identity, they found that the gene expression pattern of differentiated ESCs reflects the midbrain identity. They also revealed that the endogenous BMPs during neural differentiation principally function to inhibit the expression of genes with a telencephalic profile. This phenomenon was evidenced by treating ESCs with a number of BMP inhibitors or Noggin.

## THE NOTCH PATHWAY

Lowell *et al.*<sup>[23]</sup> explored the role of Notch receptors and ligands in mESCs. They found that genetic manipulation that the constitutively activated Notch does not change the phenotype of stem cells. Nonetheless, these cells differentiate exclusively and promptly into the neural lineage upon abolishment of self-renewal stimuli. Conversely, genetic or pharmacological interference with Notch signaling inhibits the determination of neural fate. The neural commitment enhancing by Notch needs parallel signaling through the FGF receptor. Since expression of Notch ligand in stromal cells also induces the neural differentiation of hESCs, it indicates this pathway is conserved within pluripotent stem cells<sup>[23]</sup>. Das *et al.*<sup>[24]</sup> investigate the role of the Notch pathway by engineering a mESCs line such that there were short pulses of activated Notch. The alteration of Notch protein could be induced at the various stages of neural differentiation *in vitro*. The results show that activation of Notch signaling for 6 h specifically at day 3 during neural induction from ESCs was found to lead to dramatically increase cell proliferation. This outcome is associated with the cyclin D1 expression induced by Notch. In contrast, a decrease of cyclin D1 was observed during the development of the CNS in mouse embryos without Notch signaling. The ESCs containing a dominant negative form of cyclin D1 was found to abrogate the Notch-induced cell proliferation. These seem to indicate the presence of a special function for Notch in regard of temporal context. These findings also confirm that cyclin D1 is a crucial signaling molecule in Notch-induced differentiation/proliferation in ESCs<sup>[24]</sup>.

Downstream of the Notch pathway, the protein RBP was also show to play a key role. Main *et al.*<sup>[25]</sup> obtained RBP<sup>+/-</sup> mice using RBPJK<sup>loxP/loxP</sup> mice<sup>[26]</sup> bred with CMV-cre mice. After RBP<sup>-/-</sup> mESCs had been obtained and cultured at low density, they were found to behave in the same way as wild-type cells in terms of the origin of apical specification and neural progenitors. When ESCs undergo development through the rosette formation, RBP was found to be required for the modulation of neuronal differentiation and for the appropriate preservation of rosette structure. Utilizing inhibitors of Notch and/or loss-of-function analysis of Notch signaling resulted in the disintegration of neural rosettes and an acceleration of neuronal differentiation. Rosette integrity was

also found to demand Rho kinase activity and actin polymerization in addition to requiring normal Notch signaling. However, it is worth noting that rosette maintenance is not required as a prior condition for regular neuronal differentiation. Various results demonstrate that Notch signaling also plays a role in the maintenance and organization of polarity during early nervous system development<sup>[25]</sup>. Furthermore, it was also showed that Hes1-high ESCs are inclined to a mesodermal fate, whereas Hes1-low ESCs are inclined to a neural fate<sup>[27]</sup>. Kobayashi *et al.*<sup>[28]</sup> further showed that Hes1-low and Hes1-high ESCs are respectively correlated with cells that have undergone activation and inactivation of Notch signaling. Although Notch and Hes1 function in the same direction in most other cell types, the abovementioned results show that both signaling leads to opposite effect during ESCs differentiation. That is, Hes1 would seem to be not the downstream signaling molecule of Notch pathway during ESCs differentiation.

It was known that both activation of the Sonic Hedgehog (Shh) pathway and inhibition of the Notch pathway induce the neural differentiation during the neural tube development *in vivo*. To distinguish the effect of Shh and/or Notch signaling on ESCs-derived EBs, Crawford and Roelink used N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), an inhibitor of Notch, to investigate how critical the Shh signaling is in the EBs formation and neural differentiation *in vitro*. It was found that DAPT led to promote neuronal differentiation. On the other hand, more interneurons were identified in the absence of Shh. They also found that the effect of DAPT on EBs with an activated Shh signaling is associated with the premature disappearance of markers involved in the ventral neuronal precursors<sup>[29]</sup>.

## THE FGF PATHWAY

Chen *et al.*<sup>[30]</sup> showed that FGF family includes FGF1, FGF2, and FGF4, but not FGF8b, are able to enhance the neurogenesis of mESCs during serum-free neural induction. They found that the enhanced neurogenesis by FGF is not mediated through a promotion of the proliferation of Sox1<sup>+</sup> cells or *via* a rescue of apoptosis. It was found to involve the inactivation of JNK-1 and Erk-2, but did not involve p38 MAPK, which is known to inhibit neural formation by inhibiting ESCs differentiation. Furthermore, ESCs that lacked FGF4 or have been treated with a FGF receptor inhibitor were found to be resistant to neural and mesodermal induction<sup>[30]</sup>. Kunath *et al.*<sup>[31]</sup> found that activated Erk 1/2 induced by FGF4 is a stimulus by which naive ESCs are able to be released from the self-renewal program. FGF4 is capable of initiating differentiation activity. The important role of Erk downstream signaling was further explored by an examination of Erk2 deficient



ESCs, which fail to proceed to either mesodermal or neural differentiation and retain their pluripotency. On the other hand, FGF2 which functions for the maintenance of epiblast stem cells and hESCs was found to inhibit development of early neural cells by epiblast intermediates. Nevertheless, FGF2 alone is sufficient to enhance self-renewal of epiblast stem cells. Conversely, FGF8, the endogenous inducer for embryonic neural differentiation, promotes more homogenous neural induction that is accompanied by transient self-renewal of early neural cells. They also found that completely blocking of FGF signaling in epiblast cells enhances prompt neural induction as well as the succeeding neurogenesis. Therefore, FGF signaling pathway seems to play a variety of roles during the different stages of ESCs differentiation<sup>[31]</sup>.

Dang *et al.*<sup>[32]</sup> investigated the downstream molecules involved in FGF signaling. Sox1, enhancing the colony formation of definitive neural stem cells, is regularly used as a marker of neural precursors. Under typically culture conditions of ESCs in which the medium contains leukemia inhibitory factor (LIF) and serum, overexpression of *Zfhx1b* in these cells is essential to initiate the expression of Sox1. When mESCs were initiated to the neural differentiation, a prompt increase of *Zfhx1b* gene expression is observed and can be further potentiated by FGF signaling<sup>[32]</sup>. In the same content, utilizing siRNA to knockdown *Zfhx1b* in ESCs leads to decrease the developmental capability of these neural cells although the initial transition of ESCs to a neural cell fate is not affected. Taken together, these findings show that intercellular FGF signaling induces *Zfhx1b* and this is able to promote the development of definitive neural stem cells after an initial neural specification event has occurred.

## THE CYTOKINE PATHWAY

Under culture conditions, LIF is used to prevent the differentiation of mESCs. However, He *et al.*<sup>[33]</sup> found that utilizing inhibitors to abolish the STAT3 signaling which was activated by LIF could block the neuronal differentiation of ESCs. Furthermore, inhibition of the MEK signaling which was activated by LIF decreased the differentiation of ESCs into glial cells. During ESCs differentiation, LIF enhanced proliferation of cells and inhibited apoptosis of cells. In addition, LIF promoted the determination of neural progenitors although inhibited the differentiation of mesoderm and extraembryonic endoderm fates. Foshay *et al.*<sup>[34]</sup> further examine the role of STAT3. The loss of STAT3, brought about by generating STAT3 dominant negative ESCs, led to the production of significantly fewer neural stem cells and this was associated with a decrease in the expression of the neural stem cell marker nestin. Further investigation revealed that the Sox2 promoter is directly regulated by STAT3. The decreased expression of Sox2 would induce the expression of

nestin and result in a commitment to a neural stem cells fate. Using mouse embryonal carcinoma P19 cells, Pacherník *et al.*<sup>[35]</sup> demonstrated that the effects of LIF on inducing neural differentiation were inhibited by blocking the JAK2/STAT3 signaling pathway. This is also partially true for the pro-neural effects of RA. Conversely, inhibition of the MEK1/Erk signaling pathway was found not to show any such effect. These data suggest that the cooperation between LIF and other factors, such as RA, may eventually converge into the STAT3 signaling pathway.

## THE HEDGEHOG PATHWAY

Maye *et al.*<sup>[36]</sup> demonstrated that mutants in the signaling molecules of the Hedgehog pathway within ESCs could lead to defect in the generation of neuroectoderm-forming EBs. These mutant ESCs are incapable to produce nestin positive neural stem cells or differentiate into mature neurons in respond to RA treatment. Moreover, ESCs lacking *Odf1*, a gene that causes Oral-Facial-Digital 1 (OFD1) Syndrome when mutated, were found to show an increased tendency on neuronal differentiation. Furthermore, the OFD1 mutant ESCs-derived neurons are unable to differentiate into V3 interneurons, a cell type dependent on Hedgehog signaling<sup>[37]</sup>. These findings show a role for Hedgehog signaling in producing the neuronal and glial progenitors derived from ESCs.

## THE JNK/MAPK PATHWAY

Amura *et al.*<sup>[38]</sup> showed that there is an essential role for the JNK pathway during ESCs neurogenesis. ESCs lines containing homozygous destruction of the *JNK1*, *JNK2*, or *JNK3* genes were introduced into differentiation protocol *via* the procedure at the EB formation. The outcome shows that neural differentiation was observed in wild-type EB cultures, *JNK2*<sup>-/-</sup> EB cultures, and *JNK3*<sup>-/-</sup> EB cultures except in *JNK1*<sup>-/-</sup> EB cultures. The identified inhibitors of ESCs neurogenesis, Wnt-4 and Wnt-6, were found to be increased their expression in *JNK1*<sup>-/-</sup> cultures as compared to wild-type, *JNK2*<sup>-/-</sup>, and *JNK3*<sup>-/-</sup> cultures. Furthermore, a genetic approach using JNK knockout ESCs has revealed a role for JNK1 in neural differentiation that involves repression of Wnt expression using a murine ESCs model<sup>[38]</sup>. Na *et al.*<sup>[39]</sup> reexamined the role of Erk 1/2 in hESCs by using a chemically defined culture system. The results demonstrate that when the activity of Erk 1/2 is inhibited, the differentiation of neurons and mesendoderm is inhibited. However, these cells are still able to differentiate after BMP stimulation.

## OTHER MOLECULES/FACTORS

### Calcium, calcium receptor, and calcineurin

A number of studies have shown that calcium



homeostasis is a crucial factor in determination of neural fate. This has been thoroughly discussed in a previous review<sup>[40]</sup>. It is worth noting that utilizing proteomic analysis or gene screening during neural induction in mESCs, four  $\text{Ca}^{2+}$ -related proteins, namely neuronatin, translationally controlled tumor protein, pyruvate dehydrogenase E1/E2 subunits, and calreticulin, have been found to be altered in expression<sup>[41,42]</sup>. When neuronal cells are differentiated from ESCs lacking the  $\text{Ca}^{2+}$  release channel type 2 ryanodine receptors ( $\text{RyR2}^{-/-}$ ), Yu *et al.*<sup>[43]</sup> found that the rate of neurogenesis was significantly blocked. Meanwhile, the expression of NeuroD, a neuronal transcription factor, and the activity of intracellular  $\text{Ca}^{2+}$  signaling were also inhibited in the  $\text{RyR2}^{-/-}$  deficient mESCs. Moreover, neuronal differentiation in  $\text{RyR2}^{+/+}$  cells enhanced by activation of L-type  $\text{Ca}^{2+}$  channels or of GABA receptors was inhibited by RyR inhibitors. Therefore it would seem that cooperation between RyR2 channels and L-type  $\text{Ca}^{2+}$  is important for activity-dependent neurogenesis. Recently, Cho *et al.*<sup>[44]</sup> found that neural induction is dependent on a  $\text{Ca}^{2+}$ -activated phosphatase, calcineurin. They also have shown that calcium entry mediated by FGF stimulation activates calcineurin, which then directly and specifically dephosphorylates BMP-regulated Smad1/5 proteins.

### Estrogen receptor

Utilizing an estrogen receptor (ER) agonist, Zhang *et al.*<sup>[45]</sup> found that  $\text{ER}\beta$ , but not  $\text{ER}\alpha$ , stimulated calcium oscillations in neurons derived from ESCs. The increase of calcium oscillations and the phosphorylation of PKC, AKT and Erk1/2 induced by the  $\text{ER}\beta$  agonist in ESCs derived neurons could be blocked by nifedipine, an inhibitor of L-type calcium channels. The result demonstrates that  $\text{ER}\beta$  could modulate neuron activity via L-type voltage gated calcium channels.

### Hox protein

In order to investigate the role of the *Hox* gene in neuronal differentiation, Bami *et al.*<sup>[46]</sup> used a mESCs cellular model by combining efficient neural differentiation with inducible *Hoxb1* expression. The profile of gene expression indicates that *Hoxb1* could function as both activator and repressor in the short term, whereas as a repressor in the long term. Such a pattern of *Hoxb1* activity was observed in the regulation of mESCs after RA induction.

### Ceramide

It has been previously showed that bioactive lipids are important regulators of stem cell survival and differentiation<sup>[47]</sup>. It was found that the sphingolipid ceramide and its derivative, such as sphingosine-1-phosphate, are able to function synergistically during ESCs differentiation and the guided differentiation of mESCs toward neural and glial lineages<sup>[48]</sup>.

### Glycosaminoglycan

mESCs that lack heparan sulfate (HS) cannot process into neural specification whereas this phenomenon can be recovered by adding a highly sulfated glycosaminoglycan, one kind of soluble heparin<sup>[49]</sup>. Pickford *et al.*<sup>[50]</sup> demonstrated that specific heparin polysaccharides or HS support the formation of Sox11 neural progenitor cells from wild-type ESCs. They also found that a number of receptor tyrosine kinases were affected by HS during the differentiation.

### Ginsenoside Rg1

Ginsenoside Rg1, a saponin and major component in ginseng, has been shown to possess neuroprotective effects. Wu *et al.*<sup>[51]</sup> explored the effect of Rg1 on the promotion of mESCs differentiation towards the neuronal lineage. They found Rg1 increased the phosphorylation of Akt and Erk 1/2 in a time dependent pattern through glucocorticoid receptor. Treatment with either LY294002, an inhibitor of PI3K, or U0126, an inhibitor of MEK, blocked the Rg1-induced neuronal differentiation.

### Opioids

Kim *et al.*<sup>[52]</sup> measured  $\mu$ -opioid receptor and  $\kappa$ -opioid receptor expression in various cell types including ESCs and neural progenitors induced by RA. In the RA-induced ES cells, a biphasic profile of Erk activation after opioid stimulation was observed. Nevertheless, the proliferation of the neural progenitors was inhibited after opioid stimulation in which this phenomenon was Erk independent. The findings indicate that opioids could have opposite effects on ESCs self-renewal and ESCs differentiation.

### Two pore channel 2

The nicotinic adenine acid dinucleotide phosphate (NAADP), located on membranes of lysosome, has a potent effect on mobilizing endogenous  $\text{Ca}^{2+}$ . Two pore channel 2 (TPC2), voltage-gated ion channels, is shown to be the receptor of NAADP. Zhang *et al.*<sup>[53]</sup> found that expression of TPC2 was decreased dramatically when the ESCs entry differentiation towards neural progenitor cells. During the late stages of neurogenesis, the expression of TPC2 reoccurred. Analysis of loss-of-function mutants of TPC2 found that TPC2 knockdown in mice accelerated mESCs differentiation into neural progenitors. This contrasted with the situation where there was TPC2 gain-of-function in a mouse model; this revealed that gain-of-function inhibited mESCs from entering the early neural differentiation. These findings suggest that TPC2 signaling plays a vital role in regulating the differentiation of mESCs into the neural lineage.

### Nitric oxide

Employing various approaches, including ESC-derived neural precursor cells, Arnhold *et al.*<sup>[54]</sup> studied the



role of nitric oxide in initiating the differentiation of neurons. They found that specific blocking of the NOS- II isoform was able to bring about the inhibition of neurite outgrowth.

### Chemically defined medium

When chemically defined medium (CDM) is used for growth, ESCs differentiation is highly neurogenic. Neural differentiation in CDM is shown to be dependent on endogenous FGF signaling. This process is able to be inhibited by BMP4 or LiCl in which they simulate Wnt pathway. The neural differentiation in CDM could be terminated by blocking Hedgehog activity endogenously. Therefore, a common developmental mechanism could be processing since the profile change of gene expression in stem cells cultivation in CDM and the ones in the early embryos are extremely similar<sup>[55]</sup>.

### Cell-Cell interactions

Parekkadan *et al.*<sup>[56]</sup> observed that the presence of a previously specified Sox1-GFP<sup>+</sup> cell in contact with undifferentiated ESCs was able to initiate a similar specification. This induction relied on the age of previously specified cells before co-culture. Further search for the cell adhesion molecules, it was found that connexin (Cx)-43 expression was associated with the age-dependent effect of cell contact in the experiments of cell pair. Both aberrant neuroectodermal specification and lineage commitment were seen in ESCs in which Cx-43 was knockout. Such an observation highlights the important role of gap junction signaling in the neuronal development.

### Physical stimuli

Interestingly, physical stimuli are also able to affect the differentiation of ESCs and these phenomena have gained some attention recently. Piacentini *et al.*<sup>[57]</sup> reported that the percentages of cells expressing type III  $\beta$ -tubulin, microtubule-associated protein 2, and calcium channel proteins (Cav1) were dramatically increased when differentiating neural stem cells are exposed to extremely low-frequency electromagnetic fields (ELFEFs, 1 mT, 50 Hz). An obviously increase in spontaneous firing were also found in these ELFEF-exposed neurons. Furthermore, they found that stimulation of ELFEF during the early differentiation could induce an increase of cells expressing CREB phosphorylation by which it is calcium channel dependent<sup>[57]</sup>. In another study, Maioli *et al.*<sup>[58]</sup> created a Radio Electric Asymmetric Conveyor (REAC) that is able to deliver wireless fidelity radiofrequency (Wi-Fi RF) at 2.4 GHz. This radio wave is delivered by immersing the conveyor electrodes into the culture medium. Using such a device allows mESCs to be exposed to REAC and in such circumstances it was found that transcription of genes involved

in differentiation, such as neuronal commitment (neurogenin1), were upregulated, while other genes, such as *Sox2*, *Oct4*, and *Nanog*, were downregulated. These findings mean that the physical environment is also able to regulate the fate of stem cells.

## CONCLUSION

Some canonical pathways involved in cell size such as Hippo/Yap pathways and/or growth such as PI3K/Akt pathways seem to have little relationship with the initiation of neuronal differentiation from ESCs *in vitro*. The PI3K/Akt pathway is viewed as important to the maintenance of neuronal survival, but not to the differentiation process. In this context, Watanabe *et al.*<sup>[59]</sup> show that the membrane bound Akt through myristoylation (myr-Akt) could maintain mESCs at the undifferentiated status without supplement of LIF in the medium. Once the myr-Akt was deleted, the dependence of LIF and ability of differentiation were recovered. They found that the PI3K/Akt signaling could regulate "stemness" of many stem cell systems. Zhao *et al.*<sup>[60]</sup> also found that insulin can rescue ESCs-derived neural progenitor cells from hypoxia-induced cell death. Such an effect is able to be inhibited by LY294002, an inhibitor of the phosphatidylinositol 3-kinase (PI3K). Nevertheless, Chuang *et al.*<sup>[61]</sup> have recently reported that the mTOR pathway, a downstream pathway of PI3K, would seem to play a role in ESCs-derived neuronal differentiation. In order to reveal the role of raptor/mTOR in neurons differentiated from ESCs, we established raptor gene-trap mESCs and raptor knockdown mESCs using raptor RNAi infection followed by puromycin selection. Embryonic body growth in both cases was greatly reduced and the result was an unsuccessful differentiation of neurons. Furthermore, treatment with 1  $\mu$ mol/L rapamycin over 48 to 72 h of treatment starting at the point when neuronal precursors began to differentiate from mESCs was found to bring about a gradual loss of neuritis together with a shrinkage of the soma and a decreased ratio of neurite length to cell number. Knockdown of raptor during neuronal differentiation from mESCs also resulted in a gradual loss of neurites and cell body shrinkage. The loss of neurite density that results from rapamycin treatment is able to be reversed by overexpression of S6K T389E. Therefore, raptor/mTORC1/S6K would seem to play a critical role in the differentiation and survival of neurons derived from mESCs<sup>[61]</sup>. Therefore, it seems likely that the mTOR pathway plays a pivotal role in neuronal differentiation of ES cells *in vitro*.

To comprehensive understand these pathways will definitely contribute greatly to stem cell biology and translational medicine. In conclusion, the pathways outlined here are simple and linear. However, it is still unclear how these pathways crosstalk with each other and/or what is the level of



interplay between the pathways both temporally and spatially. The integration of these pathways into a comprehensive network will probably require more incisive investigative approaches. Generating specific types of neurons from ESCs *in vitro* has created high expectations in terms of possible medical treatments and/or potential cures for neuronal pathological diseases. Knowledge obtained by the research related to ESCs derived neurons *in vitro* should also provide a technical basis for regenerative medicine, applied medical research, and drug discovery.

## REFERENCES

- Zimmerman LB**, De Jesús-Escobar JM, Harland RM. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 1996; **86**: 599-606 [PMID: 8752214 DOI: 10.1016/S0092-8674(00)80133-6]
- Stern CD**. Neural induction: old problem, new findings, yet more questions. *Development* 2005; **132**: 2007-2021 [PMID: 15829523 DOI: 10.1242/dev.01]
- Tropepe V**, Hitoshi S, Sirard C, Mak TW, Rossant J, van der Kooy D. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* 2001; **30**: 65-78 [PMID: 11343645 DOI: 10.1016/S0896-6273(01)00263-X]
- Wataya T**, Ando S, Muguruma K, Ikeda H, Watanabe K, Eiraku M, Kawada M, Takahashi J, Hashimoto N, Sasai Y. Minimization of exogenous signals in ES cell culture induces rostral hypothalamic differentiation. *Proc Natl Acad Sci USA* 2008; **105**: 11796-11801 [PMID: 18697938 DOI: 10.1073/pnas.0803078105]
- Chambers SM**, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 2009; **27**: 275-280 [PMID: 19252484 DOI: 10.1038/nbt.1529]
- Evans MJ**, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; **292**: 154-156 [PMID: 7242681 DOI: 10.1038/292154a0]
- Martin GR**, Evans MJ. Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies *in vitro*. *Proc Natl Acad Sci USA* 1975; **72**: 1441-1445 [PMID: 1055416 DOI: 10.1073/pnas.72.4.1441]
- Brüstle O**, Spiro AC, Karraam K, Choudhary K, Okabe S, McKay RD. In vitro-generated neural precursors participate in mammalian brain development. *Proc Natl Acad Sci USA* 1997; **94**: 14809-14814 [PMID: 9405695 DOI: 10.1073/pnas.94.26.14809]
- Shan ZY**, Shen JL, Li QM, Wang Y, Huang XY, Guo TY, Liu HW, Lei L, Jin LH. pCREB is involved in neural induction of mouse embryonic stem cells by RA. *Anat Rec (Hoboken)* 2008; **291**: 519-526 [PMID: 18383274 DOI: 10.1002/ar.20686]
- Theus MH**, Wei L, Francis K, Yu SP. Critical roles of Src family tyrosine kinases in excitatory neuronal differentiation of cultured embryonic stem cells. *Exp Cell Res* 2006; **312**: 3096-3107 [PMID: 16859680 DOI: 10.1016/j.yexcr.2006.06.022]
- Tonge PD**, Andrews PW. Retinoic acid directs neuronal differentiation of human pluripotent stem cell lines in a non-cell-autonomous manner. *Differentiation* 2010; **80**: 20-30 [PMID: 20427117 DOI: 10.1016/j.diff.2010.04.001]
- Li Z**, Theus MH, Wei L. Role of ERK 1/2 signaling in neuronal differentiation of cultured embryonic stem cells. *Dev Growth Differ* 2006; **48**: 513-523 [PMID: 17026715 DOI: 10.1111/j.1440-169X.2006.00889.x]
- Glaser T**, de Oliveira SL, Cheffer A, Beco R, Martins P, Fornazari M, Lameu C, Junior HM, Coutinho-Silva R, Ulrich H. Modulation of mouse embryonic stem cell proliferation and neural differentiation by the P2X7 receptor. *PLoS One* 2014; **9**: e96281 [PMID: 24798220 DOI: 10.1371/journal.pone.0096281]
- Verani R**, Cappuccio I, Spinsanti P, Gradini R, Caruso A, Magnotti MC, Motolese M, Nicoletti F, Melchiorri D. Expression of the Wnt inhibitor Dickkopf-1 is required for the induction of neural markers in mouse embryonic stem cells differentiating in response to retinoic acid. *J Neurochem* 2007; **100**: 242-250 [PMID: 17064353 DOI: 10.1111/j.1471-4159.2006.04207.x]
- Otero JJ**, Fu W, Kan L, Cuadra AE, Kessler JA. Beta-catenin signaling is required for neural differentiation of embryonic stem cells. *Development* 2004; **131**: 3545-3557 [PMID: 15262888 DOI: 10.1242/dev.01218]
- Papadimou E**, Moiana A, Goffredo D, Koch P, Bertuzzi S, Brüstle O, Cattaneo E, Conti L. p66(ShcA) adaptor molecule accelerates ES cell neural induction. *Mol Cell Neurosci* 2009; **41**: 74-84 [PMID: 19386228 DOI: 10.1016/j.mcn.2009.01.010]
- Engberg N**, Kahn M, Petersen DR, Hansson M, Serup P. Retinoic acid synthesis promotes development of neural progenitors from mouse embryonic stem cells by suppressing endogenous, Wnt-dependent nodal signaling. *Stem Cells* 2010; **28**: 1498-1509 [PMID: 20665854 DOI: 10.1002/stem.479]
- Stavridis MP**, Collins BJ, Storey KG. Retinoic acid orchestrates fibroblast growth factor signalling to drive embryonic stem cell differentiation. *Development* 2010; **137**: 881-890 [PMID: 20179094 DOI: 10.1242/dev.043117]
- Sonntag KC**, Simantov R, Björklund L, Cooper O, Pruszk J, Kowalke F, Gilmartin J, Ding J, Hu YP, Shen MM, Isacson O. Context-dependent neuronal differentiation and germ layer induction of Smad4-/- and Cripto-/- embryonic stem cells. *Mol Cell Neurosci* 2005; **28**: 417-429 [PMID: 15737733 DOI: 10.1016/j.mcn.2004.06.003]
- Gambara K**, Aberdam E, Virolle T, Aberdam D, Rouleau M. BMP-4 induces a Smad-dependent apoptotic cell death of mouse embryonic stem cell-derived neural precursors. *Cell Death Differ* 2006; **13**: 1075-1087 [PMID: 16311513 DOI: 10.1038/sj.cdd.4401799]
- Fei T**, Xia K, Li Z, Zhou B, Zhu S, Chen H, Zhang J, Chen Z, Xiao H, Han JD, Chen YG. Genome-wide mapping of SMAD target genes reveals the role of BMP signaling in embryonic stem cell fate determination. *Genome Res* 2010; **20**: 36-44 [PMID: 19926752 DOI: 10.1101/gr.092114.109]
- Bertacchi M**, Pandolfini L, Murenu E, Viegi A, Capsoni S, Cellerino A, Messina A, Casarosa S, Cremisi F. The positional identity of mouse ES cell-generated neurons is affected by BMP signaling. *Cell Mol Life Sci* 2013; **70**: 1095-1111 [PMID: 23069989 DOI: 10.1007/s00018-012-1182-3]
- Lowell S**, Benchoua A, Heavey B, Smith AG. Notch promotes neural lineage entry by pluripotent embryonic stem cells. *PLoS Biol* 2006; **4**: e121 [PMID: 16594731 DOI: 10.1371/journal.pbio.0040121]
- Das D**, Lanner F, Main H, Andersson ER, Bergmann O, Sahlgren C, Heldring N, Hermanson O, Hansson EM, Lendahl U. Notch induces cyclin-D1-dependent proliferation during a specific temporal window of neural differentiation in ES cells. *Dev Biol* 2010; **348**: 153-166 [PMID: 20887720 DOI: 10.1016/j.ydbio.2010.09.018]
- Main H**, Radenkovic J, Jin SB, Lendahl U, Andersson ER. Notch signaling maintains neural rosette polarity. *PLoS One* 2013; **8**: e62959 [PMID: 23675446 DOI: 10.1371/journal.pone.0062959]
- Han H**, Tanigaki K, Yamamoto N, Kuroda K, Yoshimoto M, Nakahata T, Ikuta K, Honjo T. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* 2002; **14**: 637-645 [PMID: 12039915 DOI: 10.1093/intimm/14.5.637]
- Kobayashi T**, Mizuno H, Imayoshi I, Furusawa C, Shirahige K, Kageyama R. The cyclic gene Hes1 contributes to diverse differentiation responses of embryonic stem cells. *Genes Dev* 2009; **23**: 1870-1875 [PMID: 19684110 DOI: 10.1101/gad.1823109]
- Kobayashi T**, Kageyama R. Hes1 regulates embryonic stem cell differentiation by suppressing Notch signaling. *Genes Cells* 2010; **15**: 689-698 [PMID: 20545770 DOI: 10.1111/j.1365-2443.2010.01413.x]



- 29 **Crawford TQ**, Roelink H. The notch response inhibitor DAPT enhances neuronal differentiation in embryonic stem cell-derived embryoid bodies independently of sonic hedgehog signaling. *Dev Dyn* 2007; **236**: 886-892 [PMID: 17295317 DOI: 10.1002/dvdy.21083]
- 30 **Chen CW**, Liu CS, Chiu IM, Shen SC, Pan HC, Lee KH, Lin SZ, Su HL. The signals of FGFs on the neurogenesis of embryonic stem cells. *J Biomed Sci* 2010; **17**: 33 [PMID: 20429889 DOI: 10.1186/1423-0127-17-33]
- 31 **Kunath T**, Saba-El-Leil MK, Almousailleakh M, Wray J, Meloche S, Smith A. FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development* 2007; **134**: 2895-2902 [PMID: 17660198 DOI: 10.1242/dev.02880]
- 32 **Dang LT**, Wong L, Tropepe V. Zfhx1b induces a definitive neural stem cell fate in mouse embryonic stem cells. *Stem Cells Dev* 2012; **21**: 2838-2851 [PMID: 22594450 DOI: 10.1089/scd.2011.0593]
- 33 **He Z**, Li JJ, Zhen CH, Feng LY, Ding XY. Effect of leukemia inhibitory factor on embryonic stem cell differentiation: implications for supporting neuronal differentiation. *Acta Pharmacol Sin* 2006; **27**: 80-90 [PMID: 16364214 DOI: 10.1111/j.1745-7254.2006.00254.x]
- 34 **Foshay KM**, Gallicano GI. Regulation of Sox2 by STAT3 initiates commitment to the neural precursor cell fate. *Stem Cells Dev* 2008; **17**: 269-278 [PMID: 18447642 DOI: 10.1089/scd.2007.0098]
- 35 **Pacherník J**, Horváth V, Kubala L, Dvorák P, Kozubík A, Hampl A. Neural differentiation potentiated by the leukaemia inhibitory factor through STAT3 signalling in mouse embryonal carcinoma cells. *Folia Biol (Praha)* 2007; **53**: 157-163 [PMID: 17976305]
- 36 **Maye P**, Becker S, Siemen H, Thorne J, Byrd N, Carpentino J, Grabel L. Hedgehog signaling is required for the differentiation of ES cells into neurectoderm. *Dev Biol* 2004; **265**: 276-290 [PMID: 14697369 DOI: 10.1016/j.ydbio.2003.09.027]
- 37 **Hunkapiller J**, Singla V, Seol A, Reiter JF. The ciliogenic protein Oral-Facial-Digital 1 regulates the neuronal differentiation of embryonic stem cells. *Stem Cells Dev* 2011; **20**: 831-841 [PMID: 20873986 DOI: 10.1089/scd.2010.0362]
- 38 **Amura CR**, Marek L, Winn RA, Heasley LE. Inhibited neurogenesis in JNK1-deficient embryonic stem cells. *Mol Cell Biol* 2005; **25**: 10791-10802 [PMID: 16314504 DOI: 10.1128/mcb.25.24.10791-10802.2005]
- 39 **Na J**, Furue MK, Andrews PW. Inhibition of ERK1/2 prevents neural and mesodermal differentiation and promotes human embryonic stem cell self-renewal. *Stem Cell Res* 2010; **5**: 157-169 [PMID: 20675210 DOI: 10.1016/j.scr.2010.06.002]
- 40 **Leclerc C**, Néant I, Moreau M. Early neural development in vertebrates is also a matter of calcium. *Biochimie* 2011; **93**: 2102-2111 [PMID: 21742011 DOI: 10.1016/j.biochi.2011.06.032]
- 41 **Lin HH**, Bell E, Uwanogho D, Perfect LW, Noristani H, Bates TJ, Snetkov V, Price J, Sun YM. Neuronatin promotes neural lineage in ESCs via Ca(2+) signaling. *Stem Cells* 2010; **28**: 1950-1960 [PMID: 20872847 DOI: 10.1002/stem.530]
- 42 **Wang D**, Gao L. Proteomic analysis of neural differentiation of mouse embryonic stem cells. *Proteomics* 2005; **5**: 4414-4426 [PMID: 16222718 DOI: 10.1002/pmic.200401304]
- 43 **Yu HM**, Wen J, Wang R, Shen WH, Duan S, Yang HT. Critical role of type 2 ryanodine receptor in mediating activity-dependent neurogenesis from embryonic stem cells. *Cell Calcium* 2008; **43**: 417-431 [PMID: 17767953 DOI: 10.1016/j.ceca.2007.07.006]
- 44 **Cho A**, Tang Y, Davila J, Deng S, Chen L, Miller E, Wernig M, Graef IA. Calcineurin signaling regulates neural induction through antagonizing the BMP pathway. *Neuron* 2014; **82**: 109-124 [PMID: 24698271 DOI: 10.1016/j.neuron.2014.02.015]
- 45 **Zhang L**, Blackman BE, Schonemann MD, Zogovic-Kapsalis T, Pan X, Tagliaferri M, Harris HA, Cohen I, Pera RA, Mellon SH, Weiner RI, Leitman DC. Estrogen receptor beta-selective agonists stimulate calcium oscillations in human and mouse embryonic stem cell-derived neurons. *PLoS One* 2010; **5**: e11791 [PMID: 20668547 DOI: 10.1371/journal.pone.0011791]
- 46 **Bami M**, Episkopou V, Gavalas A, Gouti M. Directed neural differentiation of mouse embryonic stem cells is a sensitive system for the identification of novel Hox gene effectors. *PLoS One* 2011; **6**: e20197 [PMID: 21637844 DOI: 10.1371/journal.pone.0020197]
- 47 **Bieberich E**, Silva J, Wang G, Krishnamurthy K, Condie BG. Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants. *J Cell Biol* 2004; **167**: 723-734 [PMID: 15545317 DOI: 10.1083/jcb.200405144]
- 48 **Bieberich E**. Ceramide and sphingosine-1-phosphate signaling in embryonic stem cell differentiation. *Methods Mol Biol* 2012; **874**: 177-192 [PMID: 22528448 DOI: 10.1007/978-1-61779-800-9\_14]
- 49 **Johnson CE**, Crawford BE, Stavridis M, Ten Dam G, Wat AL, Rushton G, Ward CM, Wilson V, van Kuppevelt TH, Esko JD, Smith A, Gallagher JT, Merry CL. Essential alterations of heparan sulfate during the differentiation of embryonic stem cells to Sox1-enhanced green fluorescent protein-expressing neural progenitor cells. *Stem Cells* 2007; **25**: 1913-1923 [PMID: 17464092 DOI: 10.1634/stemcells.2006-0445]
- 50 **Pickford CE**, Holley RJ, Rushton G, Stavridis MP, Ward CM, Merry CL. Specific glycosaminoglycans modulate neural specification of mouse embryonic stem cells. *Stem Cells* 2011; **29**: 629-640 [PMID: 21308866 DOI: 10.1002/stem.610]
- 51 **Wu J**, Pan Z, Cheng M, Shen Y, Yu H, Wang Q, Lou Y. Ginsenoside Rg1 facilitates neural differentiation of mouse embryonic stem cells via GR-dependent signaling pathway. *Neurochem Int* 2013; **62**: 92-102 [PMID: 23063465 DOI: 10.1016/j.neuint.2012.09.016]
- 52 **Kim E**, Clark AL, Kiss A, Hahn JW, Wesselschmidt R, Coscia CJ, Belcheva MM. Mu- and kappa-opioids induce the differentiation of embryonic stem cells to neural progenitors. *J Biol Chem* 2006; **281**: 33749-33760 [PMID: 16954126 DOI: 10.1074/jbc.M603862200]
- 53 **Zhang ZH**, Lu YY, Yue J. Two pore channel 2 differentially modulates neural differentiation of mouse embryonic stem cells. *PLoS One* 2013; **8**: e66077 [PMID: 23776607 DOI: 10.1371/journal.pone.0066077]
- 54 **Arnhold S**, Fassbender A, Klinz FJ, Kruttwig K, Löhnig B, Andressen C, Addicks K. NOS-II is involved in early differentiation of murine cortical, retinal and ES cell-derived neurons-an immunocytochemical and functional approach. *Int J Dev Neurosci* 2002; **20**: 83-92 [PMID: 12034139 DOI: 10.1016/S0736-5748(02)00020-5]
- 55 **Bouhon IA**, Kato H, Chandran S, Allen ND. Neural differentiation of mouse embryonic stem cells in chemically defined medium. *Brain Res Bull* 2005; **68**: 62-75 [PMID: 16325006 DOI: 10.1016/j.brainresbull.2005.08.022]
- 56 **Parekkadan B**, Berdichevsky Y, Irimia D, Leeder A, Yarmush G, Toner M, Levine JB, Yarmush ML. Cell-cell interaction modulates neuroectodermal specification of embryonic stem cells. *Neurosci Lett* 2008; **438**: 190-195 [PMID: 18467031 DOI: 10.1016/j.neulet.2008.03.094]
- 57 **Piacentini R**, Ripoli C, Mezzogori D, Azzena GB, Grassi C. Extremely low-frequency electromagnetic fields promote in vitro neurogenesis via upregulation of Ca(v)1-channel activity. *J Cell Physiol* 2008; **215**: 129-139 [PMID: 17941084 DOI: 10.1002/jcp.21293]
- 58 **Maioli M**, Rinaldi S, Santaniello S, Castagna A, Pigliaru G, Gualini S, Fontani V, Ventura C. Radiofrequency energy loop primes cardiac, neuronal, and skeletal muscle differentiation in mouse embryonic stem cells: a new tool for improving tissue regeneration. *Cell Transplant* 2012; **21**: 1225-1233 [PMID: 21975035 DOI: 10.3727/096368911X600966]
- 59 **Watanabe S**, Umehara H, Murayama K, Okabe M, Kimura T, Nakano T. Activation of Akt signaling is sufficient to maintain pluripotency in mouse and primate embryonic stem cells. *Oncogene* 2006; **25**: 2697-2707 [PMID: 16407845 DOI: 10.1038/sj.onc.1209307]
- 60 **Zhao Y**, Xiao Z, Gao Y, Chen B, Zhao Y, Zhang J, Dai J. Insulin rescues ES cell-derived neural progenitor cells from apoptosis by differential regulation of Akt and ERK pathways. *Neurosci Lett* 2007; **429**: 49-54 [PMID: 17980966 DOI: 10.1016/j.neulet.2007.09.076]



- 61 **Chuang JH**, Tung LC, Yin Y, Lin Y. Differentiation of glutamatergic neurons from mouse embryonic stem cells requires raptor S6K

signaling. *Stem Cell Res* 2013; **11**: 1117-1128 [PMID: 23988668 DOI: 10.1016/j.scr.2013.08.003]

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## Importance of the stem cell microenvironment for ophthalmological cell-based therapy

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*vitro*. However, for diseases of the eye, obtaining the adequate number of cells for clinical transplantation is difficult due to the small size of tissue donors and the frequent needs of long-term amplification of cells *in vitro*, which results in low cell viability after transplantation. In addition, the transplanted cells often develop fibrosis or degrade and have very low survival. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPS) are also promising candidates for cell therapy. Unfortunately, the differentiation of ESCs can bring immune rejection, tumorigenicity and undesired differentiated cells, limiting its clinical application. Although iPS cells can avoid the risk of immune rejection caused by ES cell differentiation post-transplantation, the low conversion rate, the risk of tumor formation and the potentially unpredictable biological changes that could occur through genetic manipulation hinder its clinical application. Thus, the desired clinical effect of cell therapy is impaired by these factors. Recent research findings recognize that the reason for low survival of the implanted cells not only depends on the seeded cells, but also on the cell microenvironment, which determines the cell survival, proliferation and even reverse differentiation. When used for cell therapy, the transplanted cells need a specific three-dimensional structure to anchor and specific extra cellular matrix components in addition to relevant cytokine signaling to transfer the required information to support their growth. These structures present in the matrix in which the stem cells reside are known as the stem cell microenvironment. The microenvironment interaction with the stem cells provides the necessary homeostasis for cell maintenance and growth. A large number of studies suggest that to explore how to reconstruct the stem cell microenvironment and strengthen its combination with the transplanted cells are key steps to successful cell therapy. In this review, we will describe the interactions of the stem cell microenvironment with the stem cells, discuss the importance of the stem cell microenvironment for cell-based therapy in ocular diseases, and introduce the progress of stem cell-based

### Abstract

Cell therapy is a promising treatment for diseases that are caused by cell degeneration or death. The cells for clinical transplantation are usually obtained by culturing healthy allogeneic or exogenous tissue *in*



therapy for ocular diseases.

**Key words:** Microenvironment; Niche; Stem cell; Cell-based therapy; Ocular diseases; Ophthalmology

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**Core tip:** Cell therapy is a promising treatment for the diseases caused by cell degeneration or death. However, the transplanted cells often develop fibrosis or are absorbed and cannot survive long. It is not simply because of seed cells, but also due to the cell microenvironment. How to reconstruct the stem cell microenvironment and strengthen its combination with the transplanted cells is the key to successful cell therapy. We will discuss the importance of the stem cell microenvironment for cell-based therapy in ocular diseases and introduce the progress of cell therapy for ocular diseases.

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## INTRODUCTION

Limbal stem cell deficiency, corneal endothelial decompensation, corneal grafts endothelial decompensation, retinitis pigmentosa, age-related macular degeneration, Stargardt disease and other hereditary retinal diseases are all caused by cell degeneration or death. There is no effective clinical treatment currently available. Unlike traditional medicine or surgical therapy, cell therapy is a promising treatment and can treat the abnormal cells most directly and efficiently.

The road to cell therapy has been a long and tortuous process. In the early days, most of the efforts to obtain sufficient therapeutic seed cells were based on establishing cell lines or improving tissue culture methods with the expectation that they will provide therapeutic effects after the cell transplantation. The former long-term passaged cell lines were mainly obtained by transgenic or nuclear atypia. Because of biosecurity risks and allograft immune rejection, cells obtained by this method are mainly used for basic research<sup>[1]</sup>. The cells for clinical transplantation are usually obtained by culturing healthy allogeneic or exogenous tissue *in vitro*. It is hard to get large tissue from the eye to obtain a sufficient amount of cells and the culture often needs to go through long-term amplification *in vitro*, resulting in low cell viability after transplantation. Cells often develop fibrosis or are absorbed and cannot survive long. Thus, the desired clinical effect of the cell therapy cannot be accessed

and so it was shelved for a while.

Considerable progress has been made in the induction and differentiation of embryonic stem cells (ESCs) since the last century and it brought new vitality to cell therapy. However, the differentiation of ESCs can bring immune rejection, tumorigenicity and differentiation of uncertainty, which limits its clinical application. Although induced pluripotent stem cells (iPS) can avoid the risk of immune rejection caused by ESC differentiation post-transplantation, the low conversion rate and the risk of tumor formation still exists and the potentially unpredictable biological danger achieved through genetic manipulation hinders its clinical application. Thus, cell therapy returns to the transplantation of the cultured autologous cells, especially the enriched stem cells and the methods and the results have been greatly improved.

With the development of research, it is increasingly recognized that the reason the implanted cells cannot survive long-term in cell therapy is not simply because of seed cells, but also because of the cell microenvironment which determines cell survival, proliferation and even reverse differentiation. The birth of Dolly the sheep is the best example. In a good embryo environment, mature breast cells can be re-developed into a new individual sheep. From a biological point of view, whether it is a cell or an organism, it must exist in its surroundings with exchange material, energy and information. In terms of cell therapy, the transplanted cells need a specific three-dimensional structure to anchor and specific extra cellular matrix (ECM) components and cytokine biological information transfer to support their growth. These structures are present in the matrix in which the stem cells located and are called the stem cell microenvironment. The microenvironment interacts with the stem cells and they are interdependent, mutually promote and complement each other, working together to maintain the stem cell homeostasis<sup>[2-5]</sup>. A large number of studies suggest that exploring how to strengthen the organic combination of the transplanted cells and the stem cell niche and reconstructing the stem cell microenvironment is the key to successful cell therapy.

In this review, we will recount the interactions of the stem cell microenvironment with the stem cells, discuss the importance of the stem cell microenvironment for cell-based therapy in ocular diseases, and introduce the progress of stem cell treatment for ocular diseases.

## THE ROLE OF THE STEM CELL MICROENVIRONMENT

### *Obtaining autologous cells for cell therapy aided by the stem cell microenvironment*

In the process of inducing embryonic stem cells and iPS cell differentiation, it was discovered that the adult cells can induce embryonic stem cells to differentiate



and the embryonic stem cells can promote somatic cell proliferation, repair the defects of co-cultured cells, or even reverse the differentiation state of somatic cells<sup>[6-9]</sup> by improving the microenvironment *via* secreting a variety of factors and cell interactions, *etc.*

We began to work on the induction and differentiation of embryonic stem cells and epidermal stem cells<sup>[10-12]</sup> in 1997 and found that when the adult cells were treated with supernatant from cultured embryonic stem cells or co-cultured with the embryonic stem cells, the aging process of adult cells can be slowed down or even reversed into progenitor cells and their self-renewal and proliferation capacity can be increased significantly<sup>[13-18]</sup>, whereas the adult cells can also induce embryonic stem cells to differentiate. We found a phenomenon that the corneal epithelial cells maintain long-term proliferative capacity and tissue-specific cell phenotype by factors secreted from murine ESCs. Rabbit corneal epithelial cells, cat corneal endothelial cells, rabbit skin epithelial cells and rabbit conjunctiva epithelial cells grew very well in culture medium with addition of ESC conditioned medium. These corneal epithelial cells were serially subcultured for more than 20 passages and maintained high cell purity, cobblestone-like morphology, enhanced colony forming efficiency, normal diploid and capacity to regenerate a functional stratified corneal epithelial equivalent. The rabbit corneal epithelial cells cultured in the embryonic stem cell microenvironment can be continuously passaged over 55 generations in 22 wk, gradually restoring its precursor characteristics, such as: decreased corneal epithelial cell specific differentiation markers K3/K12 expression, increased corneal epithelial precursor cell markers *P63* and *ABCG2* expression, but the expression of *Oct-4* was not detected, indicating that the embryonic stem cell microenvironment treated cells obtained a strong proliferative capacity without the potential tumorigenicity and uncertain differentiation<sup>[13-18]</sup>. Zhang *et al.*<sup>[13]</sup> also found that the proliferation and maturation of the dendrite cells co-cultured with the bone marrow mesenchymal cells were able to be significantly promoted, with the enhanced precursor cell marker expression and reduced expression of differentiation markers, so that the mature dendritic cells were reversed to the original progenitor cell stage. Pearton *et al.*<sup>[9]</sup> reported that mouse embryonic skin can induce the terminal rabbit central corneal epithelial cells to reverse to the limbal stem cells by gradually losing specific marker K12 and K3. These results strongly suggest that the stem cell microenvironment can significantly regulate adult cell proliferation. It has the potential to become a more effective and safe method to access autologous seed cells with high proliferative activity which are close to pluripotent stem cells or transient amplifying cells without uncertain differentiation direction or tumorigenicity and render them more

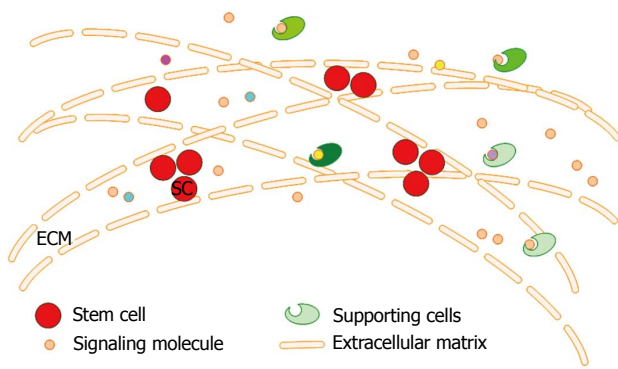
suitable for clinical use.

### **Repair of the stem cell microenvironment is the basis for long-term efficient cell-based therapy**

Stem cell microenvironment is the general term of the three-dimensional structure and a variety of signaling molecules (growth factors and their receptors, hormones and signaling molecules) present in the stroma where the stem cells reside and it can regulate the fate (proliferation/differentiation) of the stem cells. Because of its specific three-dimensional structure, it is vividly called niches (niche), which consists of three components: the extracellular matrix (ECM), niche cells (supporting cells, stem cells) and soluble factors derived from the niche cells (Figure 1). The proliferation and differentiation of stem cells are pre-programmed by themselves and are also affected by the microenvironment where they are residing. The stem cell microenvironment can anchor stem cells *in vivo* and regulate the self-renewal and production of their progeny cells through cell-cell, cell-ECM and cytokine-cell interactions. The different macromolecules or properties of the cells and ECM interact with each other in a complex and dynamic network<sup>[19,20]</sup>. Nowadays, there is increasing evidence showing that the ECM is not only the supportive scaffold but also plays a fundamental role in cell biology. It plays important roles in the development of the cells and can regulate their behavior<sup>[21]</sup> by the production, degradation of its components and the remodeling of the structure<sup>[22,23]</sup> and the direct and indirect signaling properties<sup>[21]</sup>. The polarity, division and migration of the cells can be influenced by the physical properties of the ECM, such as rigidity, porosity, topography and insolubility<sup>[24]</sup>. Cytokines play an important role in exchanging information from cell-cell and cell-ECM. The changes of the extracellular matrix components also affect the differentiation of the stem cells and the induced differentiation *in vitro* is accomplished by mimicking the cell microenvironment. So, it is difficult to obtain a long lasting therapeutic effect in cell-based therapy without the support of a good stem cell microenvironment, even when excellent cells are transplanted.

The importance of stem cell microenvironment in tissue engineering has also been verified. How to rebuild the stem cell microenvironment becomes the biggest challenge currently for constructing tissue engineered tissues and organs. In the past, because the scaffolds for tissue engineering of organs and tissues had no such sophisticated stem cell microenvironment, the desired therapeutic effect could not be achieved and the structure and function could not be completely recovered after transplantation. In 2009, we introduced phospholipase A2, which can specifically hydrolyze the phospholipids of the corneal stroma cell membrane, can destroy the cell structure and be used to prepare acellular porcine corneal stroma acellular porcine corneal stroma





**Figure 1** Schematic overview of the stem cell microenvironment components. ECM: Extra cellular matrix; SC: Stem cell .

(APCS) for biological tissue engineering cornea<sup>[25]</sup>. The natural corneal collagen structure and 80% of the extracellular matrix components can be retained by this means. This APCS not only has good biocompatibility and biomechanical strength, but also keeps the limbal stem cells microenvironment necessary for their long-term proliferation. The grafts of APCS maintained good biomechanical strength and high transparency post-transplantation in animal experiments and it can help to rebuild the limbal stem cell microenvironment<sup>[26-30]</sup>. Nakayama *et al.*<sup>[31]</sup>, Ott *et al.*<sup>[32]</sup>, Petersen *et al.*<sup>[33]</sup>, Uygun *et al.*<sup>[34]</sup> and Conrad *et al.*<sup>[35]</sup> have also reported the use of acellular matrix as a scaffold in tissue engineering in kidneys, lungs, heart, trachea and bladder. As Song *et al.*<sup>[36]</sup> stated, using an acellular matrix scaffold with natural extracellular matrix to build tissue engineering products can mediate organ development, reparation and regeneration which cannot be accessed by synthetic materials. There are further experiments that proved that the acellular materials with extracellular matrix play an important role in the regulation of stem cells<sup>[37]</sup>. These studies showed that a decellularized tissue with the natural ECM scaffold can induce the stem cells to differentiate into cell types present in certain tissue<sup>[31]</sup> and thus the decellularized organ has already been used in tissue engineering and cell therapy<sup>[31,36]</sup>.

The microenvironment has a great effect on the fate of stem cells. In recombination experiments, hair follicle stem cells were induced to differentiate into corneal epithelial cells when they were cultured in a limbus-specific-like niche<sup>[38]</sup>. These cells grow into stratified epithelium and express the cornea-specific markers K12 and Pax6 while the epidermal specific K10 obviously down-regulated<sup>[38]</sup>. While in other studies the rabbit epithelial cells from the central cornea differentiated into epidermal keratinocytes when recombined with mouse embryonic dermis, it lost corneal-specific marker K3/K12 together with the down-regulation of Pax6 and expressed keratinocyte marker K5/K14<sup>[9]</sup>. All of these changes showed that

the microenvironment has an important regulation role in the fate of the stem cells.

### **Stem cells can sustain and repair the structure and the function of the niche**

On the other hand, evidence *in vivo* showed the relevance of the ECM and the stem cell behavior in that the altered properties or the aged niches' ability of maintaining stem cells' stemness can be reduced<sup>[39]</sup> and it further affected stem cell proliferation and updated, which created a vicious cycle. Scientists from Harvard University attempted to study the relationship between human aging and microenvironmental changes between stem cells via the stem cell microenvironment (niche) and they found that altering the aged microenvironment with the young microenvironment can improve the ability of the proliferation and renewal of the stem cells. The study also found that adding "insulin-like growth factor-1" (IGF-1) can also improve the state of the aging stem cell microenvironment.

Cell receptors can directly mediate the interactions between the stem cells and the ECM where they are located. It was also found that integrins, a large family of heterodimeric transmembrane receptors, are important receptors for the ECM-stem cell interactions and can regulate cell survival, migration, proliferation and differentiation by connecting the ECM to the intracellular cytoskeleton<sup>[40]</sup> as well as the adhesion, anchorage and homing of the stem cells. Different kinds of integrins bind to different ECM components or different cell surface adhesion molecules and receptors<sup>[41-43]</sup>. Integrins can regulate the self-renewal and proliferation of the stem cells by directly activating focal adhesion kinase (FAK) and the phosphoinositide 3-kinase (PI3K) signaling pathway<sup>[40,44,45]</sup>. The  $\alpha\beta1$  integrin can bind to the ECM protein laminin and help the spermatogonial stem cells home in to the testicular niche<sup>[46]</sup> and NSCs (neural stem cells) adhere to the vascular niche<sup>[47]</sup>. The  $\alpha9$  integrin is essential for the proliferation of the HSC<sup>[48]</sup> and NSC microenvironment<sup>[49]</sup> *via* binding protein tenascin-C in the ECM. The  $\alpha4$ ,  $\alpha6$ ,  $\alpha9$  and  $\beta1$  integrin chains are also important to the HSCs in their homing in to the bone marrow niche<sup>[50-53]</sup>. HSC homing and proliferation are also regulated by  $\alpha\nu\beta3$  integrin<sup>[54-56]</sup>. Furthermore,  $\beta1$  integrins can control the balance between symmetric and asymmetric divisions in skin and brain, as well as the differentiation and self-renewal of the stem cells<sup>[57-60]</sup> by regulating the activity of the Notch pathway and EGF receptor<sup>[61,62]</sup>. They are also important for the proliferation of intestinal stem cells (ISCs) *via* the Hedgehog signaling pathway<sup>[63]</sup>. Signaling pathways of the growth factors and cytokines, such as IL-3 and TGF- $\beta$ <sup>[43,64-67]</sup>, can also be regulated by integrins and these signaling pathways can regulate the expression of integrins conversely<sup>[46]</sup>. Therefore, the receptors



have specific activities in a certain kind of stem cell microenvironment.

Some ECM components can regulate their availability by setting a biochemical gradient and binding growth factors<sup>[21]</sup>. On the one hand, the ECM can also make the growth factors insoluble, unavailable or not bioactive and serve as the reservoir for them, like proteoglycans, collagens, fibronectin and vitronectin, which bind VEGFs, FGFs, HGFs, TGF- $\beta$  and BMPs. On the other hand, ECM proteins and proteoglycans can be induced to be soluble and remodeled to release and distribute growth factors under the action of enzymes, such as metalloproteinase<sup>[21]</sup>. The function NSC can be favored by the ECM components in its niche by promoting growth factor activity *via* capturing FGF-2 from it<sup>[68,69]</sup>. Similarly, in the procedure of the regulation of muscle satellite cells, all kinds of growth factors bind to the cell surface or the basal lamina proteoglycan and then they can be activated in a certain signaling pathway<sup>[70]</sup>.

The biophysical properties of the ECM can determine the behavior of stem cells. The balance of the internal forces generated by cell cytoskeleton tension and the external forces from the compression of the neighboring cells and the stiffness of the surrounding ECM maintains the shape of the cells and makes them stay in their anatomical localization<sup>[71]</sup>, which can regulate the cell behavior finally<sup>[72-74]</sup>. Recently, the YAP/TAZ transcriptional factors were found to have key biological effects on the ECM elasticity, cell geometry and cytoskeletal modulation<sup>[71,74,75]</sup> among the mechanotransduction pathways (Wnt/ $\beta$ -catenin, PI3K/Akt, TGF- $\beta$ , Ras/MAPK, and RhoA/ROCK pathways). In fact, ECM organization and composition can regulate tissue stiffness and then have an influence on the stem cell behavior<sup>[72,76]</sup>. The human mesenchymal stem cells can express organ-specific transcription factors and differentiate into myoblasts, neurons and osteoblasts<sup>[77]</sup> when they are cultured on ECMs with similar stiffness of the muscle, brain or bone respectively. Culture on hydrogel with the same elastic modulus as the bone marrow can increase the self-renewal ability and maintain multipotency of the hMSCs (human mesenchymal stem cells) compared to those cultured on stiffer substrates<sup>[78]</sup>. NSCs cultured on hydrogel with similar stiffness to the brain tissue gain the neuronal differentiation potential, while the stiffer gels make them differentiate into glial cells<sup>[79]</sup>. That the stiffness gradients of the hippocampus regulate NSC behavior *in vivo* confirmed that ECM and its biomechanical properties play important roles in the fate of the stem cell<sup>[80]</sup>. This opens new insights in to the role of ECM mechanical properties in the stem cell microenvironment.

**Stem cells in ocular tissues:** Several studies showed the conjunctiva epithelial stem cell niche located in the fornix by growth potential assays, label-retention analyses and keratin expression detection<sup>[81-84]</sup>. They have bipotential to differentiate

into both epithelial cells and goblet cells<sup>[85]</sup>.

Corneal epithelial stem cells resident at the basal limbal epithelium and called limbal stem cells were first applied to clinical use in ophthalmology. Tung-Tien Sun's group did immunostaining with monoclonal antibodies against the corneal-specific K3<sup>[86]</sup> and showed the negative expression of K3/K12 in the limbal basal layer, which gave rise to a number of experiments that verified that the corneal stem cells were located in the limbus<sup>[86-88]</sup>. These label-retaining limbal cells<sup>[89]</sup> have a higher proliferative potential<sup>[90]</sup> and colony-forming ability<sup>[91]</sup> compared with central ones.

People made efforts to find specific molecules markers of the limbal stem cells. p63<sup>[92]</sup>, vimentin<sup>[93-95]</sup>,  $\alpha$ -enolase<sup>[96,97]</sup>,  $\alpha$ 9 $\beta$ 1 integrin<sup>[98]</sup>, tenascin-C and EMILIN1<sup>[99]</sup>, ABCG2<sup>[100-103]</sup> and ABCB5<sup>[104]</sup> are all highly expressed in the basal layer of limbal epithelium but none of them are the specific markers of the limbal stem cells as expected. This is because the early differentiating cells<sup>[105,106]</sup> still have the stem cell markers and show intermediate profiles between stem and differentiated cells until the stem cell markers are down-regulated with the expression of the differentiated phenotype<sup>[107]</sup>. Thus, we can only enrich the stem cells while separating them with these molecules markers<sup>[108]</sup>.

Since it is complex and difficult to characterize the corneal stem cells, people try to identify them by analyzing their niches and the regulatory functions of the niche.

The limbus is a specific region which is characterized by the palisades of Vogt with the papillae-like projections and the vascular net in the peripheral cornea<sup>[109]</sup>. It enables the epithelial cells to interact with ECM and chemical signals diffused from the vascular network<sup>[110]</sup>. Some studies showed that the limbus has a specific anatomic structure such as the niche, the LEC (limbal epithelial crypt) or LC (limbal crypt), which consists of a cord or finger of cells that are located between the palisades of the limbal stroma and extends radically to the conjunctiva stroma<sup>[92,93]</sup>. The high expression of K14<sup>[111]</sup>, ABCG2<sup>[112]</sup> and p63<sup>[92,93]</sup> in cells at the LEC<sup>[112]</sup>/LC<sup>[113]</sup> suggest that they are the microenvironment of the limbal stem cells but the LEC/LC structure has not been found in other species besides humans and pigs<sup>[94]</sup>.

This tissue with unique cellular properties can synthesize different kinds of ECM substrates. Several studies on the ECM components of the cornea were performed regarding the aspect of the biochemical and immunological characteristics. Corneal stroma comprises collagen type I-VI<sup>[114-117]</sup>, glycosaminoglycans (chondroitin, heparin, dermatan) and keratan sulfates<sup>[118-122]</sup>, fibronectin and laminin<sup>[105,123]</sup> and hyaluronic acid<sup>[124]</sup> and the limbal epithelial cells are more likely to adhere to a rougher surface than those in the central cornea<sup>[125]</sup>. To further study the interaction between corneal stem cells and their



microenvironment and the different functions between the central cornea and the limbus, the corneal basement membrane components were analyzed by several studies. These studies found that the conjunctiva, limbal and central corneal epithelia have a heterogeneous composition of the basal membrane (BM)<sup>[126]</sup>. Some studies reported that there was no collagen IV in the central cornea BM<sup>[127]</sup>, while others had the controversial results that collagen IV presented both in the limbal and the central corneal BM<sup>[126]</sup>. Later, people found that collagen IV  $\alpha 1$  (IV) and  $\alpha 2$  (IV) chains show more intense staining at the corneal limbus and the  $\alpha 3$  (IV) chain shows an abrupt decrease at the limbus<sup>[128,129]</sup>, while collagen types IV ( $\alpha 3$ - $\alpha 4$  chains) and XII are only expressed in the central cornea<sup>[128]</sup>. Then, other components of the limbal BM were studied further. Laminin  $\alpha 2$ - $\alpha 5$ ,  $\beta 1$ - $\beta 3$ ,  $\gamma 1$ - $\gamma 3$ , nidogen-1, -2, SPARC/BM-40, as well as agrin are preferentially expressed in the limbal BM<sup>[128]</sup>, which colocalized with the ABCG2/p63/K19-positive and K3/Cx43/desmoglein/integrin- $\alpha 2$ -negative stem cells and early progenitor cell clusters<sup>[128,129]</sup>. The BM components, such as type XVI collagen, fibulin-2, tenascin-C/R, vitronectin, bamacan, chondroitin sulfate and versican, are colocalized with the putative vimentin-positive late progenitor cells<sup>[128-130]</sup> at the limbus. On the contrary, type V collagen, fibrillin-1 and 2, and thrombospondin-1 were almost only found in the corneal BM<sup>[128]</sup>; others, such as type IV collagen  $\alpha 5$  and  $\alpha 6$  chains, collagen types VII, XV, XVII and XVIII, laminin-111, laminin-332, laminin chains  $\alpha 3$ ,  $\beta 3$  and  $\gamma 2$ , fibronectin, matrilin-2 and 4, and perlecan, were expressed throughout the epithelial layer on the ocular surface<sup>[129,130]</sup>. All these studies showed that the BM at the limbus has a specific ECM composition which is different from that in the peripheral or central cornea. This suggested that the EMC at the LEC/LC created a microenvironment that regulates stem cells and their progeny by supporting stemness while inhibiting the differentiation and preserving the proliferative abilities in limbal cells.

The stem cells of the corneal endothelium and the trabecular meshwork are believed to be located at the transition zone between the peripheral corneal endothelium and the anterior non-filtering portion of the trabecular meshwork<sup>[131]</sup>. Corneal stroma stem cells are located in the limbal stroma, play roles in visualization and have corresponded to the limbal niche cells<sup>[132]</sup>.

At the early stage, the stem cells of the lens were assumed to be the label-retaining cells which are located at the anterior central region of the lens<sup>[133]</sup>. Yamamoto *et al.*<sup>[134]</sup> concluded that the germinative zone of the lens epithelium contains transient amplifying cells with the positive expression of proliferation markers, such as A1, B1, C and D1 cyclins and PCNA (proliferating cell nuclear antigen), and can be labeled by BrdU (5-bromo-2'-deoxyuridine). On the

other hand, other studies showed that they were probably located in the region anterior to the germinative zone. However, Remington *et al.*<sup>[135]</sup> assumed that lens stem cells resided in the ciliary body because the lens is non-vascular, its epithelium does not have the morphology of other stem cells and no type of tumors are derived from the lens. Thus, the existence of the lens stem cells remains controversial and needs to be elucidated.

Previously, it was believed that there were no stem cells in the mammalian retina since it cannot regenerate<sup>[136]</sup> but von Leithner *et al.*<sup>[137]</sup> found retinal precursors in the peripheral retinal pigment epithelium later. However, the cells from the pigmented ciliary margin were later found to have the ability to form spherical colonies and produce various types of differentiated retinal cell<sup>[138]</sup>. These results gave the evidence that retinal stem cells are located in the pigmented ciliary margin epithelium.

## CELL-BASED THERAPY IN OCULAR DISEASES

### *Progress in the research of stem cell therapy for corneal diseases*

Limbal stem cell deficiency can be caused by a myriad of insults that present with the following pathological states: damaged corneal barrier function, persistent corneal epithelium defects or recurrent corneal erosions, chronic inflammation associated with corneal stromal scarring, visualization, conjunctivalization and eventually blindness. Some researchers speculate that this occurs due to gradual deterioration of the limbal stromal niches<sup>[139]</sup>. Thus, limbal epithelial stem cell transplantation and the reestablishment of the limbal stem cell microenvironment are necessary for ocular surface reconstruction in these diseases<sup>[139]</sup>.

Autologous or allogenic limbal transplantation has achieved good clinical efficiency in the treatment of limbal stem cell deficiency. However, in successful cases post limbal transplantation, there is a doubt as to whether there is a causal relationship between the survival of the donor limbal stem cells and the clinical effect. Shimazaki *et al.*<sup>[140]</sup> detected the presence of donor-derived epithelial cells in 60% of cases (10 eyes/9 cases) post human limbal transplantation with fluorescence in situ hybridization assay, with 77.8% by RFLP analysis. At the same time, the authors stated that there was no difference in the postoperative clinical outcomes whether the presence of survived donor-derived cells was detected. Thus, more studies should be carried out to confirm the clinical significance of the survival of donor cells. Furthermore, it was reported that donor-derived cells were undetected in cases with objective clinical improvement after three to five years post limbal transplantation, even with DNA fingerprinting analysis<sup>[141]</sup>. This suggested that the survival of



donor-derived cells is not essential for improvements of the clinical symptoms. These results indicated that limbal transplantation might simply serve as the corneal stroma transplantation. Presumably, limbal transplantation improves the residual stroma stem cell microenvironment, making residual stem cells in the patient regenerate on the ocular surface and allowing for improvement of the ocular surface<sup>[142]</sup>. Therefore, some scholars believe that the essence of limbal transplantation may be the restoration of the limbal stroma resulting in increased stability of the limbal stem cell "niche"<sup>[143]</sup>. So, limbal epithelial stem cell deficiency treatment lies in the application of various methods to restore the normal limbus matrix.

Although traditional corneal transplantation, limbal transplantation, has already achieved good results in ocular surface reconstruction for corneal diseases, there still several problems that have hindered clinical application, such as the shortage of donors, the immune rejection post-transplantation and other issues. Therefore, the construction of tissue engineering cornea *in vitro* with appropriate biomaterials and synthetic materials is the hope for solving these questions. How to get a sufficient amount of high activity seed cells for tissue engineering has been the challenge for tissue engineering product construction and cell therapy. The microenvironment plays an important role in the survival and development of cells and tissues. The microenvironment or simulated microenvironment can effectively induce ES and iPS differentiation in a certain direction and the embryonic microenvironment also has the effect of reverse differentiation of the adult cells. We have considered two aspects in our studies: (1) cellular microenvironment: embryonic stem cell microenvironment culture systems can make the differentiated corneal epithelial cells, conjunctiva epithelial cells<sup>[15]</sup> and even human corneal endothelial cells<sup>[17]</sup> obtain a strong proliferation ability and can be passaged long-term with de-differentiation cell marker expression, normal cell morphology and karyotype, but no tumorigenicity. Our preliminary findings showed that the ES microenvironment may inhibit the apoptosis of the cells by activating telomerase *via* integrin the b1-FAK-PI3K/Akt, telomerase-p21-mitochondrial axis and FAK/Wnt signaling pathway<sup>[18,144]</sup>; and (2) stromal microenvironment: the APCS limbal produced by lipase (not existing protease digestion)<sup>[25,30]</sup> can retain the normal extracellular matrix, collagen lamellar micro ultrastructure. It can repair and maintain the stemness and proliferation ability of the limbal stem cells after it is transplanted to the limbal stem cell deficiency rabbit model. In short, the microenvironment can be used to obtain sufficiently pure seed cells with strong proliferation capability but no immunogenicity. The microenvironment plays an important role in cell therapy and is the basis for the long-term efficacy of the treatment. Establishment of seed cells using the microenvironment will make cell therapy return to

autologous cell transplantation. Maintaining the specific three-dimensional structure and the extracellular matrix to promote the proliferation and long-term survival of the transplanted cells<sup>[145]</sup> has great meaning. Professor Ott *et al.*<sup>[32]</sup> stated that acellular matrix has a natural extracellular matrix which can mediate and guide organ development and mediate repair and regeneration. The key steps for the best clinical efficacy of cell transplantation are the long-term survival of the seed cells and the reconstruction of the stem cell niche.

### **Progress in the research of stem cell therapy for the retinal and optic nerve diseases**

**Retinal stem cells and optic nerve repair:** Retinal diseases such as age-related macular degeneration, Leber congenital amaurosis and cone rod dystrophy are caused by lesions of retinal neuronal cells, which have an irreversible pathological process of degeneration and damage of the retinal neuronal cells, causing serious visual impairment or even blindness which currently cannot be effectively treated. Since the stem cells have self-renewal and multi-differentiation potential abilities, using stem cells as donor cells for retinal diseases treatment has become a hot topic.

In 2000, Wirtschafter *et al.*<sup>[83]</sup> found that groups of self-renewing cells in the ciliary epithelium of the adult mice can form neurospheres when they were cultured *in vitro*. They can be induced to differentiate into specific types of neuronal cells in the retina, such as the rod cells, bipolar cells and glial cells, indicating that retinal stem cells exist in adult mammalian eyes. Ballios *et al.*<sup>[146]</sup> found that retinal stem cells (retinal stem cell, RSC) also exist in the ciliary margin zone in people of different ages. These cells have proliferative capacity *in vitro* and can be induced to differentiate into different retinal neurons. Some of these stem cells can migrate and integrate into the host retina and can even differentiate into photoreceptor cells. These studies suggested that RSCs from different sources of animals or humans can survive and migrate to the host retina layers after transplantation. Although the implanted RSCs can migrate into the retina and differentiate into a variety of retinal cells, most transplanted RSCs remain in the subretinal space or the vitreous body, while less can be integrated and induced to differentiation, so the efficiency is not ideal.

Meyer *et al.*<sup>[147]</sup> transplanted GFP-labeled ESCs into mouse vitreous after induced differentiation and found that the transplanted cells can migrate into the entire retina layers and differentiate into retinal neurons cells with the expression of markers such as NeuN, calretinin and cPKC- $\alpha$ . In 2010, Parameswaran *et al.*<sup>[148]</sup>, using a similar method as embryonic stem cell differentiation, completed the differentiation of mouse iPS cells into retinal ganglion cells, which not only highly express the retinal ganglion cells



regulation gene *Ath5*, *Wtl*, *Brn3b*, *Rpfl* and *Irx2*, but also can specifically project upwards to the superior colliculus with the synapse structure formation which has a sensitive tetrodotoxin voltage-dependent sodium current. This fully proved that iPS cells can differentiate into retinal ganglion cells. In glaucoma and traumatic optic neuropathy, the regeneration of retinal ganglion cells may be the only way to restore vision. The finding that iPS cells can differentiate into retinal ganglion cells provides a new method for the treatment of such diseases.

Arnhold *et al.*<sup>[149]</sup> found that the cones cannot survive without healthy rod cells. Therefore, it is difficult to achieve the aim of the treatment by simply transplanting the induced iPS cells into the retina of patients with retinitis pigmentosa because the rod cells will eventually die.

### Stem cell treatment for retinitis pigmentosa:

Retinal pigment epithelium (RPE) is a single layer of epithelium with polarity and a rich pigment that is located between the neural retina and choriocapillaris layer. It can support the metabolism and activities of the retinal photoreceptor cells and phagocytose the outer segment photoreceptors. Recent studies show that the retinal pigment epithelium also has self-renewing stem cells that can be induced to form other cell types under suitable conditions.

RPE degeneration diseases caused by age-related macular degeneration (AMD), hereditary retinal degeneration, macular dystrophy and Stargardt disease result in the death of photoreceptors and neural retina and eventually cause blindness. There is currently no effective treatment for retinitis pigmentosa. The transplantation of the induced differentiated stem cells to establish the retinal pigment epithelium membrane *in vivo* has been studied extensively but it is still far from functional reconstruction. Thus, looking for new ways to stimulate the repair of RPE is an important research direction for the future. With the rise of regenerative medicine research, stem cell transplantation as a regenerative therapy becomes a hotspot for research. In the past decade, scholars induced bone marrow mesenchymal stem cells, iPS cells and embryonic stem cells to differentiate into retinal pigment epithelial cells but the process of differentiation into RPE cells is not clear and only a small fraction of cells can be differentiated into RPE cells. So, the research of finding the desired factors to directly induce cell differentiation is still very popular.

In 2010, Geron biopharmaceutical company (Geron, United States) sponsored the world's first clinical trial on using hESC to repair damaged nerves. Currently, the United States Food and Drug Administration has approved the ACT company (Advanced Stem Cell Technologies) to carry out two Phase II clinical trials on using embryonic stem cells to treat macular degeneration in the United States, dry AMD and juvenile macular dystrophy (Stargardt disease). This

time, they induced hESCs to differentiate into retinal epithelial cells with purity over 99%. Approximately 50000 retinal pigment epithelial cells were isolated and injected into the retina of two patients. Four months later, the researchers found that RPE had been completely replaced by the injected retinal epithelial cells. They measured the visual acuity of the two female patients and the data confirmed that the injected cells survived and largely improved their vision. Early data suggest that hESC therapy is not only safe but also efficient. The research paper was published in the world's oldest and most respected peer-reviewed medical journal, "The Lancet"<sup>[150]</sup>. These results from ACT have brought new optimistic hope for stem cell research. This same study found that it is crucial that the transplanted cells can attach to the Bruch's membrane and integrate into the host RPE layer and survive to have a successful therapeutic effect<sup>[149]</sup>.

## CONCLUSION

In summary, recent studies show that real cell therapy is not a simple supplement of cells. The interaction, interdependence, mutual promotion and supplementation between the transplanted cells and the microenvironment are more important. The microenvironment of the recipient can regulate the transplanted cell behavior and decide their fate. The transplanted cells cannot survive long-term without the support from the microenvironment of the recipient. The survived transplanted cells can not only completely replace the recipient's cells, but also supplement the sufficient quantity and function of the recipient's cells. More importantly, they are involved in the cellular microenvironment reconstruction so the stem cells obtain a stronger self-renewal capacity and proliferation ability. Therefore, in order to improve clinical cell therapy, we should pay more attention to the characteristics and components of each stem cell microenvironment and put efforts into understanding the regulation mechanisms of the stem cell microenvironment<sup>[151]</sup>.

## REFERENCES

1. Liu J, Song G, Wang Z, Huang B, Gao Q, Liu B, Xu Y, Liang X, Ma P, Gao N, Ge J. Establishment of a corneal epithelial cell line spontaneously derived from human limbal cells. *Exp Eye Res* 2007; **84**: 599-609 [PMID: 17223104 DOI: 10.1016/j.exer.2006.11.014]
2. Discher DE, Mooney DJ, Zandstra PW. Growth factors, matrices, and forces combine and control stem cells. *Science* 2009; **324**: 1673-1677 [PMID: 19556500 DOI: 10.1126/science.1171643]
3. Peerani R, Zandstra PW. Enabling stem cell therapies through synthetic stem cell-niche engineering. *J Clin Invest* 2010; **120**: 60-70 [PMID: 20051637 DOI: 10.1172/JCI41158]
4. Pera MF, Tam PP. Extrinsic regulation of pluripotent stem cells. *Nature* 2010; **465**: 713-720 [PMID: 20535200 DOI: 10.1038/nature09228]
5. Watt FM, Fujiwara H. Cell-extracellular matrix interactions in normal and diseased skin. *Cold Spring Harb Perspect Biol* 2011; **3**:



- a005124 [PMID: 21441589 DOI: 10.1101/cshperspect.a005124]
- 6 **Chou YF**, Chen HH, Eijpe M, Yabuuchi A, Chenoweth JG, Tesar P, Lu J, McKay RD, Geijsen N. The growth factor environment defines distinct pluripotent ground states in novel blastocyst-derived stem cells. *Cell* 2008; **135**: 449-461 [PMID: 18984157 DOI: 10.1016/j.cell.2008.08.035]
- 7 **Guo Y**, Graham-Evans B, Broxmeyer HE. Murine embryonic stem cells secrete cytokines/growth modulators that enhance cell survival/anti-apoptosis and stimulate colony formation of murine hematopoietic progenitor cells. *Stem Cells* 2006; **24**: 850-856 [PMID: 16339641 DOI: 10.1634/stemcells.2005-0457]
- 8 **Jiang XX**, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, Mao N. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005; **105**: 4120-4126 [PMID: 15692068 DOI: 10.1182/blood-2004-02-0586]
- 9 **Pearton DJ**, Yang Y, Dhoulally D. Transdifferentiation of corneal epithelium into epidermis occurs by means of a multistep process triggered by dermal developmental signals. *Proc Natl Acad Sci USA* 2005; **102**: 3714-3719 [PMID: 15738417 DOI: 10.1073/pnas.0500344102]
- 10 **Wang Z**, Ge J, Chen J, Huang B. [Preliminary experimental study on commitment differentiation of embryonic stem cells induced by corneal limbal stroma in vitro]. *Yanke Xuebao* 1999; **15**: 195-198 [PMID: 12579666]
- 11 **Wang Z**, Ge J, Huang B, Gao Q, Liu B, Wang L, Yu L, Fan Z, Lu X, Liu J. Differentiation of embryonic stem cells into corneal epithelium. *Sci China C Life Sci* 2005; **48**: 471-480 [PMID: 16315598 DOI: 10.1360/04yc0050]
- 12 **Gao N**, Wang Z, Huang B, Ge J, Lu R, Zhang K, Fan Z, Lu L, Peng Z, Cui G. Putative epidermal stem cell convert into corneal epithelium-like cell under corneal tissue in vitro. *Sci China C Life Sci* 2007; **50**: 101-110 [PMID: 17393090 DOI: 10.1007/s11427-007-0006-4]
- 13 **Zhang B**, Liu R, Shi D, Liu X, Chen Y, Dou X, Zhu X, Lu C, Liang W, Liao L, Zenke M, Zhao RC. Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2-dependent regulatory dendritic cell population. *Blood* 2009; **113**: 46-57 [PMID: 18832657 DOI: 10.1182/blood-2008-04-154138]
- 14 **Orkin SH**, Hochedlinger K. Chromatin connections to pluripotency and cellular reprogramming. *Cell* 2011; **145**: 835-850 [PMID: 21663790 DOI: 10.1016/j.cell.2011.05.019]
- 15 **Liu Y**, Ding Y, Ma P, Wu Z, Duan H, Liu Z, Wan P, Lu X, Xiang P, Ge J, Wang Z. Enhancement of long-term proliferative capacity of rabbit corneal epithelial cells by embryonic stem cell conditioned medium. *Tissue Eng Part C Methods* 2010; **16**: 793-802 [PMID: 19842914 DOI: 10.1089/ten.TEC.2009.0380]
- 16 **Zhan W**, Liu Z, Liu Y, Ke Q, Ding Y, Lu X, Wang Z. Modulation of rabbit corneal epithelial cells fate using embryonic stem cell extract. *Mol Vis* 2010; **16**: 1154-1161 [PMID: 20664691]
- 17 **Lu X**, Chen D, Liu Z, Li C, Liu Y, Zhou J, Wan P, Mou YG, Wang Z. Enhanced survival in vitro of human corneal endothelial cells using mouse embryonic stem cell conditioned medium. *Mol Vis* 2010; **16**: 611-622 [PMID: 20383337]
- 18 **Zhou J**, Chen F, Xiao J, Li C, Liu Y, Ding Y, Wan P, Wang X, Huang J, Wang Z. Enhanced functional properties of corneal epithelial cells by coculture with embryonic stem cells via the integrin  $\beta$ 1-FAK-PI3K/Akt pathway. *Int J Biochem Cell Biol* 2011; **43**: 1168-1177 [PMID: 21550417 DOI: 10.1016/j.biocel.2011.04.010]
- 19 **Ozbek S**, Balasubramanian PG, Chiquet-Ehrismann R, Tucker RP, Adams JC. The evolution of extracellular matrix. *Mol Biol Cell* 2010; **21**: 4300-4305 [PMID: 21160071 DOI: 10.1091/mbc.E10-03-0251]
- 20 **Watt FM**, Huck WT. Role of the extracellular matrix in regulating stem cell fate. *Nat Rev Mol Cell Biol* 2013; **14**: 467-473 [PMID: 23839578 DOI: 10.1038/nrm3620]
- 21 **Hynes RO**. The extracellular matrix: not just pretty fibrils. *Science* 2009; **326**: 1216-1219 [PMID: 19965464 DOI: 10.1126/science.1176009]
- 22 **Page-McCaw A**, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat Rev Mol Cell Biol* 2007; **8**: 221-233 [PMID: 17318226]
- 23 **Lu P**, Takai K, Weaver VM, Werb Z. Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harb Perspect Biol* 2011; **3**: a005058 [PMID: 21917992 DOI: 10.1101/cshperspect.a005058]
- 24 **Lu P**, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol* 2012; **196**: 395-406 [PMID: 22351925 DOI: 10.1083/jcb.201102147]
- 25 **Wu Z**, Zhou Y, Li N, Huang M, Duan H, Ge J, Xiang P, Wang Z. The use of phospholipase A(2) to prepare acellular porcine corneal stroma as a tissue engineering scaffold. *Biomaterials* 2009; **30**: 3513-3522 [PMID: 19321202 DOI: 10.1016/j.biomaterials.2009.03.003]
- 26 **Zhou Y**, Wu Z, Ge J, Wan P, Li N, Xiang P, Gao Q, Wang Z. Development and characterization of acellular porcine corneal matrix using sodium dodecylsulfate. *Cornea* 2011; **30**: 73-82 [PMID: 20861730 DOI: 10.1097/ICO.0b013e3181dc8184]
- 27 **Wu Z**, Zhou Q, Duan H, Wang X, Xiao J, Duan H, Li N, Li C, Wan P, Liu Y, Song Y, Zhou C, Huang Z, Wang Z. Reconstruction of auto-tissue-engineered lamellar cornea by dynamic culture for transplantation: a rabbit model. *PLoS One* 2014; **9**: e93012 [PMID: 24705327 DOI: 10.1371/journal.pone.0093012]
- 28 **Xiao J**, Duan H, Liu Z, Wu Z, Lan Y, Zhang W, Li C, Chen F, Zhou Q, Wang X, Huang J, Wang Z. Construction of the recellularized corneal stroma using porous acellular corneal scaffold. *Biomaterials* 2011; **32**: 6962-6971 [PMID: 21719100 DOI: 10.1016/j.biomaterials.2011.05.084]
- 29 **Liu Z**, Zhou Q, Zhu J, Xiao J, Wan P, Zhou C, Huang Z, Qiang N, Zhang W, Wu Z, Quan D, Wang Z. Using genipin-crosslinked acellular porcine corneal stroma for cosmetic corneal lens implants. *Biomaterials* 2012; **33**: 7336-7346 [PMID: 22795849 DOI: 10.1016/j.biomaterials.2012.06.080]
- 30 **Huang M**, Li N, Wu Z, Wan P, Liang X, Zhang W, Wang X, Li C, Xiao J, Zhou Q, Liu Z, Wang Z. Using acellular porcine limbal stroma for rabbit limbal stem cell microenvironment reconstruction. *Biomaterials* 2011; **32**: 7812-7821 [PMID: 21784513 DOI: 10.1016/j.biomaterials.2011.07.012]
- 31 **Nakayama KH**, Batchelder CA, Lee CI, Tarantal AF. Decellularized rhesus monkey kidney as a three-dimensional scaffold for renal tissue engineering. *Tissue Eng Part A* 2010; **16**: 2207-2216 [PMID: 20156112 DOI: 10.1089/ten.TEA.2009.0602]
- 32 **Ott HC**, Clippinger B, Conrad C, Schuetz C, Pomerantseva I, Ikonomou L, Kotton D, Vacanti JP. Regeneration and orthotopic transplantation of a bioartificial lung. *Nat Med* 2010; **16**: 927-933 [PMID: 20628374 DOI: 10.1038/nm.2193]
- 33 **Petersen TH**, Calle EA, Zhao L, Lee EJ, Gui L, Raredon MB, Gavrilov K, Yi T, Zhuang ZW, Breuer C, Herzog E, Niklason LE. Tissue-engineered lungs for in vivo implantation. *Science* 2010; **329**: 538-541 [PMID: 20576850 DOI: 10.1126/science.1189345]
- 34 **Uygun BE**, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F, Hertl M, Nahmias Y, Yarmush ML, Uygun K. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat Med* 2010; **16**: 814-820 [PMID: 20543851 DOI: 10.1038/nm.2170]
- 35 **Conrad C**, Schuetz C, Clippinger B, Vacanti JP, Markmann JF, Ott HC. Bio-engineered endocrine pancreas based on decellularized pancreatic matrix and mesenchymal stem cell/islet cell coculture. *J Am Coll Surgeons* 2010; **211**: S62 [DOI: 10.1016/j.jamcollsurg.2010.06.161]
- 36 **Song JJ**, Ott HC. Organ engineering based on decellularized matrix scaffolds. *Trends Mol Med* 2011; **17**: 424-432 [PMID: 21514224 DOI: 10.1016/j.molmed.2011.03.005]
- 37 **Soto-Gutierrez A**, Yagi H, Uygun BE, Navarro-Alvarez N, Uygun K, Kobayashi N, Yang YG, Yarmush ML. Cell delivery: from cell transplantation to organ engineering. *Cell Transplant* 2010; **19**: 655-665 [PMID: 20525441 DOI: 10.3727/096368910X508753]
- 38 **Blazewiska EA**, Schlötzer-Schrehardt U, Zenkel M, Bachmann B, Chankiewicz E, Jacobi C, Kruse FE. Corneal limbal



- microenvironment can induce transdifferentiation of hair follicle stem cells into corneal epithelial-like cells. *Stem Cells* 2009; **27**: 642-652 [PMID: 19074417 DOI: 10.1634/stemcells.2008-0721]
- 39 **Kurtz A**, Oh SJ. Age related changes of the extracellular matrix and stem cell maintenance. *Prev Med* 2012; **54** Suppl: S50-S56 [PMID: 22285947]
  - 40 **Legate KR**, Wickström SA, Fässler R. Genetic and cell biological analysis of integrin outside-in signaling. *Genes Dev* 2009; **23**: 397-418 [PMID: 19240129 DOI: 10.1101/gad.1758709]
  - 41 **Hynes RO**. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002; **110**: 673-687 [PMID: 12297042]
  - 42 **Barczyk M**, Carracedo S, Gullberg D. Integrins. *Cell Tissue Res* 2010; **339**: 269-280 [PMID: 19693543 DOI: 10.1007/s00441-009-0834-6]
  - 43 **Brizzi MF**, Tarone G, Defilippi P. Extracellular matrix, integrins, and growth factors as tailors of the stem cell niche. *Curr Opin Cell Biol* 2012; **24**: 645-651 [PMID: 22898530 DOI: 10.1016/j.ccb.2012.07.001]
  - 44 **Kim SH**, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. *J Endocrinol* 2011; **209**: 139-151 [PMID: 21307119 DOI: 10.1530/JOE-10-0377]
  - 45 **Buitenhuis M**. The role of PI3K/protein kinase B (PKB/c-akt) in migration and homing of hematopoietic stem and progenitor cells. *Curr Opin Hematol* 2011; **18**: 226-230 [PMID: 21519240 DOI: 10.1097/MOH.0b013e32834760e5]
  - 46 **Kanatsu-Shinohara M**, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, Raducanu A, Nakatsuji N, Fässler R, Shinohara T. Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. *Cell Stem Cell* 2008; **3**: 533-542 [PMID: 18983968 DOI: 10.1016/j.stem.2008.08.002]
  - 47 **Shen Q**, Wang Y, Kokovay E, Lin G, Chuang SM, Goderie SK, Roysam B, Temple S. Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. *Cell Stem Cell* 2008; **3**: 289-300 [PMID: 18786416 DOI: 10.1016/j.stem.2008.07.026]
  - 48 **Nakamura-Ishizu A**, Okuno Y, Omatsu Y, Okabe K, Morimoto J, Uede T, Nagasawa T, Suda T, Kubota Y. Extracellular matrix protein tenascin-C is required in the bone marrow microenvironment primed for hematopoietic regeneration. *Blood* 2012; **119**: 5429-5437 [PMID: 22553313 DOI: 10.1182/blood-2011-11-393645]
  - 49 **Kazanis I**, Belhadi A, Faissner A, Ffrench-Constant C. The adult mouse subependymal zone regenerates efficiently in the absence of tenascin-C. *J Neurosci* 2007; **27**: 13991-13996 [PMID: 18094237]
  - 50 **Potocnik AJ**, Brakebusch C, Fässler R. Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow. *Immunity* 2000; **12**: 653-663 [PMID: 10894165]
  - 51 **Qian H**, Tryggvason K, Jacobsen SE, Ekblom M. Contribution of alpha6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with alpha4 integrins. *Blood* 2006; **107**: 3503-3510 [PMID: 16439681 DOI: 10.1182/blood-2005-10-3932]
  - 52 **Grassinger J**, Haylock DN, Storan MJ, Haines GO, Williams B, Whitty GA, Vinson AR, Be CL, Li S, Sørensen ES, Tam PP, Denhardt DT, Sheppard D, Choong PF, Nilsson SK. Thrombin-cleaved osteopontin regulates hemopoietic stem and progenitor cell functions through interactions with alpha9beta1 and alpha4beta1 integrins. *Blood* 2009; **114**: 49-59 [PMID: 19417209 DOI: 10.1182/blood-2009-01-197988]
  - 53 **Schreiber TD**, Steil M, Essl M, Abele H, Geiger K, Müller CA, Aicher WK, Klein G. The integrin alpha9beta1 on hematopoietic stem and progenitor cells: involvement in cell adhesion, proliferation and differentiation. *Haematologica* 2009; **94**: 1493-1501 [PMID: 19608669 DOI: 10.3324/haematol.2009.006072]
  - 54 **Yoshihara H**, Arai F, Hosokawa K, Hagiwara T, Takubo K, Nakamura Y, Gomei Y, Iwasaki H, Matsuoka S, Miyamoto K, Miyazaki H, Takahashi T, Suda T. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell* 2007; **1**: 685-697 [PMID: 18371409 DOI: 10.1016/j.stem.2007.10.020]
  - 55 **Wang Z**, Li G, Tse W, Bunting KD. Conditional deletion of STAT5 in adult mouse hematopoietic stem cells causes loss of quiescence and permits efficient nonablative stem cell replacement. *Blood* 2009; **113**: 4856-4865 [PMID: 19258595 DOI: 10.1182/blood-2008-09-181107]
  - 56 **Umamoto T**, Yamato M, Ishihara J, Shiratsuchi Y, Utsumi M, Morita Y, Tsukui H, Terasawa M, Shibata T, Nishida K, Kobayashi Y, Petrich BG, Nakauchi H, Eto K, Okano T. Integrin- $\alpha\beta 3$  regulates thrombopoietin-mediated maintenance of hematopoietic stem cells. *Blood* 2012; **119**: 83-94 [PMID: 22096247 DOI: 10.1182/blood-2011-02-335430]
  - 57 **Raymond K**, Deugnier MA, Faraldo MM, Glukhova MA. Adhesion within the stem cell niches. *Curr Opin Cell Biol* 2009; **21**: 623-629 [PMID: 19535237 DOI: 10.1016/j.ccb.2009.05.004]
  - 58 **Marthiens V**, Kazanis I, Moss L, Long K, Ffrench-Constant C. Adhesion molecules in the stem cell niche—more than just staying in shape? *J Cell Sci* 2010; **123**: 1613-1622 [PMID: 20445012 DOI: 10.1242/jcs.054312]
  - 59 **Suh HN**, Han HJ. Collagen I regulates the self-renewal of mouse embryonic stem cells through  $\alpha 2 \beta 1$  integrin- and DDR1-dependent Bmi-1. *J Cell Physiol* 2011; **226**: 3422-3432 [PMID: 21344393 DOI: 10.1002/jcp.22697]
  - 60 **Chen S**, Lewallen M, Xie T. Adhesion in the stem cell niche: biological roles and regulation. *Development* 2013; **140**: 255-265 [PMID: 23250203 DOI: 10.1242/dev.083139]
  - 61 **Campos LS**, Decker L, Taylor V, Skarnes W. Notch, epidermal growth factor receptor, and beta1-integrin pathways are coordinated in neural stem cells. *J Biol Chem* 2006; **281**: 5300-5309 [PMID: 16332675 DOI: 10.1074/jbc.M511886200]
  - 62 **Briskien C**, Duss S. Stem cells and the stem cell niche in the breast: an integrated hormonal and developmental perspective. *Stem Cell Rev* 2007; **3**: 147-156 [PMID: 17873347 DOI: 10.1007/s12015-007-0019-1]
  - 63 **Jones RG**, Li X, Gray PD, Kuang J, Clayton F, Samowitz WS, Madison BB, Gumucio DL, Kuwada SK. Conditional deletion of beta1 integrins in the intestinal epithelium causes a loss of Hedgehog expression, intestinal hyperplasia, and early postnatal lethality. *J Cell Biol* 2006; **175**: 505-514 [PMID: 17088430 DOI: 10.1083/jcb.200602160]
  - 64 **Defilippi P**, Rosso A, Dentelli P, Calvi C, Garbarino G, Tarone G, Pegoraro L, Brizzi MF. {beta}1 Integrin and IL-3R coordinately regulate STAT5 activation and anchorage-dependent proliferation. *J Cell Biol* 2005; **168**: 1099-1108 [PMID: 15795318 DOI: 10.1083/jcb.200405116]
  - 65 **Uberti B**, Dentelli P, Rosso A, Defilippi P, Brizzi MF. Inhibition of  $\beta 1$  integrin and IL-3R $\beta$  common subunit interaction hinders tumour angiogenesis. *Oncogene* 2010; **29**: 6581-6590 [PMID: 20802515 DOI: 10.1038/onc.2010.384]
  - 66 **Margadant C**, Sonnenberg A. Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing. *EMBO Rep* 2010; **11**: 97-105 [PMID: 20075988 DOI: 10.1038/embor.2009.276]
  - 67 **Ivaska J**, Heino J. Cooperation between integrins and growth factor receptors in signaling and endocytosis. *Annu Rev Cell Dev Biol* 2011; **27**: 291-320 [PMID: 21663443 DOI: 10.1146/annurev-cellbio-092910-154017]
  - 68 **Kerever A**, Schnack J, Vellinga D, Ichikawa N, Moon C, Arikawa-Hirasawa E, Efrid JT, Mercier F. Novel extracellular matrix structures in the neural stem cell niche capture the neurogenic factor fibroblast growth factor 2 from the extracellular milieu. *Stem Cells* 2007; **25**: 2146-2157 [PMID: 17569787 DOI: 10.1634/stemcells.2007-0082]
  - 69 **Douet V**, Kerever A, Arikawa-Hirasawa E, Mercier F. Fractone-heparan sulphates mediate FGF-2 stimulation of cell proliferation in the adult subventricular zone. *Cell Prolif* 2013; **46**: 137-145 [PMID: 23510468 DOI: 10.1111/cpr.12023]
  - 70 **Cosgrove BD**, Sacco A, Gilbert PM, Blau HM. A home away from home: challenges and opportunities in engineering in vitro muscle satellite cell niches. *Differentiation* 2009; **78**: 185-194 [PMID: 19751902 DOI: 10.1016/j.diff.2009.08.004]



- 71 **Halder G**, Dupont S, Piccolo S. Transduction of mechanical and cytoskeletal cues by YAP and TAZ. *Nat Rev Mol Cell Biol* 2012; **13**: 591-600 [PMID: 22895435 DOI: 10.1038/nrm3416]
- 72 **Mammoto T**, Ingber DE. Mechanical control of tissue and organ development. *Development* 2010; **137**: 1407-1420 [PMID: 20388652 DOI: 10.1242/dev.024166]
- 73 **Mammoto A**, Mammoto T, Ingber DE. Mechanosensitive mechanisms in transcriptional regulation. *J Cell Sci* 2012; **125**: 3061-3073 [PMID: 22797927 DOI: 10.1242/jcs.093005]
- 74 **Hao J**, Zhang Y, Wang Y, Ye R, Qiu J, Zhao Z, Li J. Role of extracellular matrix and YAP/TAZ in cell fate determination. *Cell Signal* 2014; **26**: 186-191 [PMID: 24216612 DOI: 10.1016/j.cellsig.2013.11.006]
- 75 **Sun Y**, Chen CS, Fu J. Forcing stem cells to behave: a biophysical perspective of the cellular microenvironment. *Annu Rev Biophys* 2012; **41**: 519-542 [PMID: 22404680 DOI: 10.1146/annurev-biophys-042910-155306]
- 76 **DuFort CC**, Paszek MJ, Weaver VM. Balancing forces: architectural control of mechanotransduction. *Nat Rev Mol Cell Biol* 2011; **12**: 308-319 [PMID: 21508987 DOI: 10.1038/nrm3112]
- 77 **Engler AJ**, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell* 2006; **126**: 677-689 [PMID: 16923388 DOI: 10.1016/j.cell.2006.06.044]
- 78 **Winer JP**, Janmey PA, McCormick ME, Funaki M. Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. *Tissue Eng Part A* 2009; **15**: 147-154 [PMID: 18673086 DOI: 10.1089/ten.tea.2007.0388]
- 79 **Saha K**, Keung AJ, Irwin EF, Li Y, Little L, Schaffer DV, Healy KE. Substrate modulus directs neural stem cell behavior. *Biophys J* 2008; **95**: 4426-4438 [PMID: 18658232 DOI: 10.1529/biophysj.108.132217]
- 80 **Keung AJ**, de Juan-Pardo EM, Schaffer DV, Kumar S. Rho GTPases mediate the mechanosensitive lineage commitment of neural stem cells. *Stem Cells* 2011; **29**: 1886-1897 [PMID: 21956892 DOI: 10.1002/stem.746]
- 81 **Wei ZG**, Wu RL, Lavker RM, Sun TT. In vitro growth and differentiation of rabbit bulbar, fornix, and palpebral conjunctival epithelia. Implications on conjunctival epithelial transdifferentiation and stem cells. *Invest Ophthalmol Vis Sci* 1993; **34**: 1814-1828 [PMID: 8473120]
- 82 **Wei ZG**, Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells are preferentially located in corneal epithelium: implications on conjunctival epithelial homeostasis. *Invest Ophthalmol Vis Sci* 1995; **36**: 236-246 [PMID: 7822151]
- 83 **Wirtschafter JD**, McLoon LK, Ketcham JM, Weinstock RJ, Cheung JC. Palpebral conjunctival transient amplifying cells originate at the mucocutaneous junction and their progeny migrate toward the fornix. *Trans Am Ophthalmol Soc* 1997; **95**: 417-29; discussion 429-32 [PMID: 9440182]
- 84 **Harun MH**, Sepian SN, Chua KH, Ropilah AR, Abd Ghafar N, Che-Hamzah J, Bt Hj Idrus R, Annuar FH. Human forniceal region is the stem cell-rich zone of the conjunctival epithelium. *Hum Cell* 2013; **26**: 35-40 [PMID: 21748521 DOI: 10.1007/s13577-011-0025-0]
- 85 **Wei ZG**, Lin T, Sun TT, Lavker RM. Clonal analysis of the in vivo differentiation potential of keratinocytes. *Invest Ophthalmol Vis Sci* 1997; **38**: 753-761 [PMID: 9071229]
- 86 **Liu CY**, Zhu G, Westerhausen-Larson A, Converse R, Kao CW, Sun TT, Kao WW. Cornea-specific expression of K12 keratin during mouse development. *Curr Eye Res* 1993; **12**: 963-974 [PMID: 7508359 DOI: 10.3109/02713689309029222]
- 87 **Schermer A**, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986; **103**: 49-62 [PMID: 2424919 DOI: 10.1083/jcb.103.1.49]
- 88 **Wu RL**, Zhu G, Galvin S, Xu C, Haseba T, Chaloin-Dufau C, Dhoulailly D, Wei ZG, Lavker RM, Kao WY. Lineage-specific and differentiation-dependent expression of K12 keratin in rabbit corneal/limbal epithelial cells: cDNA cloning and northern blot analysis. *Differentiation* 1994; **55**: 137-144 [PMID: 7511548 DOI: 10.1046/j.1432-0436.1994.5520137.x]
- 89 **Cotsarelis G**, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell* 1989; **57**: 201-209 [PMID: 2702690 DOI: 10.1016/0092-8674(89)90958-6]
- 90 **Pellegrini G**, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P, De Luca M. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J Cell Biol* 1999; **145**: 769-782 [PMID: 10330405 DOI: 10.1083/jcb.145.4.769]
- 91 **Kruse FE**, Tseng SC. Serum differentially modulates the clonal growth and differentiation of cultured limbal and corneal epithelium. *Invest Ophthalmol Vis Sci* 1993; **34**: 2976-2989 [PMID: 7689546]
- 92 **Pellegrini G**, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F, De Luca M. p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* 2001; **98**: 3156-3161 [PMID: 11248048 DOI: 10.1073/pnas.061032098]
- 93 **Kasper M**. Patterns of cytokeratins and vimentin in guinea pig and mouse eye tissue: evidence for regional variations in intermediate filament expression in limbal epithelium. *Acta Histochem* 1992; **93**: 319-332 [PMID: 1382351 DOI: 10.1016/S0065-1281(11)80231-X]
- 94 **Lauweryns B**, van den Oord JJ, De Vos R, Missotten L. A new epithelial cell type in the human cornea. *Invest Ophthalmol Vis Sci* 1993; **34**: 1983-1990 [PMID: 7684031]
- 95 **Lauweryns B**, van den Oord JJ, Missotten L. The transitional zone between limbus and peripheral cornea. An immunohistochemical study. *Invest Ophthalmol Vis Sci* 1993; **34**: 1991-1999 [PMID: 8387976]
- 96 **Zieske JD**, Bukusoglu G, Yankauckas MA. Characterization of a potential marker of corneal epithelial stem cells. *Invest Ophthalmol Vis Sci* 1992; **33**: 143-152 [PMID: 1730535]
- 97 **Zieske JD**, Bukusoglu G, Yankauckas MA, Wasson ME, Keutmann HT. Alpha-enolase is restricted to basal cells of stratified squamous epithelium. *Dev Biol* 1992; **151**: 18-26 [PMID: 1577187 DOI: 10.1016/0012-1606(92)90209-Y]
- 98 **Pajooheh-Ganji A**, Ghosh SP, Stepp MA. Regional distribution of alpha9beta1 integrin within the limbus of the mouse ocular surface. *Dev Dyn* 2004; **230**: 518-528 [PMID: 15188436 DOI: 10.1002/dvdy.20050]
- 99 **Hoye AM**, Couchman JR, Wewer UM, Fukami K, Yoneda A. The newcomer in the integrin family: integrin  $\alpha 9$  in biology and cancer. *Adv Biol Regul* 2012; **52**: 326-339 [PMID: 22781746 DOI: 10.1016/j.jbior.2012.03.004]
- 100 **Chen Z**, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells* 2004; **22**: 355-366 [PMID: 15153612 DOI: 10.1634/stemcells.22-3-355]
- 101 **Watanabe K**, Nishida K, Yamato M, Umemoto T, Sumide T, Yamamoto K, Maeda N, Watanabe H, Okano T, Tano Y. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. *FEBS Lett* 2004; **565**: 6-10 [PMID: 15135043 DOI: 10.1016/j.febslet.2004.03.064]
- 102 **Budak MT**, Alpdogan OS, Zhou M, Lavker RM, Akinci MA, Wolosin JM. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci* 2005; **118**: 1715-1724 [PMID: 15811951 DOI: 10.1242/jcs.02279]
- 103 **de Paiva CS**, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells* 2005; **23**: 63-73 [PMID: 15625123 DOI: 10.1634/stemcells.2004-0093]
- 104 **Ksander BR**, Kolovou PE, Wilson BJ, Saab KR, Guo Q, Ma J, McGuire SP, Gregory MS, Vincent WJ, Perez VL, Cruz-Guilloty F, Kao WW, Call MK, Tucker BA, Zhan Q, Murphy GF, Lathrop KL, Alt C, Mortensen LJ, Lin CP, Zieske JD, Frank MH, Frank NY. ABCB5 is a limbal stem cell gene required for corneal development and repair. *Nature* 2014; **511**: 353-357 [PMID: 25030174 DOI: 10.1038/nature13426]



- 105 **Noisa P**, Ramasamy TS, Lamont FR, Yu JS, Sheldon MJ, Russell A, Jin X, Cui W. Identification and characterisation of the early differentiating cells in neural differentiation of human embryonic stem cells. *PLoS One* 2012; **7**: e37129 [PMID: 22615918 DOI: 10.1371/journal.pone.0037129]
- 106 **Naujok O**, Lenzen S. A critical re-evaluation of CD24-positivity of human embryonic stem cells differentiated into pancreatic progenitors. *Stem Cell Rev* 2012; **8**: 779-791 [PMID: 22529013 DOI: 10.1007/s12015-012-9362-y]
- 107 **Lehrer MS**, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci* 1998; **111** (Pt 19): 2867-2875 [PMID: 9730979]
- 108 **Notta F**, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 2011; **333**: 218-221 [PMID: 21737740 DOI: 10.1126/science.1201219]
- 109 **Townsend WM**. The limbal palisades of Vogt. *Trans Am Ophthalmol Soc* 1991; **89**: 721-756 [PMID: 1808821]
- 110 **Ecoffier T**, Yuen D, Chen L. Differential distribution of blood and lymphatic vessels in the murine cornea. *Invest Ophthalmol Vis Sci* 2010; **51**: 2436-2440 [PMID: 20019372 DOI: 10.1167/iovs.09-4505]
- 111 **Meller D**, Pires RT, Tseng SC. Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures. *Br J Ophthalmol* 2002; **86**: 463-471 [PMID: 11914219 DOI: 10.1136/bjo.86.4.463]
- 112 **Dua HS**, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol* 2005; **89**: 529-532 [PMID: 15834076 DOI: 10.1136/bjo.2004.049742]
- 113 **Shortt AJ**, Secker GA, Munro PM, Khaw PT, Tuft SJ, Daniels JT. Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells. *Stem Cells* 2007; **25**: 1402-1409 [PMID: 17332511 DOI: 10.1634/stemcells.2006-0580]
- 114 **Lee RE**, Davison PF. The collagens of the developing bovine cornea. *Exp Eye Res* 1984; **39**: 639-652 [PMID: 6394353 DOI: 10.1016/0014-4835(84)90063-0]
- 115 **Linsenmayer TF**, Bruns RR, Mentzer A, Mayne R. Type VI collagen: immunohistochemical identification as a filamentous component of the extracellular matrix of the developing avian corneal stroma. *Dev Biol* 1986; **118**: 425-431 [PMID: 3539660 DOI: 10.1016/0012-1606(86)90013-8]
- 116 **Linsenmayer TF**, Fitch JM, Mayne R. Extracellular matrices in the developing avian eye: type V collagen in corneal and noncorneal tissues. *Invest Ophthalmol Vis Sci* 1984; **25**: 41-47 [PMID: 6365824]
- 117 **Pratt BM**, Madri JA. Immunolocalization of type IV collagen and laminin in nonbasement membrane structures of murine corneal stroma. A light and electron microscopic study. *Lab Invest* 1985; **52**: 650-656 [PMID: 3892156]
- 118 **Funderburgh JL**, Chandler JW. Proteoglycans of rabbit corneas with nonperforating wounds. *Invest Ophthalmol Vis Sci* 1989; **30**: 435-442 [PMID: 2925315]
- 119 **Funderburgh JL**, Caterson B, Conrad GW. Keratan sulfate proteoglycan during embryonic development of the chicken cornea. *Dev Biol* 1986; **116**: 267-277 [PMID: 2942429 DOI: 10.1016/0012-1606(86)90130-2]
- 120 **Funderburgh JL**, Caterson B, Conrad GW. Distribution of proteoglycans antigenically related to corneal keratan sulfate proteoglycan. *J Biol Chem* 1987; **262**: 11634-11640 [PMID: 2957372]
- 121 **Hyldahl L**, Aspinall R, Watt FM. Immunolocalization of keratan sulphate in the human embryonic cornea and other human foetal organs. *J Cell Sci* 1986; **80**: 181-191 [PMID: 2941445]
- 122 **Hart GW**. Biosynthesis of glycosaminoglycans during corneal development. *J Biol Chem* 1976; **251**: 6513-6521 [PMID: 988023]
- 123 **Fitch JM**, Birk DE, Linsenmayer C, Linsenmayer TF. Stromal assemblies containing collagen types IV and VI and fibronectin in the developing embryonic avian cornea. *Dev Biol* 1991; **144**: 379-391 [PMID: 2010037 DOI: 10.1016/0012-1606(91)90430-B]
- 124 **Toole BP**, Trelstad RL. Hyaluronate production and removal during corneal development in the chick. *Dev Biol* 1971; **26**: 28-35 [PMID: 5111769 DOI: 10.1016/0012-1606(71)90104-7]
- 125 **Gipson IK**. The epithelial basement membrane zone of the limbus. *Eye (Lond)* 1989; **3** (Pt 2): 132-140 [PMID: 2515978 DOI: 10.1038/eye.1989.21]
- 126 **Kolega J**, Manabe M, Sun TT. Basement membrane heterogeneity and variation in corneal epithelial differentiation. *Differentiation* 1989; **42**: 54-63 [PMID: 2695378 DOI: 10.1111/j.1432-0436.1989.tb00607.x]
- 127 **Cleutjens JP**, Havenith MG, Kasper M, Vallinga M, Bosman FT. Absence of type IV collagen in the centre of the corneal epithelial basement membrane. *Histochem J* 1990; **22**: 688-694 [PMID: 2079442 DOI: 10.1007/BF01047454]
- 128 **Schlötzer-Schrehardt U**, Dietrich T, Saito K, Sorokin L, Sasaki T, Paulsson M, Kruse FE. Characterization of extracellular matrix components in the limbal epithelial stem cell compartment. *Exp Eye Res* 2007; **85**: 845-860 [PMID: 17927980 DOI: 10.1016/j.exer.2007.08.020]
- 129 **Kabosova A**, Azar DT, Bannikov GA, Campbell KP, Durbeek M, Ghohestani RF, Jones JC, Kenney MC, Koch M, Ninomiya Y, Patton BL, Paulsson M, Sado Y, Sage EH, Sasaki T, Sorokin LM, Steiner-Champlaud MF, Sun TT, Sundararaj N, Timpl R, Virtanen I, Ljubimov AV. Compositional differences between infant and adult human corneal basement membranes. *Invest Ophthalmol Vis Sci* 2007; **48**: 4989-4999 [PMID: 17962449 DOI: 10.1167/iovs.07-0654]
- 130 **Ljubimov AV**, Burgeson RE, Butkowski RJ, Michael AF, Sun TT, Kenney MC. Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. *Lab Invest* 1995; **72**: 461-473 [PMID: 7723285]
- 131 **Yu WY**, Sheridan C, Grierson I, Mason S, Kearns V, Lo AC, Wong D. Progenitors for the corneal endothelium and trabecular meshwork: a potential source for personalized stem cell therapy in corneal endothelial diseases and glaucoma. *J Biomed Biotechnol* 2011; **2011**: 412743 [PMID: 22187525 DOI: 10.1155/2011/412743]
- 132 **Li GG**, Chen SY, Xie HT, Zhu YT, Tseng SC. Angiogenesis potential of human limbal stromal niche cells. *Invest Ophthalmol Vis Sci* 2012; **53**: 3357-3367 [PMID: 22538425 DOI: 10.1167/iovs.11-9414]
- 133 **Zhou M**, Leiberman J, Xu J, Lavker RM. A hierarchy of proliferative cells exists in mouse lens epithelium: implications for lens maintenance. *Invest Ophthalmol Vis Sci* 2006; **47**: 2997-3003 [PMID: 16799045 DOI: 10.1167/iovs.06-0130]
- 134 **Yamamoto N**, Majima K, Marunouchi T. A study of the proliferating activity in lens epithelium and the identification of tissue-type stem cells. *Med Mol Morphol* 2008; **41**: 83-91 [PMID: 18592162 DOI: 10.1007/s00795-008-0395-x]
- 135 **Remington SG**, Meyer RA. Lens stem cells may reside outside the lens capsule: an hypothesis. *Theor Biol Med Model* 2007; **4**: 22 [PMID: 17559656 DOI: 10.1186/1742-4682-4-22]
- 136 **Perron M**, Harris WA. Retinal stem cells in vertebrates. *Bioessays* 2000; **22**: 685-688 [PMID: 10918298 DOI: 10.1002/1521-1878(200008)22:8<685::AID-BIES1>3.0.CO;2-C]
- 137 **von Leithner PL**, Ciurtin C, Jeffery G. Microscopic mammalian retinal pigment epithelium lesions induce widespread proliferation with differences in magnitude between center and periphery. *Mol Vis* 2010; **16**: 570-581 [PMID: 20360994]
- 138 **Tropepe V**, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, McInnes RR, van der Kooy D. Retinal stem cells in the adult mammalian eye. *Science* 2000; **287**: 2032-2036 [PMID: 10720333 DOI: 10.1126/science.287.5460.2032]
- 139 **Lavker RM**, Tseng SC, Sun TT. Corneal epithelial stem cells at the limbus: looking at some old problems from a new angle. *Exp Eye Res* 2004; **78**: 433-446 [PMID: 15106923 DOI: 10.1016/j.exer.2003.09.008]
- 140 **Shimazaki J**, Kaido M, Shinozaki N, Shimmura S, Munkhbat B, Hagihara M, Tsuji K, Tsubota K. Evidence of long-term survival of donor-derived cells after limbal allograft transplantation. *Invest Ophthalmol Vis Sci* 1999; **40**: 1664-1668 [PMID: 10393033]

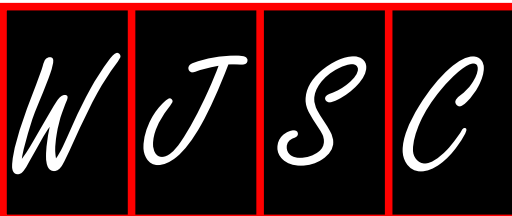


- 141 **Williams KA**, Brereton HM, Aggarwal R, Sykes PJ, Turner DR, Russ GR, Coster DJ. Use of DNA polymorphisms and the polymerase chain reaction to examine the survival of a human limbal stem cell allograft. *Am J Ophthalmol* 1995; **120**: 342-350 [PMID: 7661206 DOI: 10.1016/S0002-9394(14)72164-6]
- 142 **Mills JC**, Gordon JI. The intestinal stem cell niche: there grows the neighborhood. *Proc Natl Acad Sci USA* 2001; **98**: 12334-12336 [PMID: 11675485 DOI: 10.1073/pnas.231487198]
- 143 **Zeng Y**, Yang J, Huang K, Lee Z, Lee X. A comparison of biomechanical properties between human and porcine cornea. *J Biomech* 2001; **34**: 533-537 [PMID: 11266678 DOI: 10.1016/S0021-9290(00)00219-0]
- 144 **Liu Z**, Wan P, Duan H, Zhou J, Tan B, Liu Y, Zhou Q, Zhou C, Huang Z, Tian B, Li C, Wang Z. ES micro-environment enhances stemness and inhibits apoptosis in human limbal stem cells via the maintenance of telomerase activity. *PLoS One* 2013; **8**: e53576 [PMID: 23326460 DOI: 10.1371/journal.pone.0053576]
- 145 **Sellaro TL**, Ravindra AK, Stolz DB, Badyak SF. Maintenance of hepatic sinusoidal endothelial cell phenotype in vitro using organ-specific extracellular matrix scaffolds. *Tissue Eng* 2007; **13**: 2301-2310 [PMID: 17561801 DOI: 10.1089/ten.2006.0437]
- 146 **Ballios BG**, Clarke L, Coles BL, Shoichet MS, Van Der Kooy D. The adult retinal stem cell is a rare cell in the ciliary epithelium whose progeny can differentiate into photoreceptors. *Biol Open* 2012; **1**: 237-246 [PMID: 23213414 DOI: 10.1242/bio.2012027]
- 147 **Meyer JS**, Katz ML, Maruniak JA, Kirk MD. Embryonic stem cell-derived neural progenitors incorporate into degenerating retina and enhance survival of host photoreceptors. *Stem Cells* 2006; **24**: 274-283 [PMID: 16123383 DOI: 10.1634/stemcells.2005-0059]
- 148 **Parameswaran S**, Balasubramanian S, Babai N, Qiu F, Eudy JD, Thoreson WB, Ahmad I. Induced pluripotent stem cells generate both retinal ganglion cells and photoreceptors: therapeutic implications in degenerative changes in glaucoma and age-related macular degeneration. *Stem Cells* 2010; **28**: 695-703 [PMID: 20166150 DOI: 10.1002/stem.320]
- 149 **Arnhold S**, Absenger Y, Klein H, Addicks K, Schraermeyer U. Transplantation of bone marrow-derived mesenchymal stem cells rescue photoreceptor cells in the dystrophic retina of the rhodopsin knockout mouse. *Graefes Arch Clin Exp Ophthalmol* 2007; **245**: 414-422 [PMID: 16896916 DOI: 10.1007/s00417-006-0382-7]
- 150 **Schwartz SD**, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, Klimanskaya I, Lanza R. Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet* 2012; **379**: 713-720 [PMID: 22281388 DOI: 10.1016/S0140-6736(12)60028-2]
- 151 **Chen FM**, Wu LA, Zhang M, Zhang R, Sun HH. Homing of endogenous stem/progenitor cells for in situ tissue regeneration: Promises, strategies, and translational perspectives. *Biomaterials* 2011; **32**: 3189-3209 [PMID: 21300401 DOI: 10.1016/j.biomaterials.2010.12.032]

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## Using induced pluripotent stem cells as a tool for modelling carcinogenesis

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### Abstract

Cancer is a highly heterogeneous group of diseases that despite improved treatments remain prevalent accounting for over 14 million new cases and 8.2 million deaths per year. Studies into the process of carcinogenesis are limited by lack of appropriate models for the development and pathogenesis of the disease based on human tissues. Primary culture of patient samples can help but is difficult to grow for a number of tissues. A potential opportunity to overcome these barriers is based on the landmark study by Yamanaka which demonstrated the ability of four factors;

Oct4, Sox2, Klf4, and c-Myc to reprogram human somatic cells in to pluripotency. These cells were termed induced pluripotent stem cells (iPSCs) and display characteristic properties of embryonic stem cells. This technique has a wide range of potential uses including disease modelling, drug testing and transplantation studies. Interestingly iPSCs also share a number of characteristics with cancer cells including self-renewal and proliferation, expression of stem cell markers and altered metabolism. Recently, iPSCs have been generated from a number of human cancer cell lines and primary tumour samples from a range of cancers in an attempt to recapitulate the development of cancer and interrogate the underlying mechanisms involved. This review will outline the similarities between the reprogramming process and carcinogenesis, and how these similarities have been exploited to generate iPSC models for a number of cancers.

**Key words:** Induced pluripotent stem cells; Cancer; Model; Reprogramming

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**Core tip:** Human induced pluripotent stem cells (iPSCs) represent a novel method for studying the mechanisms of cancer development and progression. Recently, a number of studies have generated iPSCs from human cancer cells and cell lines, which can then be used as a model for carcinogenesis. This review outlines the similarities that exist between pluripotent and malignant cells and summarizes available studies that have generated iPSC models of cancer.

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## INTRODUCTION

Cancer is a heterogeneous group of diseases that accounts for an estimated 14.1 million new cases and 8.2 million deaths annually, with the number of cases expected to increase to 24 million annually by 2035<sup>[1]</sup>. Surgical removal, chemotherapy and/or radiotherapy have become the mainstay treatment for cancer. In recent decades research has focused on improving current drugs and developing targeted therapies specific to the defining biological properties of tumours. Despite the development of these therapeutic interventions, the death rate from cancer remains high. One major reason for this is the limitations of current representative pre-clinical models for human carcinogenesis, including the difficulty in growing and expanding primary cultures. Induced pluripotent stem cells (iPSCs) offer a relevant and unlimited system to study the development and progression of cancer. This review will discuss the current models for carcinogenesis, similarities between cancer and stem cells, and recent iPSC models of human cancers.

## CURRENT MODELS

Cell lines developed from human tumours are often used to study carcinogenesis. These are inexpensive and can be maintained over lengthy periods; however prolonged culture can alter the characteristics of cells resulting in them becoming less representative of primary tumours<sup>[2]</sup>. Alterations in gene expression with prolonged *in vitro* culture are also associated with the use of cell lines, with studies using microarray data from snap-frozen normal human tissue, primary tumour biopsy tissue and tumour-derived cell lines identifying that only 2% of tissue specific and 5% of tumour specific genes were expressed when compared to their equivalent tissue or tumour<sup>[3]</sup>. Primary cell cultures better represent inter-patient heterogeneity which exists due to differences in tumorigenic cell properties and numbers, variation in cell of origin and frequency of mutations<sup>[4,5]</sup>, however cultures have a limited life span and are difficult to obtain, maintain and expand. A number of animal models for carcinogenesis exist and have greatly increased our knowledge of cancer. However, animal models are not fully representative of carcinogenesis in the human setting due to inherent species differences including organism size and longevity as well as cancer susceptibility<sup>[6]</sup>.

## STEM CELLS IN DISEASE MODELLING

Stem cells are defined by their ability for self-renewal and differentiation into a range of cell types. Their ability to replicate indefinitely overcomes the limitations of current human tissue models as they are able to generate a limitless supply of human cells. Somatic stem cells are present within many organs

and are defined by their ability for both self-renewal and differentiation to maintain homeostasis<sup>[7]</sup>. These cells could be used to model development and disease; however adult stem cells comprise rare populations that are not easily identifiable.

The first human embryonic stem cell line was derived from human blastocysts in 1998 by Thomson *et al*<sup>[8]</sup>. Stem cells have two defining features; self-renewal and indefinite proliferation, meaning a limitless supply of tissue can be derived from these cells. Due to these properties, it is hoped that stem cells can be used as a system for disease modelling and drug discovery. However, research using human embryonic stem cells (hESCs) is hampered due to the ethical issues surrounding ESCs and the stringent restrictions enforced as a result.

An alternative to hESCs and adult stem cells are induced pluripotent stem cells (iPSCs), generated in a landmark study by Takahashi *et al*<sup>[9]</sup>. From a screen of 24 candidates, 4 factors were identified which were able to reprogram mouse somatic cells to pluripotency; Oct3/4, Sox2, c-Myc and Klf4. These cells showed characteristics of ESCs including morphology, marker expression and the ability to form all three embryonic germ layers<sup>[9]</sup>. Subsequently, the same four factors were shown to also have the ability to reprogram human adult dermal fibroblasts to iPSCs<sup>[10]</sup>. An alternative cocktail of factors consisting of Oct4, Sox2, Nanog and Lin28 was also shown to generate iPSCs from human fibroblasts<sup>[11]</sup>. Human iPSCs possess a number of features that are typical of ESCs including self-renewal and expression of ESC marker genes. Importantly, as for hESCs, iPSCs have the ability to differentiate both *in vitro*, via the formation of embryoid bodies (EBs) comprising all three germ layers and *in vivo*, as demonstrated by the formation of teratomas<sup>[10]</sup>. The use of iPSCs also resolves the ethical issues associated with hESCs, a controversial topic due to the use of blastocyst stage human embryos to derive the cells<sup>[12]</sup>. As iPSCs can be generated from somatic cells, they also show great potential for developing patient specific models of diseases, which can be used to study the underlying mechanisms of disease development and the efficacy of treatments<sup>[13]</sup>.

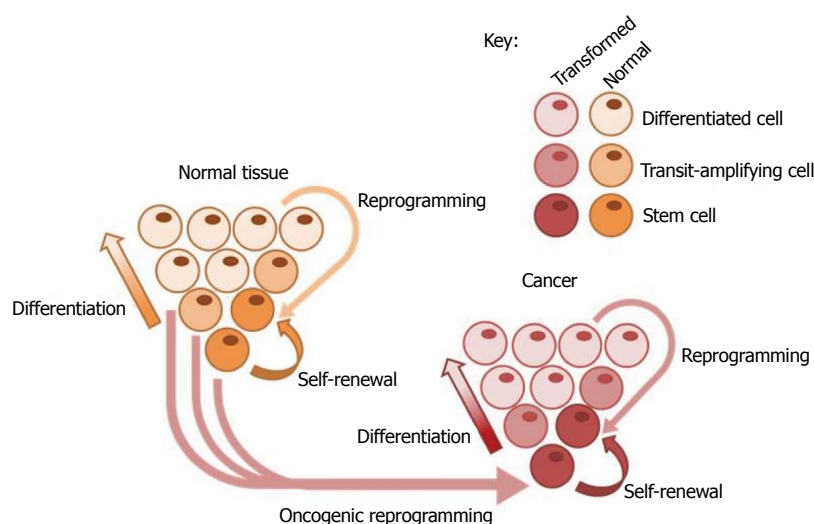
## REPROGRAMMING VS CARCINOGENESIS

A number of similarities exist between the processes of reprogramming and carcinogenesis. Cancer cells have a number of defined characteristics including sustained proliferative signaling and replicative immortality<sup>[14]</sup>. Stem cells also possess this intrinsic ability for both self-renewal and proliferation, highlighting their similarity to cancer cells.

### Cancer stem cells

Tumours, like normal tissue, are heterogeneous populations of cells, varying in phenotype, function





**Figure 1** Schematic illustrating normal stem cell expansion and the cancer stem cell model. Normal stem, transit-amplifying or differentiated cells can undergo oncogenic reprogramming resulting in generation of cancer stem cells (CSCs). Non-CSCs also demonstrate plasticity and can dedifferentiate to CSCs.

and gene expression<sup>[15]</sup>. Furthermore, studies from a number of cancers have shown that not all cells can regenerate tumours upon injection into immunodeficient mice, a functional assay which is now used to identify cells termed cancer stem cells (CSCs)<sup>[16]</sup>. CSCs can be defined as tumour cells which have the ability for both self-renewal to maintain the stem cell pool, and differentiation to the heterogeneous cell types which maintain the tumour<sup>[17]</sup>, and therefore share the quintessential properties of normal stem cells (Figure 1). Importantly, due to these properties, CSCs which survive chemotherapy are able to re-establish tumours<sup>[18]</sup>. Whilst the origin of CSCs is not fully known, it has been suggested that CSCs could arise as a result of mutations in stem, transit-amplifying, differentiated or cancer cells leading to acquisition of malignant and/or stem cell properties<sup>[19]</sup>. Understanding the process by which CSCs are generated and how they maintain tumours is critical to develop therapies which target this cell population and therefore prevent tumour recurrence.

CSCs were originally identified in acute myeloid leukemia, however putative CSCs have now been identified in solid cancers including breast, colon, brain, prostate, and lung cancer<sup>[20]</sup>. These cells could be used to study tumours however they are difficult to isolate, particularly in solid tumours which are very heterogeneous and therefore are not fully represented by tissue samples taken from a single site within the tumour<sup>[21]</sup>. Reprogramming of primary cells of interest derived from patients into iPSCs could allow generation of disease- and patient-specific models to study carcinogenesis and to further interrogate the CSC model. An interesting addition to the CSC model is the concept of tumour cell plasticity. A number of studies have shown that non-CSCs can dedifferentiate and acquire properties of stem cells, converting them to CSCs which can then maintain the tumour. In colorectal cancer, Wnt signaling induced by the tumour microenvironment activates CSC properties in differentiated cancer cells<sup>[22]</sup>, whilst transformed

human mammary epithelial cells can spontaneously convert to cells with a CSC phenotype both *in vitro* and *in vivo*<sup>[23]</sup>.

#### **Cell reprogramming, clonal expansion and evolution**

Carcinogenesis is a multi-step process consisting of initiation, promotion, conversion to malignancy and progression<sup>[24]</sup>. Cancer is known to undergo clonal expansion throughout this process, resulting from an initial change or mutation in a single cell which confers a growth advantage allowing for enhanced proliferation and expansion of this population. Further mutations then occur within cells forming subpopulations which expand and continue to accumulate changes and mutations which further alter proliferation, cause loss of differentiation and enhance invasive potential, resulting in a malignancy which is genetically and phenotypically divergent from the normal tissue counterpart<sup>[25]</sup>. Clonality is also a defining feature of stem cells, and is often used to measure the ability of such cells to self-renew<sup>[26]</sup>.

#### **Role of epithelial-mesenchymal transition and its significance**

Epithelial-mesenchymal transition (EMT) is defined as a conversion of cells from an epithelial to mesenchymal phenotype with loss of epithelial properties such as apical-basal polarization and cell-cell adhesion and gain of mesenchymal properties such as motility, degradation of the extracellular matrix and resulting invasiveness<sup>[27,28]</sup>. This process is critical for normal development- during gastrulation for formation of the mesoderm, and also at subsequent stages such as neural crest formation and heart development<sup>[29]</sup>. Similarly, EMT occurs during differentiation of ESCs<sup>[30]</sup>, whilst its opposing process mesenchymal-epithelial transition is necessary to reprogram cells to pluripotency<sup>[31]</sup>. EMT has also been implicated in cancer with roles in a number of cancer hallmarks. The EMT inducers SNAIL1 and SNAIL2 correlate with relapse and survival in breast, ovarian and colorectal



cancers, suggesting that EMT is associated with poor outcome<sup>[28]</sup>. Expression of EMT genes is also associated with cancer progression as these expression profiles are identified at the invasive front of a number of cancers including colon carcinoma, papillary thyroid carcinoma and some breast carcinomas<sup>[28]</sup>. Furthermore, an EMT expression profile was shown to associate with development of metastasis in cutaneous malignant melanoma<sup>[32]</sup>.

TGF $\beta$ , an inducer of EMT, has a dual role in cancer, acting as a tumour suppressor and activator of apoptosis whilst also promoting immune tolerance, invasion and metastasis<sup>[33]</sup>. TWIST1/2 proteins, which also act as EMT inducers, are increased in a number of human cancers and prevent senescence in cancer cells by inhibition of the p53 and Rb pathways<sup>[34]</sup>. Finally, EMT has also been implicated in resistance to chemotherapies, with evidence that a number of chemoresistant cell lines are induced to undergo EMT<sup>[35,36]</sup>.

### Role of reprogramming factors in cancer

Transcription factors which play a critical role in reprogramming cells to pluripotency have also been identified in human cancers. Oct4 is a transcription factor with roles in embryogenesis<sup>[37]</sup> which is expressed in all testicular germ cell tumours and also pre-malignant carcinoma *in situ* lesions<sup>[38]</sup>, as well as in breast, pancreas and colon cancer cDNA panels<sup>[39]</sup>. Furthermore, induced expression of Oct4 in the somatic cells of mice results in epithelial dysplasia, providing further evidence of a role for stem cell genes in carcinogenesis<sup>[40]</sup>.

Immunohistochemical staining of a panel of oral squamous cell carcinoma (OSCC) patients identified an increased incidence of Oct4 expression with advanced stages of the disease, with enhanced nuclear staining in higher grade oral cancers<sup>[41]</sup>. Transduction of primary human breast epithelial cultures with Oct4 generated colonies with self-renewal capacity, which similarly to oral squamous cells with overexpressed Oct4 showed enhanced expression of mesenchymal markers and a loss of epithelial markers. These cells were also highly tumorigenic, forming poorly differentiated, high grade tumours after injection into nude mice<sup>[42]</sup>. Oct4 has also been implicated in cervical cancer with increasing numbers of Oct4 expressing cells in both carcinoma *in situ* and invasive cervical cancer compared to normal cervical tissue, suggesting a role for Oct4 in the development of cervical cancer. Overexpression of Oct4 in cervical cancer cell lines resulted in increased tumour volume and weight upon injection into mice, and decreased apoptosis both *in vitro* and *in vivo* compared to controls<sup>[43]</sup>.

MYC encodes the Myc transcription factor which regulates genes involved in cell growth and proliferation<sup>[44]</sup>. Myc is a known oncogene and one of the most commonly altered genes in human cancers with copy number

alterations in 14% of cancer samples<sup>[45]</sup>. Translocation of c-Myc from chromosome 8 to chromosome 2, 14 or 22 in Burkitt lymphoma cells was the first evidence for the role of Myc in cancer development<sup>[46]</sup>. Myc translocation is also common in multiple myeloma where the oncogene is fused to an IgH or IgL locus in early carcinogenesis<sup>[47]</sup>. Abnormalities at the c-Myc or I-Myc locus have been identified in 19/20 multiple myeloma cell lines and 50% of advanced primary cancers<sup>[48]</sup>. Finally, Myc has also been implicated as a downstream target of deregulated Notch signalling which is apparent in T cell leukaemia, with the two molecules concurrently regulating leukaemic cell growth and proliferation<sup>[49]</sup>.

Myc has also been implicated in solid tumours. c-Myc amplification occurs in 25% of primary breast carcinomas with protein overexpression in 45% of breast tumours, and was significantly correlated with poorly differentiated and highly proliferative tumours<sup>[50]</sup>. Overexpression of c-Myc mRNA is also thought to occur in 60%-80% of human colorectal adenocarcinomas<sup>[51]</sup> and has been shown to enhance colon cancer cell angiogenesis *via* inhibition of HIF-1 $\alpha$  degradation and promotion of VEGF expression<sup>[52]</sup>.

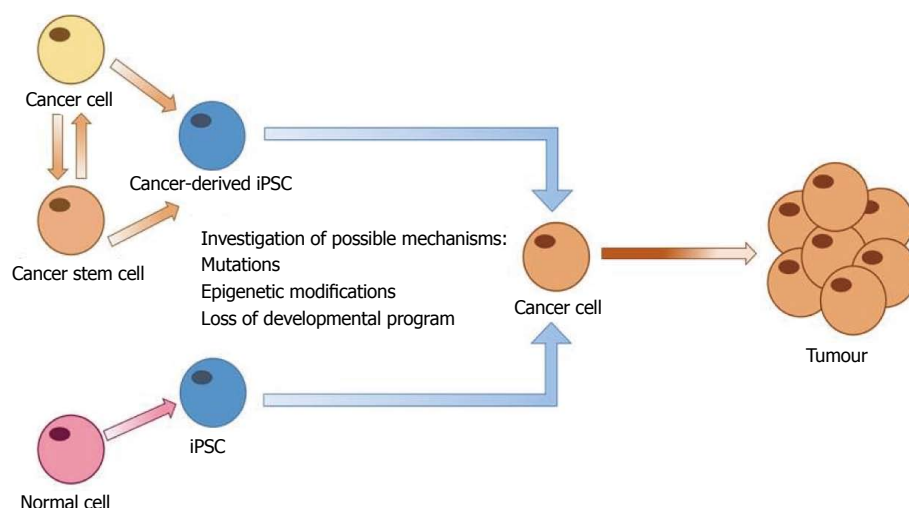
Myc is overexpressed or activated in over 50% of human cancers, but in most human cells cannot independently induce tumorigenesis, instead cooperating with other events such as loss of p53 or overexpression of Bcl2 to bypass normal cell checkpoints and initiate carcinogenesis<sup>[53]</sup>. Interestingly, the level of Myc expression has been shown to produce varying effects, with low level deregulated Myc resulting in proliferation alone whilst high levels of Myc overexpression are required to activate tumour suppressor mechanisms such as ARF induction leading to cellular apoptosis<sup>[54]</sup>.

As is the case with Oct4, Nanog expression correlates with poor survival in OSCC and also in nasopharyngeal carcinoma if co-expressed with Oct4<sup>[41,55]</sup>. High levels of Nanog expression are also present in breast, lung, ovarian and colon cancer cell lines, and furthermore in cervical cancer Nanog expression increases with progression from cervical intraepithelial neoplasia to invasive cervical carcinoma and is associated with poorer prognosis. Inhibition of Nanog by siRNA decreased proliferation in a mouse model of colon cancer, implicating Nanog as a potential therapeutic target in the disease<sup>[56]</sup>.

Sox2 is contained within the most significantly amplified peak in lung and esophageal squamous cell carcinoma (SSC), identifying it as an oncogene for these cancers<sup>[57]</sup>. Sox2 is also expressed in both mouse and human pre-neoplastic skin lesions and squamous cell carcinomas, but not in normal epidermis. Deletion of Sox2 in melanoma or SSC caused regression of tumours, and identified a number of genes involved in proliferation, stemness and cell survival which are regulated by Sox2, providing further evidence of a role for Sox2 in carcinogenesis<sup>[58]</sup>.

A number of studies have suggested a role for





**Figure 2** Generation of induced pluripotent stem cells from both normal and malignant patient tissue could be used to model carcinogenesis and elucidate the mechanisms which underlie the stages of cancer development. iPSC: Induced pluripotent stem cell.

typical stem cell genes in carcinogenesis. An 11-gene stem cell-like signature found in primary prostate tumours can be used as a predictive tool for prostate, breast and lung cancer patients, amongst other cancer types<sup>[59]</sup>. Construction of a gene module map by Chang *et al.*<sup>[60]</sup> identified modules specific to adult and murine ESCs and found that the ESC module was activated in human epithelial cancers. Activation of these genes was associated with poorly differentiated breast tumours with an increased risk of metastasis and death, and was also associated with an increased risk of death in lung adenocarcinomas. The c-Myc oncogene was subsequently shown to induce the ESC-like module both *in vitro* and *in vivo*<sup>[60]</sup>, consistent with the well-described role of c-Myc in stem cell self-renewal<sup>[61]</sup>. An ESC signature developed by Weinberg's group showed enrichment of stem cell genes in poorly differentiated and larger breast tumours and again this was associated with a poorer survival. The ESC signature was also associated with high grade gliomas and bladder carcinomas<sup>[62]</sup>.

### Metabolism

Altered metabolism has been implicated in cancer due to the "Warburg effect" whereby cancer cells use glycolysis for energy production rather than mitochondrial oxidative phosphorylation<sup>[63]</sup>. Aerobic glycolysis occurs in cultured cancer cells and pluripotent stem cells<sup>[64]</sup>. This is mediated by uncoupling proteins (UCPs) which uncouple oxidative phosphorylation from glycolysis, including UCP2 which is thought to be important for pluripotency<sup>[65]</sup> and is also increased in most human color cancers<sup>[66]</sup> as well as showing high expression in 90% of ovarian carcinomas and 94% of breast carcinomas<sup>[67]</sup>. Recent studies have found that during induction of pluripotency in somatic cells, genes involved in glycolysis are upregulated whilst those involved in oxidative pathways are downregulated, mediating a switch from oxidative phosphorylation to glycolysis<sup>[68]</sup> in a similar manner to that seen in carcinogenesis.

## CURRENT MODELS OF CARCINOGENESIS

The generation of iPSCs from human somatic cells heralds a new era in disease modelling, allowing the development of patient specific models. As previously mentioned, despite improvements in cancer treatment the disease is still a major cause of morbidity and mortality. The lack of a relevant model to study the development of cancers and their progression has limited research which is suitable for translation to the clinical setting. Generation of iPSCs from human cancer cells represents an opportunity to develop *in vitro* models of carcinogenesis for specific cancer types (Figure 2).

Recently, a number of studies have generated iPSCs from cancer cells with the hope of developing such a model (summarized in Table 1). One of the earliest attempts to generate iPSCs from malignant cells used retroviral transduction of Oct3/4, Sox2, Klf4 and c-Myc into cancer cell lines from pancreatic, liver, stomach and colorectal cancers. These cancer-derived iPSCs had slower proliferation and increased sensitivity to chemotherapeutic agents in comparison to their parental cells<sup>[69]</sup>.

### iPSCs and chronic myeloid leukemia

Carette *et al.*<sup>[70]</sup> generated iPSCs from the KBM7 chronic myeloid leukemia (CML) cell line. Interestingly, despite the sensitivity of the KBM7 cell line to imatinib, the iPSCs generated lost their BCR-ABL dependence and became resistant to imatinib<sup>[70]</sup>, the tyrosine kinase inhibitor which targets the BCR-ABL protein which defines CML<sup>[71]</sup>. In a further study, Kumano *et al.*<sup>[72]</sup> derived iPSCs from primary cultures from two CML patients and showed stem cell morphology and markers along with the ability to differentiate into haematopoietic progenitors which expressed the BCR-ABL fusion protein. Again, these iPSCs were generated from imatinib-sensitive patients but became resistant. Once differentiated, immature cells (CD34<sup>+</sup>38<sup>+</sup>90<sup>+</sup>45<sup>+</sup>) were identified which were resistant



**Table 1** Current induced pluripotent stem cells models of carcinogenesis

Malignancy	Study	Ref.
PDAC	Model of early PDAC and progression	Kim <i>et al</i> <sup>[73]</sup> , 2013
CML	iPSCs from CML cell lines and primary patient samples	Carette <i>et al</i> <sup>[70]</sup> , 2010 Kumano <i>et al</i> <sup>[72]</sup> , 2012
JML	Derived iPSCs from 2 JML patients	Gandre-Babbe <i>et al</i> <sup>[73]</sup> , 2013
Gastrointestinal cancer	Generated iPSCs from multiple GI cancer cell lines	Miyoshi <i>et al</i> <sup>[69]</sup> , 2010
Glioblastoma	iPSCs generated from glioblastoma-derived neural stem cells	Stricker <i>et al</i> <sup>[76]</sup> , 2014; Stricker <i>et al</i> <sup>[77]</sup> , 2013

iPSC: Induced pluripotent stem cells; PDAC: Pancreatic ductal adenocarcinoma; CML: Chronic myeloid leukaemia; JML: Juvenile myelomonocytic leukemia.

to imatinib and demonstrated phenotypic similarities to CML stem cells. This cell population may therefore be useful to interrogate the role of stem cells in CML and the mechanisms underlying the development of resistance<sup>[72]</sup>.

#### **iPSCs and pancreatic ductal adenocarcinoma**

iPSCs have also been used to study pancreatic ductal adenocarcinoma (PDAC), which currently has no suitable model. iPSC lines were generated from human tumours and injected into immunodeficient mice. After 3 mo, pancreatic intraepithelial neoplasia-like structures could be identified in 9 out of 10 teratomas, and by 9 mo solid tumours were present, suggesting that PDAC derived iPSCs can capture the process of carcinogenesis from pre-malignant lesions to the malignant phenotype. *In vitro* 3D culture of cells harvested from mice at 3 mo identified 25 proteins which were secreted by all teratomas, of which 8 have been previously reported in PDAC, pancreatic epithelial neoplasia or intraductal papillary mucinous neoplasms<sup>[73]</sup>. However, although 9 patient samples were used in the study, only 1 iPSC line with a cancer genotype was generated from a single patient, which is consistent with other studies suggesting the difficulty of reprogramming malignant cells<sup>[74]</sup>.

#### **iPSCs and juvenile myelomonocytic leukemia**

A model of juvenile myelomonocytic leukemia using iPSCs derived from two patients has also recently been generated. Cells were able to differentiate to myeloid cells which showed phenotypic similarities to the primary tumours including enhanced proliferation, suggesting they could be a useful resource to model the disease<sup>[75]</sup>.

#### **Gastrointestinal cancer and glioblastoma**

iPSCs have also been generated from a number of gastrointestinal cell lines using retroviral transduction. These cancer-derived iPSCs showed reduced tumorigenic potential *in vivo* as well as increased sensitivity to anti-cancer drugs and decreased proliferative rate<sup>[69]</sup>. This decrease in tumour forming ability is a concern for the use of cancer-derived iPSCs as it is thought that reprogramming cells removes epigenetic marks which are important for lineage identity and malignancy. However, lineage specificity may remain due to incomplete

reprogramming and can also be induced by differentiation. For example, in iPSCs derived from glioblastomas both lineage and cancer associated methylation marks were reset however during differentiation along the neural lineage cells maintained their malignant phenotype<sup>[76,77]</sup>.

#### **Modelling carcinogenesis using non-malignant cells**

An alternative method to study carcinogenesis using iPSCs is to reprogram normal cells to pluripotency and follow their development to interrogate the processes which contribute to cancer development (Figure 2). iPSCs are able to differentiate into all adult cell types and can therefore be used to model development of human tissues<sup>[78]</sup>. iPSC models of organ and tissue development can then be monitored in real-time to identify any changes which may induce the onset of carcinogenesis. Recently, an *in vitro* model of skin was developed using iPSCs differentiated to keratinocytes and fibroblasts, providing an iPSC-generated *in vitro* model of a human organ<sup>[79]</sup>.

Alternatively, iPSCs from normal somatic cells could also be manipulated to study carcinogenesis through overexpression or silencing of oncogenes, tumour suppressor genes and other factors thought to play a role in carcinogenesis, or alteration of the microenvironment. The response of cells to these changes can then be studied to determine the roles of such factors in cancer initiation and progression.

## **CONCLUSION**

Pluripotent cells and cancer cells share a number of characteristics including the ability for continual proliferation and self-renewal. iPSCs offer an opportunity to develop disease-specific models for carcinogenesis by reprogramming malignant cells. A number of studies have successfully generated iPSCs from human tumours and cancer cell lines and used them to study the underlying mechanisms of cancer. Generation of iPSCs from other cancer types is necessary to develop relevant *in vitro* models for carcinogenesis.

## **REFERENCES**

- 1 Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012



- v1.0, Cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer, 2013. Available from: URL: <http://globocan.iarc.fr>
- 2 **Kaur G**, Dufour JM. Cell lines: Valuable tools or useless artifacts. *Spermatogenesis* 2012; **2**: 1-5 [PMID: 22553484 DOI: 10.4161/spmg.19885]
  - 3 **Sandberg R**, Ernberg I. Assessment of tumor characteristic gene expression in cell lines using a tissue similarity index (TSI). *Proc Natl Acad Sci USA* 2005; **102**: 2052-2057 [PMID: 15671165 DOI: 10.1073/pnas.0408105102]
  - 4 **Lawrence MS**, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, Carter SL, Stewart C, Mermel CH, Roberts SA, Kiezun A, Hammerman PS, McKenna A, Drier Y, Zou L, Ramos AH, Pugh TJ, Stransky N, Helman E, Kim J, Sougnez C, Ambrogio L, Nickerson E, Shefler E, Cortés ML, Auclair D, Saksena G, Voet D, Noble M, DiCara D, Lin P, Lichtenstein L, Heiman DI, Fennell T, Imielinski M, Hernandez B, Hodis E, Baca S, Dulak AM, Lohr J, Landau DA, Wu CJ, Melendez-Zajgla J, Hidalgo-Miranda A, Koren A, McCarroll SA, Mora J, Lee RS, Crompton B, Onofrio R, Parkin M, Winckler W, Ardlie K, Gabriel SB, Roberts CW, Biegel JA, Stegmaier K, Bass AJ, Garraway LA, Meyerson M, Golub TR, Gordenin DA, Sunyaev S, Lander ES, Getz G. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* 2013; **499**: 214-218 [PMID: 23770567 DOI: 10.1038/nature12213]
  - 5 **Meacham CE**, Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature* 2013; **501**: 328-337 [PMID: 24048065 DOI: 10.1038/nature12624]
  - 6 **Rangarajan A**, Weinberg RA. Opinion: Comparative biology of mouse versus human cells: modelling human cancer in mice. *Nat Rev Cancer* 2003; **3**: 952-959 [PMID: 14737125]
  - 7 **Collins AT**, Habib FK, Maitland NJ, Neal DE. Identification and isolation of human prostate epithelial stem cells based on alpha(2)beta(1)-integrin expression. *J Cell Sci* 2001; **114**: 3865-3872 [PMID: 11719553]
  - 8 **Thomson JA**, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: 9804556 DOI: 10.1126/science.282.5391.1145]
  - 9 **Takahashi K**, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]
  - 10 **Takahashi K**, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861-872 [PMID: 18035408 DOI: 10.1016/j.cell.2007.11.019]
  - 11 **Yu J**, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin IL, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007; **318**: 1917-1920 [PMID: 18029452 DOI: 10.1126/science.1151526]
  - 12 **de Wert G**, Mummery C. Human embryonic stem cells: research, ethics and policy. *Hum Reprod* 2003; **18**: 672-682 [PMID: 12660256 DOI: 10.1093/humrep/deg143]
  - 13 **Lee G**, Papapetrou EP, Kim H, Chambers SM, Tomishima MJ, Fasano CA, Ganat YM, Menon J, Shimizu F, Viale A, Tabar V, Sadelaian M, Studer L. Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature* 2009; **461**: 402-406 [PMID: 19693009 DOI: 10.1038/nature08320]
  - 14 **Hanahan D**, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57-70 [PMID: 10647931 DOI: 10.1016/S0092-8674(00)81683-9]
  - 15 **Marte B**. Tumour heterogeneity. *Nature* 2013; **501**: 327 [PMID: 24048064 DOI: 10.1038/501327a]
  - 16 **Beck B**, Blanpain C. Unravelling cancer stem cell potential. *Nat Rev Cancer* 2013; **13**: 727-738 [PMID: 24060864 DOI: 10.1038/nrc3597]
  - 17 **Clarke MF**, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res* 2006; **66**: 9339-9344 [PMID: 16990346 DOI: 10.1158/0008-5472.can-06-3126]
  - 18 **Al-Hajj M**, Becker MW, Wicha M, Weissman I, Clarke MF. Therapeutic implications of cancer stem cells. *Curr Opin Genet Dev* 2004; **14**: 43-47 [PMID: 15108804 DOI: 10.1016/j.gde.2003.11.007]
  - 19 **Wu XZ**. Origin of cancer stem cells: the role of self-renewal and differentiation. *Ann Surg Oncol* 2008; **15**: 407-414 [PMID: 18043974 DOI: 10.1245/s10434-007-9695-y]
  - 20 **Visvader JE**, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008; **8**: 755-768 [PMID: 18784658 DOI: 10.1038/nrc2499]
  - 21 **Kreso A**, Dick JE. Evolution of the cancer stem cell model. *Cell Stem Cell* 2014; **14**: 275-291 [PMID: 24607403 DOI: 10.1016/j.stem.2014.02.006]
  - 22 **Vermeulen L**, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuijnman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G, Medema JP. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nat Cell Biol* 2010; **12**: 468-476 [PMID: 20418870 DOI: 10.1038/ncb2048]
  - 23 **Chaffer CL**, Brueckmann I, Scheel C, Kaestli AJ, Wiggins PA, Rodrigues LO, Brooks M, Reinhardt F, Su Y, Polyak K, Arendt LM, Kuperwasser C, Bieri B, Weinberg RA. Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. *Proc Natl Acad Sci USA* 2011; **108**: 7950-7955 [PMID: 21498687 DOI: 10.1073/pnas.1102454108]
  - 24 **Vincent TL**, Gatenby RA. An evolutionary model for initiation, promotion, and progression in carcinogenesis. *Int J Oncol* 2008; **32**: 729-737 [PMID: 18360700 DOI: 10.3892/ijo.32.4.729]
  - 25 **Nowell PC**. The clonal evolution of tumor cell populations. *Science* 1976; **194**: 23-28 [PMID: 959840 DOI: 10.1126/science.959840]
  - 26 **Li L**, Wang BH, Wang S, Moalim-Nour L, Mohib K, Lohnes D, Wang L. Individual cell movement, asymmetric colony expansion, rho-associated kinase, and E-cadherin impact the clonogenicity of human embryonic stem cells. *Biophys J* 2010; **98**: 2442-2451 [PMID: 20513387 DOI: 10.1016/j.bpj.2010.02.029]
  - 27 **Lamouille S**, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* 2014; **15**: 178-196 [PMID: 24556840 DOI: 10.1038/nrm3758]
  - 28 **Thiery JP**, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; **139**: 871-890 [PMID: 19945376 DOI: 10.1016/j.cell.2009.11.007]
  - 29 **Larue L**, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene* 2005; **24**: 7443-7454 [PMID: 16288291 DOI: 10.1038/sj.onc.1209091]
  - 30 **Kim YS**, Yi BR, Kim NH, Choi KC. Role of the epithelial-mesenchymal transition and its effects on embryonic stem cells. *Exp Mol Med* 2014; **46**: e108 [PMID: 25081188 DOI: 10.1038/emmm.2014.44]
  - 31 **Li R**, Liang J, Ni S, Zhou T, Qing X, Li H, He W, Chen J, Li F, Zhuang Q, Qin B, Xu J, Li W, Yang J, Gan Y, Qin D, Feng S, Song H, Yang D, Zhang B, Zeng L, Lai L, Esteban MA, Pei D. A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. *Cell Stem Cell* 2010; **7**: 51-63 [PMID: 20621050 DOI: 10.1016/j.stem.2010.04.014]
  - 32 **Alonso SR**, Tracey L, Ortiz P, Pérez-Gómez B, Palacios J, Pollán M, Linares J, Serrano S, Sáez-Castillo AI, Sánchez L, Pajares R, Sánchez-Aguilera A, Artiga MJ, Piris MA, Rodríguez-Peralto JL. A high-throughput study in melanoma identifies epithelial-mesenchymal transition as a major determinant of metastasis. *Cancer Res* 2007; **67**: 3450-3460 [PMID: 17409456 DOI: 10.1158/0008-5472.can-06-3481]
  - 33 **Massagué J**. TGFbeta in Cancer. *Cell* 2008; **134**: 215-230 [PMID: 18662538 DOI: 10.1016/j.cell.2008.07.001]
  - 34 **Ansieau S**, Bastid J, Doreau A, Morel AP, Bouchet BP, Thomas C, Fauvet F, Puisieux I, Doglioni C, Piccinin S, Maestro R, Voeltzel T, Selmi A, Valsesia-Wittmann S, Caron de Fromental C, Puisieux A. Induction of EMT by twist proteins as a collateral effect of tumor-



- promoting inactivation of premature senescence. *Cancer Cell* 2008; **14**: 79-89 [PMID: 18598946 DOI: 10.1016/j.ccr.2008.06.005]
- 35 **Kajiyama H**, Shibata K, Terauchi M, Yamashita M, Ino K, Nawa A, Kikkawa F. Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells. *Int J Oncol* 2007; **31**: 277-283 [PMID: 17611683 DOI: 10.3892/ijo.31.2.277]
  - 36 **Yang AD**, Fan F, Camp ER, van Buren G, Liu W, Somcio R, Gray MJ, Cheng H, Hoff PM, Ellis LM. Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. *Clin Cancer Res* 2006; **12**: 4147-4153 [PMID: 16857785 DOI: 10.1158/1078-0432.ccr-06-0038]
  - 37 **Jerabek S**, Merino F, Schöler HR, Cojocaru V. OCT4: dynamic DNA binding pioneers stem cell pluripotency. *Biochim Biophys Acta* 2014; **1839**: 138-154 [PMID: 24145198 DOI: 10.1016/j.bbaggm.2013.10.001]
  - 38 **Gidekel S**, Pizov G, Bergman Y, Pikarsky E. Oct-3/4 is a dose-dependent oncogenic fate determinant. *Cancer Cell* 2003; **4**: 361-370 [PMID: 14667503 DOI: 10.1016/S1535-6108(03)00270-8]
  - 39 **Monk M**, Holding C. Human embryonic genes re-expressed in cancer cells. *Oncogene* 2001; **20**: 8085-8091 [PMID: 11781821 DOI: 10.1038/sj.onc.1205088]
  - 40 **Hochedlinger K**, Yamada Y, Beard C, Jaenisch R. Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. *Cell* 2005; **121**: 465-477 [PMID: 15882627 DOI: 10.1016/j.cell.2005.02.018]
  - 41 **Chiou SH**, Yu CC, Huang CY, Lin SC, Liu CJ, Tsai TH, Chou SH, Chien CS, Ku HH, Lo JF. Positive correlations of Oct-4 and Nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. *Clin Cancer Res* 2008; **14**: 4085-4095 [PMID: 18593985 DOI: 10.1158/1078-0432.ccr-07-4404]
  - 42 **Beltran AS**, Rivenbark AG, Richardson BT, Yuan X, Quian H, Hunt JP, Zimmerman E, Graves LM, Blanford P. Generation of tumor-initiating cells by exogenous delivery of OCT4 transcription factor. *Breast Cancer Res* 2011; **13**: R94 [PMID: 21952072 DOI: 10.1186/bcr3019]
  - 43 **Wang YD**, Cai N, Wu XL, Cao HZ, Xie LL, Zheng PS. OCT4 promotes tumorigenesis and inhibits apoptosis of cervical cancer cells by miR-125b/BAK1 pathway. *Cell Death Dis* 2013; **4**: e760 [PMID: 23928699 DOI: 10.1038/cddis.2013.272]
  - 44 **Dang CV**. MYC on the path to cancer. *Cell* 2012; **149**: 22-35 [PMID: 22464321 DOI: 10.1016/j.cell.2012.03.003]
  - 45 **Beroukhi R**, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, Barretina J, Boehm JS, Dobson J, Urashima M, McHenry KT, Pinchback RM, Ligon AH, Cho YJ, Haery L, Greulich H, Reich M, Winckler W, Lawrence MS, Weir BA, Tanaka KE, Chiang DY, Bass AJ, Loo A, Hoffman C, Prensner J, Liefeld T, Gao Q, Yecies D, Signoretti S, Maher E, Kaye FJ, Sasaki H, Tepper JE, Fletcher JA, Tabernero J, Baselga J, Tsao MS, Demicheli F, Rubin MA, Janne PA, Daly MJ, Nucera C, Levine RL, Ebert BL, Gabriel S, Rustgi AK, Antonescu CR, Ladanyi M, Letai A, Garraway LA, Loda M, Beer DG, True LD, Okamoto A, Pomeroy SL, Singer S, Golub TR, Lander ES, Getz G, Sellers WR, Meyerson M. The landscape of somatic copy-number alteration across human cancers. *Nature* 2010; **463**: 899-905 [PMID: 20164920 DOI: 10.1038/nature08822]
  - 46 **Dalla-Favera R**, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc Natl Acad Sci USA* 1982; **79**: 7824-7827 [PMID: 6961453]
  - 47 **Wiener F**, Potter M. In: Kirsch IR, editor. The causes and consequences of chromosomal aberrations. New York: CRC Press, 1993: 91-124
  - 48 **Shou Y**, Martelli ML, Gabrea A, Qi Y, Brents LA, Roschke A, Dewald G, Kirsch IR, Bergsagel PL, Kuehl WM. Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. *Proc Natl Acad Sci USA* 2000; **97**: 228-233 [PMID: 10618400]
  - 49 **Palomero T**, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, Barnes KC, O'Neil J, Neuberg D, Weng AP, Aster JC, Sigaux F, Soulier J, Look AT, Young RA, Califano A, Ferrando AA. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc Natl Acad Sci USA* 2006; **103**: 18261-18266 [PMID: 17114293 DOI: 10.1073/pnas.0606108103]
  - 50 **Naidu R**, Wahab NA, Yadav M, Kutty MK. Protein expression and molecular analysis of c-myc gene in primary breast carcinomas using immunohistochemistry and differential polymerase chain reaction. *Int J Mol Med* 2002; **9**: 189-196 [PMID: 11786932 DOI: 10.3892/ijmm.9.2.189]
  - 51 **Smith DR**, Goh HS. Overexpression of the c-myc proto-oncogene in colorectal carcinoma is associated with a reduced mortality that is abrogated by point mutation of the p53 tumor suppressor gene. *Clin Cancer Res* 1996; **2**: 1049-1053 [PMID: 9816266]
  - 52 **Chen C**, Cai S, Wang G, Cao X, Yang X, Luo X, Feng Y, Hu J. c-Myc enhances colon cancer cell-mediated angiogenesis through the regulation of HIF-1 $\alpha$ . *Biochem Biophys Res Commun* 2013; **430**: 505-511 [PMID: 23237807 DOI: 10.1016/j.bbrc.2012.12.006]
  - 53 **Gabay M**, Li Y, Felsner DW. MYC activation is a hallmark of cancer initiation and maintenance. *Cold Spring Harb Perspect Med* 2014; **4** [PMID: 24890832 DOI: 10.1101/cshperspect.a014241]
  - 54 **Murphy DJ**, Junttila MR, Pouyet L, Karnezis A, Shchors K, Bui DA, Brown-Swigart L, Johnson L, Evan GI. Distinct thresholds govern Myc's biological output in vivo. *Cancer Cell* 2008; **14**: 447-457 [PMID: 19061836 DOI: 10.1016/j.ccr.2008.10.018]
  - 55 **Luo W**, Li S, Peng B, Ye Y, Deng X, Yao K. Embryonic stem cells markers SOX2, OCT4 and Nanog expression and their correlations with epithelial-mesenchymal transition in nasopharyngeal carcinoma. *PLoS One* 2013; **8**: e56324 [PMID: 23424657 DOI: 10.1371/journal.pone.0056324]
  - 56 **Noh KH**, Kim BW, Song KH, Cho H, Lee YH, Kim JH, Chung JY, Kim JH, Hewitt SM, Seong SY, Mao CP, Wu TC, Kim TW. Nanog signaling in cancer promotes stem-like phenotype and immune evasion. *J Clin Invest* 2012; **122**: 4077-4093 [PMID: 23093782 DOI: 10.1172/jci64057]
  - 57 **Bass AJ**, Watanabe H, Mermel CH, Yu S, Perner S, Verhaak RG, Kim SY, Wardwell L, Tamayo P, Gat-Viks I, Ramos AH, Woo MS, Weir BA, Getz G, Beroukhi R, O'Kelly M, Dutt A, Rozenblatt-Rosen O, Dziunycz P, Komisarof J, Chirieac LR, Lafargue CJ, Scheble V, Wilbertz T, Ma C, Rao S, Nakagawa H, Stairs DB, Lin L, Giordano TJ, Wagner P, Minna JD, Gazdar AF, Zhu CQ, Brose MS, Ceccanello I, Jr UR, Marie SK, Dahl O, Shivdasani RA, Tsao MS, Rubin MA, Wong KK, Regev A, Hahn WC, Beer DG, Rustgi AK, Meyerson M. SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. *Nat Genet* 2009; **41**: 1238-1242 [PMID: 19801978]
  - 58 **Boumahdi S**, Driessens G, Lapouge G, Rorive S, Nassar D, Le Mercier M, Delatte B, Caauwe A, Lenglez S, Nkusi E, Brohée S, Salmon I, Dubois C, del Marmol V, Fuks F, Beck B, Blanpain C. SOX2 controls tumour initiation and cancer stem-cell functions in squamous-cell carcinoma. *Nature* 2014; **511**: 246-250 [PMID: 24909994 DOI: 10.1038/nature13305]
  - 59 **Glinksy GV**, Berezovska O, Glinksy AB. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. *J Clin Invest* 2005; **115**: 1503-1521 [PMID: 15931389 DOI: 10.1172/jci23412]
  - 60 **Wong DJ**, Liu H, Ridky TW, Cassarino D, Segal E, Chang HY. Module map of stem cell genes guides creation of epithelial cancer stem cells. *Cell Stem Cell* 2008; **2**: 333-344 [PMID: 18397753 DOI: 10.1016/j.stem.2008.02.009]
  - 61 **Wilson A**, Murphy MJ, Oskarsson T, Kaloulis K, Bettess MD, Oser GM, Pasche AC, Knabenhans C, Macdonald HR, Trumpp A. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes Dev* 2004; **18**: 2747-2763 [PMID: 15545632 DOI: 10.1101/gad.313104]
  - 62 **Ben-Porath I**, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 2008; **40**: 499-507 [PMID: 18443585]
  - 63 **Warburg O**. On the origin of cancer cells. *Science* 1956; **123**:



- 309-314 [PMID: 13298683 DOI: 10.1126/science.123.3191.309]
- 64 **Zhang J**, Nuebel E, Daley GQ, Koehler CM, Teitell MA. Metabolic regulation in pluripotent stem cells during reprogramming and self-renewal. *Cell Stem Cell* 2012; **11**: 589-595 [PMID: 23122286 DOI: 10.1016/j.stem.2012.10.005]
  - 65 **Shyh-Chang N**, Zheng Y, Locasale JW, Cantley LC. Human pluripotent stem cells decouple respiration from energy production. *EMBO J* 2011; **30**: 4851-4852 [PMID: 22166995 DOI: 10.1038/emboj.2011.436]
  - 66 **Horimoto M**, Resnick MB, Konkin TA, Routhier J, Wands JR, Baffy G. Expression of uncoupling protein-2 in human colon cancer. *Clin Cancer Res* 2004; **10**: 6203-6207 [PMID: 15448008 DOI: 10.1158/1078-0432.ccr-04-0419]
  - 67 **Ayyasamy V**, Owens KM, Desouki MM, Liang P, Bakin A, Thangaraj K, Buchsbaum DJ, LoBuglio AF, Singh KK. Cellular model of Warburg effect identifies tumor promoting function of UCP2 in breast cancer and its suppression by genipin. *PLoS One* 2011; **6**: e24792 [PMID: 21935467 DOI: 10.1371/journal.pone.0024792]
  - 68 **Panopoulos AD**, Yanes O, Ruiz S, Kida YS, Diep D, Tautenhahn R, Herreras A, Batchelder EM, Plongthongkum N, Lutz M, Berggren WT, Zhang K, Evans RM, Siuzdak G, Izpisua Belmonte JC. The metabolome of induced pluripotent stem cells reveals metabolic changes occurring in somatic cell reprogramming. *Cell Res* 2012; **22**: 168-177 [PMID: 22064701 DOI: 10.1038/cr.2011.177]
  - 69 **Miyoshi N**, Ishii H, Nagai K, Hoshino H, Mimori K, Tanaka F, Nagano H, Sekimoto M, Doki Y, Mori M. Defined factors induce reprogramming of gastrointestinal cancer cells. *Proc Natl Acad Sci USA* 2010; **107**: 40-45 [PMID: 20018687 DOI: 10.1073/pnas.0912407107]
  - 70 **Carette JE**, Pruszk J, Varadarajan M, Blomen VA, Gokhale S, Camargo FD, Wernig M, Jaenisch R, Brummelkamp TR. Generation of iPSCs from cultured human malignant cells. *Blood* 2010; **115**: 4039-4042 [PMID: 20233975 DOI: 10.1182/blood-2009-07-231845]
  - 71 **Nowell PC**, Hungerford DA. Chromosome studies on normal and leukemic human leukocytes. *J Natl Cancer Inst* 1960; **25**: 85-109 [PMID: 14427847 DOI: 10.1093/jnci/25.1.85]
  - 72 **Kumano K**, Arai S, Hosoi M, Taoka K, Takayama N, Otsu M, Nagae G, Ueda K, Nakazaki K, Kamikubo Y, Eto K, Aburatani H, Nakauchi H, Kurokawa M. Generation of induced pluripotent stem cells from primary chronic myelogenous leukemia patient samples. *Blood* 2012; **119**: 6234-6242 [PMID: 22592606 DOI: 10.1182/blood-2011-07-367441]
  - 73 **Kim J**, Hoffman JP, Alpaugh RK, Rhim AD, Reichert M, Stanger BZ, Furth EE, Sepulveda AR, Yuan CX, Won KJ, Donahue G, Sands J, Gumbs AA, Zaret KS. An iPSC line from human pancreatic ductal adenocarcinoma undergoes early to invasive stages of pancreatic cancer progression. *Cell Rep* 2013; **3**: 2088-2099 [PMID: 23791528 DOI: 10.1016/j.celrep.2013.05.036]
  - 74 **Ramos-Mejia V**, Fraga MF, Menendez P. iPSCs from cancer cells: challenges and opportunities. *Trends Mol Med* 2012; **18**: 245-247 [PMID: 22521522 DOI: 10.1016/j.molmed.2012.04.001]
  - 75 **Gandre-Babbe S**, Paluru P, Aribena C, Chou ST, Bresolin S, Lu L, Sullivan SK, Tasian SK, Weng J, Favre H, Choi JK, French DL, Loh ML, Weiss MJ. Patient-derived induced pluripotent stem cells recapitulate hematopoietic abnormalities of juvenile myelomonocytic leukemia. *Blood* 2013; **121**: 4925-4929 [PMID: 23620576 DOI: 10.1182/blood-2013-01-478412]
  - 76 **Stricker S**, Pollard S. Reprogramming cancer cells to pluripotency: an experimental tool for exploring cancer epigenetics. *Epigenetics* 2014; **9**: 798-802 [PMID: 24686321 DOI: 10.4161/epi.28600]
  - 77 **Stricker SH**, Feber A, Engström PG, Carén H, Kurian KM, Takashima Y, Watts C, Way M, Dirks P, Bertone P, Smith A, Beck S, Pollard SM. Widespread resetting of DNA methylation in glioblastoma-initiating cells suppresses malignant cellular behavior in a lineage-dependent manner. *Genes Dev* 2013; **27**: 654-669 [PMID: 23512659 DOI: 10.1101/gad.212662.112]
  - 78 **Grskovic M**, Javaherian A, Strulovici B, Daley GQ. Induced pluripotent stem cells--opportunities for disease modelling and drug discovery. *Nat Rev Drug Discov* 2011; **10**: 915-929 [PMID: 22076509 DOI: 10.1038/nrd3577]
  - 79 **Itoh M**, Umegaki-Arao N, Guo Z, Liu L, Higgins CA, Christiano AM. Generation of 3D skin equivalents fully reconstituted from human induced pluripotent stem cells (iPSCs). *PLoS One* 2013; **8**: e77673 [PMID: 24147053 DOI: 10.1371/journal.pone.0077673]

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## CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture

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purification of human bone marrow mesenchymal stem cells. This marker has been shown to be specifically expressed by these cells. Thus, CD271 has been proposed as a versatile marker to selectively isolated and expand multipotent mesenchymal stem cells with both immunosuppressive and lymphohematopoietic engraftment-promoting properties. This review focuses on this marker, specifically on identification of mesenchymal stem cells from different tissues. Literature revision suggests that CD271 should not be defined as a universal marker to identify mesenchymal stem cells before culture from different sources. In the case of bone marrow or adipose tissue, CD271 could be considered a quite suitable marker; however this marker seems to be inadequate for the isolation of mesenchymal stem cells from other tissues such as umbilical cord blood or wharton's jelly among others.

**Key words:** Mesenchymal stem cells; CD271; Low-affinity nerve growth factor receptor; p75; Bone marrow

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**Core tip:** CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture.

### Abstract

Mesenchymal stem cells, due to their characteristics are ideal candidates for cellular therapy. Currently, in culture these cells are defined by their adherence to plastic, specific surface antigen expression and multipotent differentiation potential. However, the *in vivo* identification of mesenchymal stem cells, before culture, is not so well established. Pre-culture identification markers would ensure higher purity than that obtained with selection based on adherence to plastic. Up until now, CD271 has been described as the most specific marker for the characterization and

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### INTRODUCTION

In recent decades researchers have focused on regenerative medicine as a interesting option



for the treatment of different pathologies<sup>[1,2]</sup>. Mesenchymal stem cells (MSC) are ideal candidates for cellular therapy due to their characteristics<sup>[3]</sup>. MSC are viewed as clinically promising because of their differentiation capacity and pro-regenerative features<sup>[4,5]</sup>. Moreover, MSC could be used for both autologous and heterologous transplant due to their low immunogenicity<sup>[6]</sup>. Friedenstein *et al.*<sup>[7]</sup> discovered bone marrow (BM)-MSC. They demonstrated that BM contains a population of cells with a high proliferative capacity that adhere to plastic. These authors were also the first to propose the capacity of these cells to form colonies from a single cell (the fibroblast-colony forming unit F-CFU)<sup>[7-9]</sup>. Since Friedenstein *et al.*<sup>[7]</sup> described MSC in the BM in the 70's several researchers have focused their attention on this type of adult stem cell. After their initial isolation from humans, MSC have since been successfully harvested from many other species including: mouse, rat, dog and horse<sup>[10]</sup>. They have also been isolated from almost every type of tissue, including: BM, adipose tissue, liver, skeletal muscle, amniotic fluid, umbilical cord blood (UCB) or dental pulp<sup>[11-14]</sup>. Due to the heterogeneity of the results obtained by many groups, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes minimal criteria to define human MSC. They proposed three criteria to define MSC in culture: adherence to plastic, specific surface antigen expression and multipotent differentiation potential<sup>[15]</sup>. All these criteria perfectly define MSC in culture, however the *in vivo* identification of MSC, before culture, is not as well established. In 1999, Pittenger *et al.*<sup>[16]</sup> described a small percentage of MSC present in BM (0.001%-0.01%) so this could explain the difficulty in establishing the exact phenotype of MSC before culture<sup>[16]</sup>.

A suitable method of selection would allow the employment of MSC in different pathologies directly after their isolation or after their *in vitro* expansion. Pre-culture identification markers would ensure higher purity than that obtained with selection based on adherence to plastic. Many investigators direct their efforts towards find a marker or a combination of markers to ensure their selection.

Up until now, CD271 (LNGFR) has been described as one of the most specific markers for the purification of human BM-MSCs<sup>[17,18]</sup>. CD271, also known as low-affinity nerve growth factor receptor (LNGFR), nerve growth factor receptor (NGFR), or p75NTR (neurotrophin receptor), belongs to the tumor necrosis factor superfamily<sup>[19]</sup>. Taking into account that this marker has been the most commonly used in the isolation of BM-MSC this review will focus on to the identification of MSC from different tissues (Table 1).

## BM

Several independent studies have confirmed the

specificity of CD271 on MSC isolated from BM. At the beginning of the last decade, Quirici *et al.*<sup>[18]</sup>, (2002) published that the anti-CD271 antibody is specific for a population of multipotent BM cells and suggested the use of this marker as an option for the selection of MSC from BM<sup>[18]</sup>. The same year, Jones *et al.*<sup>[20]</sup>, conducted a study in which they carried out a cellular purification from BM with antibody-conjugated magnetic beads. They found that the D7-FIB-positive fraction contained not only all the CFU-F activity but also a unique population of CD45<sup>low</sup>/LNGFR<sup>+</sup> cells. These cells were adherent, proliferative, and multipotent following cell sorting and standard expansion<sup>[20]</sup>. In 2006 the same group, suggested the need to find a way to count MSC based on the use of surface markers considering that up to this point researchers were using CFU-F assays for the enumeration of MSC. Published results demonstrate the use of flow cytometry as a rapid MSC detection method and suggested *in vivo* selection of the phenotype CD45<sup>low</sup>/LNGFR<sup>+</sup>/D7FIB<sup>+</sup><sup>[21]</sup>. Following the studies started in 2002, Jones's group continued providing more data about this marker. In a comparative study, Jones *et al.*<sup>[22]</sup> demonstrated that CD271 antigen (followed by CD146, CD106, D7-FIB, CD13 and CD166) remained one of the most selective markers for enriching progenitor cells from MSC of human BM. These results are supported by Kuçi *et al.*<sup>[23,24]</sup>, who published a study which demonstrated that the CD271 is an adequate marker for the selection of multipotent BM cells with immunosuppressive properties. Afterwards, the same group published another study in which they demonstrated that CFU-F activity was found only in the CD271<sup>+</sup> cell fraction, whereas no CFU-F was observed in the CD271<sup>-</sup> population<sup>[24]</sup>. Flores-Torales *et al.*<sup>[25]</sup>, (2010) proposed the use of a single marker, CD271, for the selection of MSC from BM before culture. These authors maintain that the use of this marker would reduce costs and provide a rapid and simple way of obtaining MSC. However, a high percentage of CD271<sup>+</sup> cells in BM and synovium co-express CD34, which disqualifies CD271 as a unique marker for the isolation of MSC. Nevertheless, these studies, among others, have confirmed the usefulness of CD271 in combination with other markers such as CD45 to isolate fresh BM-MSC. Poloni *et al.*<sup>[26]</sup>, (2009) carried out a selection of CD271 positive cells and cultivated them in a media supplemented with 10% allogeneic human sera, cells maintained the capacity to differentiate and no karyotypic variations were observed. Our group utilized this marker (CD271<sup>+</sup>/CD45<sup>-</sup>) to quantify the MSC population in BM samples obtained for cell therapy using flow cytometry<sup>[27]</sup>. Recently Mabuchi *et al.*<sup>[28]</sup> performed a comprehensive screening of putative surface markers to select the most useful ones for prospectively identifying a pure MSC population in human BM. They concluded that the combination marker CD271<sup>+</sup>CD90<sup>+</sup>CD106<sup>+</sup> can



**Table 1 Summary of references documenting CD271 expression in different sources**

Source	CD271	Ref.
Bone marrow	Positive	Alvarez-Viejo <i>et al</i> <sup>[27]</sup> , 2013 Flores-Torales <i>et al</i> <sup>[25]</sup> , 2010 Jones <i>et al</i> <sup>[20]</sup> , 2002; Jones <i>et al</i> <sup>[21]</sup> , 2006; Jones <i>et al</i> <sup>[22]</sup> , 2008 Kuçi <i>et al</i> <sup>[23]</sup> , 2011; Kuçi <i>et al</i> <sup>[24]</sup> , 2010 Poloni <i>et al</i> <sup>[26]</sup> , 2009 Quirici <i>et al</i> <sup>[18]</sup> , 2002
Adipose tissue	Positive	Cuevas-Diaz Duran <i>et al</i> <sup>[31]</sup> , 2013 Quirici <i>et al</i> <sup>[33]</sup> , 2010
Umbilical cord blood	Negative	Attar <i>et al</i> <sup>[46]</sup> , 2013 Alvarez-Viejo <i>et al</i> <sup>[44]</sup> , 2013 Watson <i>et al</i> <sup>[47]</sup> , 2013
Wharton's Jelly	Low/negative	Alvarez-Viejo <i>et al</i> <sup>[44]</sup> , 2013 Margossian <i>et al</i> <sup>[35]</sup> , 2012
Placenta	Positive/negative	Battula <i>et al</i> <sup>[50]</sup> , 2008 Soncini <i>et al</i> <sup>[52]</sup> , 2007
Trabecular bone cavity	Positive	Jones <i>et al</i> <sup>[56]</sup> , 2010
Dermis	Positive	Vaculik <i>et al</i> <sup>[57]</sup> , 2012
Peripheral blood	Negative	Attar <i>et al</i> <sup>[55]</sup> , 2011

be used selectively to isolate the most potent and genetically stable MSC<sup>[28]</sup>. Therefore, CD271 may be considered a suitable marker for determining MSC in BM.

It has been demonstrated that as the age of donors increases the number and potential for differentiation of BM MSC diminish<sup>[29]</sup>. Taking into consideration that it is not easy to obtain such blood donors other sources for the obtention of MSC are required.

Adipose tissue is an interesting option for this end. MSCs can be isolated from fat tissue easily obtained by liposuction. It has been demonstrated that these cells are easily cultivated and have the capacity to differentiate into various cell lines<sup>[30]</sup>.

## ADIPOSE TISSUE

In 2002 a type of stem cell from adipose tissue was isolated for the first time: adipose-derived stem cell (ADSC)<sup>[31]</sup>. Since then, it has been demonstrated that adipose tissue provides an abundant source of MSC with similar yields to those obtained from BM. Furthermore, ADSC have a similar differentiation capability, morphology, and phenotype to MSC collected from BM<sup>[31]</sup>. It has been shown in mice that p75NRT seems to be a useful marker for ADSC isolation<sup>[32]</sup>. After this, Quirici *et al*<sup>[33]</sup> selected CD271<sup>+</sup> cells immune-magnetically from ADSCs in humans in a similar way as has been done from BM by others authors. CD271<sup>+</sup> cells showed higher clonogenic and differentiation potential compared to plastic adherent ADSC. Thus these authors demonstrated the utility of this marker in the selection of ADSC cells<sup>[33]</sup>. Cuevas-Diaz Duran *et al*<sup>[31]</sup> used this marker to analyze if there was a relationship between donor's age and CD271<sup>+</sup> cell yield in freshly isolated ADSC. They suggested that the proportion of CD271<sup>+</sup> ADSC decreased with

age; however, positive cells were present in all age groups and their frequency was higher than what has been found in BM. Therefore, CD271<sup>+</sup> cells from adipose tissue were proposed as the primary choice for tissue regeneration and autologous stem cell therapies in older subjects<sup>[31]</sup>.

The umbilical cord, placenta and placental membrane have been presented as cheap and attractive alternatives for the obtention of MSC<sup>[34]</sup>.

## UMBILICAL CORD

The UC is the structure that connects the foetus to the placenta. The UC has two arteries and a vein wrapped in a gelatinous connective tissue called Wharton's jelly (WJ). The UC has characteristics ideal for procuring of MSC, because its use would not generate ethical conflicts, it is easily obtained and it is an abundant resource which is discarded<sup>[35]</sup>. As described below, MSCs have been described from UCB and from WJ.

## UCB

UCB was found to contain different populations of stem cells, a unique feature not shared with peripheral blood. Some authors propose that UCB derived MSC show high morphological and molecular similarities to BM derived MSC including the lack of hematopoietic surface antigens<sup>[36]</sup>. The presence or absence of MSC in UCB has been quite controversial. A few researchers have denied the presence of MSC in UCB such as Yu *et al*<sup>[37]</sup>, (2004) whose results suggest that early fetal blood is rich in MSC however, the term UCB is not used. On the other hand, some authors propose the difficulty in obtaining MSC from UCB to be due to their low frequency, inferior to that in BM. Up until now, the literature does not



show interesting results regarding the obtention, isolation and purification of UCB MSCs. Some publications show that the MSC can be obtained in a low percentage of UCB units (20%-63%)<sup>[38,39]</sup>. These data bring into question the possibility of considering the UCB as a resource for acquiring MSC<sup>[40]</sup>. However, several authors have differentiated UCB-MSC *in vitro* to osteogenic, chondrogenic, neural and hepatic lineages successfully<sup>[41-43]</sup>. Our group has not been able to identify MSC from UCB using conventional methods of adherence to plastic. We observed a heterogeneous population of adherent cells, with a rounded and spindle-shaped appearance<sup>[44]</sup>. Our results are in concordance with Perdikogianni *et al.*<sup>[45]</sup>, (2008). We also used CD271 for the presence of cells from UCB expressing this marker. Our results showed no validity for this marker in this tissue and these data are in agreement with two studies published in parallel. Attar and coworkers showed that CD271 did not contribute to isolation of MSC from UCB<sup>[46]</sup>. Watson *et al.*<sup>[47]</sup>, (2013) published that, CD271 is an efficient marker for MSC isolation from BM but failed to isolate MSC from UCB. Therefore, our experience and results from the literature suggest that CD271 is not a suitable marker for the identification of MSC from UCB without culture.

## WJ

Thomas Wharton was the first to describe WJ in 1656. WJ is a gelatinous substance composed of various isoforms of collagen and proteoglycans. The principal function of WJ is to protect the arteries and veins from the compression and torsion that they can be subjected to. These provide a bidirectional flow, providing oxygen and nutrients that contribute to the adequate development of the foetus and eliminating waste and carbon dioxide<sup>[48]</sup>. It has been demonstrated that human WJ-MSC present an elevated capacity for autoregeneration and have been compared with those obtained from BM<sup>[39]</sup>. MSC derived from the discarded UC, more precisely WJ, offer a low-cost and pain-free collection method of MSC that may be cryogenically stored, and are considered extremely favorable for tissue engineering purposes<sup>[35]</sup>. Data published by our group highlight the ineffectiveness of CD271 as a marker for the isolation of MSC from WJ before culture<sup>[44]</sup>. These results are supported by data published by Margossian *et al.*<sup>[35]</sup>. They studied the expression of CD271 *in situ* on fresh fragments of WJ and they observed it to be weakly expressed<sup>[35]</sup>. Although few studies have focused on determining the effectiveness of this marker in WJ, those that have been carried out suggest that CD271 is not a suitable marker to identify MSC from WJ before culture.

## PLACENTA

Human placenta, plays a fundamental and essential

role in fetal development, nutrition, and tolerance<sup>[49]</sup>. Various reports have demonstrated that human term placenta is a plentiful source of MSC<sup>[50,51]</sup>. Considering the complexity of the structure of the placenta, Parolini *et al.*<sup>[49]</sup> published a paper in order to define, as clearly as possible, the region of origin and methods of isolation of cells derived from this tissue. This work arose out the first international Workshop on Placenta Derived Stem Cells, (March 2007). One of the main characteristics is the existence of four regions in the placenta: Amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal and chorionic trophoblastic. From these regions, the following cell populations are isolated: amniotic epithelial cells, amniotic mesenchymal stromal cells, chorionic mesenchymal stromal cells, chorionic trophoblastic stromal cells<sup>[49]</sup>.

In 2007, Soncini *et al.*<sup>[52]</sup>, achieved the isolation of amnion mesenchymal cells (AMSC) and chorion mesenchymal cells (CMSC). These MSCs were isolated by a mechanical separation followed by enzymatic digestion. AMSC and CMSC show MSC characteristics, such as adherence to plastic, fibroblastic morphology and the capacity to form colonies. Both types of cells when analysed by flow cytometry show phenotypes similar to BM-MSC. Also, AMSC and CMSC demonstrate high plasticity when cultivated in adequate differentiating media, showing that these can differentiate into fat, bone and cartilage.

In order to isolate cells with MSC characteristics from human fetal membranes, AMSC and CMSC expressing CD271 were enriched by immunomagnetic isolation. CD271<sup>+</sup> cells were demonstrated to possess higher clonogenic and osteogenic differentiation potentials than CD271-depleted fractions. Based on these findings, these authors suggest that amnion and chorion can be considered as a novel and convenient sources of adult MSC<sup>[52]</sup>. Another study published in 2007 by Battula *et al.*<sup>[50]</sup> confirmed the possibility of obtaining MSC from chorion; however, their results showed that CD271 is not an adequate marker for the identification of MSC. They demonstrated that CD271 is expressed only at negligible levels on naive placenta MSC. Nevertheless, they identified FZD9 (frizzled-9) as a novel marker for isolation of MSC from chorionic placenta and showed that cells with CFU-F capacity reside exclusively in the FZD9<sup>+</sup> population<sup>[50]</sup>. FZD proteins comprise a family (FZD1-10) of seven transmembrane-spanning receptors<sup>[53]</sup>. Based on the literature, it is still not possible to confirm CD271 as a suitable marker for the isolation of MSC from placenta.

Because of their attractiveness, researchers have attempted to isolate MSC from many tissues and their existence has been documented in several of them. Here, we discuss some studies which have referred to the use of CD271 for the identification of MSC from tissues other than those discussed above.



## OTHER SOURCES

Chong *et al.*<sup>[54]</sup> in 2012 demonstrated that MSC from peripheral blood maintain similar characteristics and have similar chondrogenic differentiation potential to those derived from BM. Based on the literature, we have found just one reference which documents that CD271 positive selection can not help isolation of MSC from granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood<sup>[55]</sup>. Another source is published by Jones *et al.*<sup>[56]</sup>, (2010). They showed that CD45<sup>low</sup>CD271<sup>+</sup> MSC are abundant in the trabecular bone cavity and indistinguishable from aspirated CD45<sup>low</sup>CD271<sup>+</sup> MSC. Moreover, MSC are found in human dermis. Vaculik *et al.*<sup>[57]</sup> distinguished dermal MSC from differentiated fibroblasts. For this they selected CD271<sup>+</sup> and SSEA-4<sup>+</sup> cells from adherent dermal cells and checked their differentiation capacity. They observed that a CD271<sup>+</sup> dermal population presented a greater potential for adipogenic, osteogenic and chondrogenic differentiation.

Besides CD271, several markers have been used in order to identify MSC before culture. Possibly one of the most commonly used markers for this purpose is Stro-1. After a complete review, Lv *et al.*<sup>[58]</sup>, concluded that Stro-1 expression appears not be a universal marker for MSC from different tissues. Another marker used to identify MSC is SSEA-4. It is an embryonic stem cell marker and it was documented in the isolation of genuine MSC from BM<sup>[59]</sup>. Conversely, other authors reported no detection of SSEA-4 expressing cells in unsorted BM<sup>[60,61]</sup>.

Taken together, the data discussed in this review suggest that CD271 would not be considered as a universal marker to identify MSC before culture. In the case of BM or adipose tissue, CD271 could be considered a quite suitable marker for the isolation of MSC. As described in this review, several independent studies confirm the specificity of this marker in different tissues. However, CD271 is not adequate in the isolation of MSC from other tissues such as UC or UCB. Moreover, in the case of placenta contradictory results have been obtained by different groups. These contradictory results could be due to variations in the methodologies used by different laboratories. Hines *et al.*<sup>[62]</sup> published an interesting study in which they demonstrated that membrane markers are notoriously dynamic and their expression can often be dependent on minor technical issues. Technical issues, indicating that the reproduction of results is the corner-stone of science<sup>[62]</sup>.

Due to the interest in MSC for their potential applications in the clinic, it is necessary to continue research in this field, in order to find a marker or markers for optimal selection and identification of MSCs without culture. This would allow the generation of purer cultures than those obtained by adherence to plastic alone and possibly direct application avoiding cultivation costs, time and risk of contamination.

## REFERENCES

- 1 **Farini A**, Sitzia C, Erratico S, Meregalli M, Torrente Y. Clinical applications of mesenchymal stem cells in chronic diseases. *Stem Cells Int* 2014; **2014**: 306573 [PMID: 24876848 DOI: 10.1155/2014/306573]
- 2 **Pikula M**, Marek-Trzonkowska N, Wardowska A, Renkielska A, Trzonkowski P. Adipose tissue-derived stem cells in clinical applications. *Expert Opin Biol Ther* 2013; **13**: 1357-1370 [PMID: 23919743 DOI: 10.1517/14712598.2013.823153]
- 3 **Chan TM**, Harn HJ, Lin HP, Chou PW, Chen JY, Ho TJ, Chiou TW, Chuang HM, Chiu SC, Chen YC, Yen SY, Huang MH, Liang BC, Lin SZ. Improved human mesenchymal stem cell isolation. *Cell Transplant* 2014; **23**: 399-406 [PMID: 24816441 DOI: 10.3727/096368914X678292]
- 4 **Das M**, Sundell IB, Koka PS. Adult mesenchymal stem cells and their potency in the cell-based therapy. *J Stem Cells* 2013; **8**: 1-16 [PMID: 24459809]
- 5 **Dorronsoro A**, Fernández-Rueda J, Fechter K, Ferrin I, Salcedo JM, Jakobsson E, Trigueros C. Human mesenchymal stromal cell-mediated immunoregulation: mechanisms of action and clinical applications. *Bone Marrow Res* 2013; **2013**: 203643 [PMID: 24187625 DOI: 10.1155/2013/203643]
- 6 **Bieback K**. Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures. *Transfus Med Hemother* 2013; **40**: 326-335 [PMID: 24273486 DOI: 10.1159/000354061]
- 7 **Friedenstein AJ**, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970; **3**: 393-403 [PMID: 5523063]
- 8 **Boxall SA**, Jones E. Markers for characterization of bone marrow multipotential stromal cells. *Stem Cells Int* 2012; **2012**: 975871 [PMID: 22666272 DOI: 10.1155/2012/975871]
- 9 **Bianco P**, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008; **2**: 313-319 [PMID: 18397751 DOI: 10.1016/j.stem.2008.03.002]
- 10 **Rastegar F**, Shenaq D, Huang J, Zhang W, Zhang BQ, He BC, Chen L, Zuo GW, Luo Q, Shi Q, Wagner ER, Huang E, Gao Y, Gao JL, Kim SH, Zhou JZ, Bi Y, Su Y, Zhu G, Luo J, Luo X, Qin J, Reid RR, Luu HH, Haydon RC, Deng ZL, He TC. Mesenchymal stem cells: Molecular characteristics and clinical applications. *World J Stem Cells* 2010; **2**: 67-80 [PMID: 21607123 DOI: 10.4252/wjsc.v2.i4.67]
- 11 **Hong SJ**, Jia SX, Xie P, Xu W, Leung KP, Mustoe TA, Galiano RD. Topically delivered adipose derived stem cells show an activated-fibroblast phenotype and enhance granulation tissue formation in skin wounds. *PLoS One* 2013; **8**: e55640 [PMID: 23383253]
- 12 **Huang GT**, Al-Habib M, Gauthier P. Challenges of stem cell-based pulp and dentin regeneration: a clinical perspective. *Endod Topics* 2013; **28**: 51-60 [PMID: 23914150]
- 13 **Judson RN**, Zhang RH, Rossi FM. Tissue-resident mesenchymal stem/progenitor cells in skeletal muscle: collaborators or saboteurs? *FEBS J* 2013; **280**: 4100-4108 [PMID: 23763717 DOI: 10.1111/febs.12370]
- 14 **Strioga M**, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. *Stem Cells Dev* 2012; **21**: 2724-2752 [PMID: 22468918 DOI: 10.1089/scd.2011.0722]
- 15 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606]
- 16 **Pittenger MF**, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-147 [PMID: 10102814]
- 17 **Bühring HJ**, Battula VL, Treml S, Schewe B, Kanz L, Vogel W.



- Novel markers for the prospective isolation of human MSC. *Ann N Y Acad Sci* 2007; **1106**: 262-271 [PMID: 17395729]
- 18 **Quirici N**, Soligo D, Bossolasco P, Servida F, Lumini C, Deliliers GL. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol* 2002; **30**: 783-791 [PMID: 12135677]
- 19 **Thomson TM**, Rettig WJ, Chesa PG, Green SH, Mena AC, Old LJ. Expression of human nerve growth factor receptor on cells derived from all three germ layers. *Exp Cell Res* 1988; **174**: 533-539 [PMID: 2828087]
- 20 **Jones EA**, Kinsey SE, English A, Jones RA, Straszynski L, Meredith DM, Markham AF, Jack A, Emery P, McGonagle D. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum* 2002; **46**: 3349-3360 [PMID: 12483742]
- 21 **Jones EA**, English A, Kinsey SE, Straszynski L, Emery P, Ponchel F, McGonagle D. Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells from human bone marrow. *Cytometry B Clin Cytom* 2006; **70**: 391-399 [PMID: 16977637]
- 22 **Jones E**, McGonagle D. Human bone marrow mesenchymal stem cells in vivo. *Rheumatology (Oxford)* 2008; **47**: 126-131 [PMID: 17986482]
- 23 **Kuçi Z**, Kuçi S, Zircher S, Koller S, Schubert R, Bönig H, Henschler R, Lieberz R, Klingebiel T, Bader P. Mesenchymal stromal cells derived from CD271(+) bone marrow mononuclear cells exert potent allosuppressive properties. *Cytotherapy* 2011; **13**: 1193-1204 [PMID: 21905954 DOI: 10.3109/14653249.2011.605118]
- 24 **Kuçi S**, Kuçi Z, Kreyenberg H, Deak E, Pütsch K, Huenecke S, Amara C, Koller S, Rettinger E, Grez M, Koehl U, Latifi-Pupovci H, Henschler R, Tonn T, von Laer D, Klingebiel T, Bader P. CD271 antigen defines a subset of multipotent stromal cells with immunosuppressive and lymphohematopoietic engraftment-promoting properties. *Haematologica* 2010; **95**: 651-659 [PMID: 20179086 DOI: 10.3324/haematol.2009.015065]
- 25 **Flores-Torales E**, Orozco-Barocio A, Gonzalez-Ramella OR, Carrasco-Yalan A, Gazarian K, Cuneo-Pareto S. The CD271 expression could be alone for establisher phenotypic marker in Bone Marrow derived mesenchymal stem cells. *Folia Histochem Cytobiol* 2010; **48**: 682-686 [PMID: 21478116 DOI: 10.2478/v10042-010-0063-6]
- 26 **Poloni A**, Maurizi G, Rosini V, Mondini E, Mancini S, Discepoli G, Biasio S, Battaglini G, Felicetti S, Berardinelli E, Serrani F, Leoni P. Selection of CD271(+) cells and human AB serum allows a large expansion of mesenchymal stromal cells from human bone marrow. *Cytotherapy* 2009; **11**: 153-162 [PMID: 19301169 DOI: 10.1080/14653240802582125]
- 27 **Álvarez-Viejo M**, Menendez-Menendez Y, Blanco-Gelaz MA, Ferrero-Gutierrez A, Fernandez-Rodriguez MA, Gala J, Otero-Hernandez J. Quantifying mesenchymal stem cells in the mononuclear cell fraction of bone marrow samples obtained for cell therapy. *Transplant Proc* 2013; **45**: 434-439 [PMID: 23375334 DOI: 10.1016/j.transproceed.2012.05.091]
- 28 **Mabuchi Y**, Morikawa S, Harada S, Niihe K, Suzuki S, Renault-Mihara F, Houlihan DD, Akazawa C, Okano H, Matsuzaki Y. LNGFR(+)THY-1(+)VCAM-1(hi+) cells reveal functionally distinct subpopulations in mesenchymal stem cells. *Stem Cell Reports* 2013; **1**: 152-165 [PMID: 24052950]
- 29 **Stolzinger A**, Jones E, McGonagle D, Scutt A. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech Ageing Dev* 2008; **129**: 163-173 [PMID: 18241911 DOI: 10.1016/j.mad.2007.12.002]
- 30 **Zuk PA**, Zhu M, Ashjian P, De Ugarte DA, Huang JJ, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; **13**: 4279-4295 [PMID: 12475952]
- 31 **Cuevas-Díaz Duran R**, González-Garza MT, Cardenas-Lopez A, Chavez-Castilla L, Cruz-Vega DE, Moreno-Cuevas JE. Age-related yield of adipose-derived stem cells bearing the low-affinity nerve growth factor receptor. *Stem Cells Int* 2013; **2013**: 372164 [PMID: 24376462 DOI: 10.1155/2013/372164]
- 32 **Yamamoto N**, Akamatsu H, Hasegawa S, Yamada T, Nakata S, Ohkuma M, Miyachi E, Marunouchi T, Matsunaga K. Isolation of multipotent stem cells from mouse adipose tissue. *J Dermatol Sci* 2007; **48**: 43-52 [PMID: 17644316]
- 33 **Quirici N**, Scavullo C, de Girolamo L, Lopa S, Arrigoni E, Deliliers GL, Brini AT. Anti-L-NGFR and -CD34 monoclonal antibodies identify multipotent mesenchymal stem cells in human adipose tissue. *Stem Cells Dev* 2010; **19**: 915-925 [PMID: 19929314 DOI: 10.1089/scd.2009.0408]
- 34 **Murphy SV**, Atala A. Amniotic fluid and placental membranes: unexpected sources of highly multipotent cells. *Semin Reprod Med* 2013; **31**: 62-68 [PMID: 23329638 DOI: 10.1055/s-0032-1331799]
- 35 **Margossian T**, Reppel L, Makdissy N, Stoltz JF, Bensoussan D, Huselstein C. Mesenchymal stem cells derived from Wharton's jelly: comparative phenotype analysis between tissue and in vitro expansion. *Biomed Mater Eng* 2012; **22**: 243-254 [PMID: 22785368 DOI: 10.3233/BME-2012-0714]
- 36 **Erices A**, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 2000; **109**: 235-242 [PMID: 10848804]
- 37 **Yu M**, Xiao Z, Shen L, Li L. Mid-trimester fetal blood-derived adherent cells share characteristics similar to mesenchymal stem cells but full-term umbilical cord blood does not. *Br J Haematol* 2004; **124**: 666-675 [PMID: 14871255]
- 38 **Bieback K**, Kern S, Klüter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells* 2004; **22**: 625-634 [PMID: 15277708]
- 39 **Kern S**, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006; **24**: 1294-1301 [PMID: 16410387]
- 40 **Peters R**, Wolf MJ, van den Broek M, Nuvolone M, Dannenmann S, Stieger B, Rapold R, Konrad D, Rubin A, Bertino JR, Aguzzi A, Heikenwalder M, Knuth AK. Efficient generation of multipotent mesenchymal stem cells from umbilical cord blood in stroma-free liquid culture. *PLoS One* 2010; **5**: e15689 [PMID: 21209896 DOI: 10.1371/journal.pone.0015689]
- 41 **Liu G**, Ye X, Zhu Y, Li Y, Sun J, Cui L, Cao Y. Osteogenic differentiation of GFP-labeled human umbilical cord blood derived mesenchymal stem cells after cryopreservation. *Cryobiology* 2011; **63**: 125-128 [PMID: 21684270 DOI: 10.1016/j.cryobiol.2011.05.005]
- 42 **Tio M**, Tan KH, Lee W, Wang TT, Udolph G. Roles of db-cAMP, IBMX and RA in aspects of neural differentiation of cord blood derived mesenchymal-like stem cells. *PLoS One* 2010; **5**: e9398 [PMID: 20195526]
- 43 **Zhang HT**, Chen H, Zhao H, Dai YW, Xu RX. Neural stem cells differentiation ability of human umbilical cord mesenchymal stromal cells is not altered by cryopreservation. *Neurosci Lett* 2011; **487**: 118-122 [PMID: 20946937 DOI: 10.1016/j.neulet.2010.10.008]
- 44 **Álvarez-Viejo M**, Menendez-Menendez Y, Blanco-Gelaz MA, Ferrero-Gutierrez A, Fernandez-Rodriguez MA, Perez-Basterrechea M, Garcia-Gala JM, Perez-Lopez S and Otero-Hernandez J. LNGFR (CD271) as Marker to Identify Mesenchymal Stem Cells from Different Human Sources: Umbilical Cord Blood, Wharton's Jelly and Bone Marrow. *J Bone Marrow Res* 2013; **1**: 132 [DOI: 10.4172/2329-8820.1000132]
- 45 **Perdikogianni C**, Dimitriou H, Stiakaki E, Martimianaki G, Kalmanti M. Could cord blood be a source of mesenchymal stromal cells for clinical use? *Cytotherapy* 2008; **10**: 452-459 [PMID: 18821358 DOI: 10.1080/14653240701883079]
- 46 **Attar A**, Ghalyanchi Langeroudi A, Vassaghi A, Ahrari I, Maharlooie MK, Monabati A. Role of CD271 enrichment in the isolation of mesenchymal stromal cells from umbilical cord blood. *Cell Biol Int* 2013; **37**: 1010-1015 [PMID: 23619775 DOI: 10.1002/cbin.10117]
- 47 **Watson JT**, Foo T, Wu J, Moed BR, Thorpe M, Schon L, Zhang Z. CD271 as a marker for mesenchymal stem cells in bone marrow versus umbilical cord blood. *Cells Tissues Organs* 2013; **197**:



- 496-504 [PMID: 23689142]
- 48 **Taghizadeh RR**, Cetrulo KJ, Cetrulo CL. Wharton's Jelly stem cells: future clinical applications. *Placenta* 2011; **32** Suppl 4: S311-S315 [PMID: 21733573 DOI: 10.1016/j.placenta.2011.06.010]
- 49 **Parolini O**, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA, Redl H, Sakuragawa N, Wolbank S, Zeisberger S, Zisch A, Strom SC. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* 2008; **26**: 300-311 [PMID: 17975221]
- 50 **Battula VL**, Trembl S, Abele H, Bühring HJ. Prospective isolation and characterization of mesenchymal stem cells from human placenta using a frizzled-9-specific monoclonal antibody. *Differentiation* 2008; **76**: 326-336 [PMID: 17924962]
- 51 **In't Anker PS**, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* 2004; **22**: 1338-1345 [PMID: 15579651]
- 52 **Soncini M**, Vertua E, Gibelli L, Zorzi F, Denegri M, Albertini A, Wengler GS, Parolini O. Isolation and characterization of mesenchymal cells from human fetal membranes. *J Tissue Eng Regen Med* 2007; **1**: 296-305 [PMID: 18038420]
- 53 **Logan CY**, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004; **20**: 781-810 [PMID: 15473860]
- 54 **Chong PP**, Selvaratnam L, Abbas AA, Kamarul T. Human peripheral blood derived mesenchymal stem cells demonstrate similar characteristics and chondrogenic differentiation potential to bone marrow derived mesenchymal stem cells. *J Orthop Res* 2012; **30**: 634-642 [PMID: 21922534 DOI: 10.1002/jor.21556]
- 55 **Attar A PZN**, Ahrari I, Khosravi Maharloo M, Monabati A. Assessing the role of CD271 isolation from GCSF mobilized peripheral blood in isolation of MSCs. *Cell J* 2011; **12**: 131
- 56 **Jones E**, English A, Churchman SM, Kouroupis D, Boxall SA, Kinsey S, Giannoudis PG, Emery P, McGonagle D. Large-scale extraction and characterization of CD271+ multipotential stromal cells from trabecular bone in health and osteoarthritis: implications for bone regeneration strategies based on uncultured or minimally cultured multipotential stromal cells. *Arthritis Rheum* 2010; **62**: 1944-1954 [PMID: 20222109]
- 57 **Vaculik C**, Schuster C, Bauer W, Iram N, Pfisterer K, Kramer G, Reinisch A, Strunk D, Elbe-Bürger A. Human dermis harbors distinct mesenchymal stromal cell subsets. *J Invest Dermatol* 2012; **132**: 563-574 [PMID: 22048731 DOI: 10.1038/jid.2011.355]
- 58 **Lv FJ**, Tuan RS, Cheung KM, Leung VY. Concise review: the surface markers and identity of human mesenchymal stem cells. *Stem Cells* 2014; **32**: 1408-1419 [PMID: 24578244 DOI: 10.1002/stem.1681]
- 59 **Gang EJ**, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RC. SSEA-4 identifies mesenchymal stem cells from bone marrow. *Blood* 2007; **109**: 1743-1751 [PMID: 17062733]
- 60 **Tormin A**, Li O, Brune JC, Walsh S, Schütz B, Ehinger M, Ditzel N, Kassem M, Scheding S. CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. *Blood* 2011; **117**: 5067-5077 [PMID: 21415267 DOI: 10.1182/blood-2010-08-304287]
- 61 **Wagner W**, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, Blake J, Schwager C, Eckstein V, Ansorge W, Ho AD. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol* 2005; **33**: 1402-1416 [PMID: 16263424]
- 62 **Hines WC**, Su Y, Kuhn I, Polyak K, Bissell MJ. Sorting out the FACS: a devil in the details. *Cell Rep* 2014; **6**: 779-781 [PMID: 24630040 DOI: 10.1016/j.celrep.2014.02.021]

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## Adult stem cells in neural repair: Current options, limitations and perspectives

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that defy doctors and researchers around the world. Stem cells can be divided into three main groups: (1) embryonic stem cells; (2) fetal stem cells; and (3) adult stem cells. In terms of their capacity for proliferation, stem cells are also classified as totipotent, pluripotent or multipotent. Adult stem cells, also known as somatic cells, are found in various regions of the adult organism, such as bone marrow, skin, eyes, viscera and brain. They can differentiate into unipotent cells of the residing tissue, generally for the purpose of repair. These cells represent an excellent choice in regenerative medicine, every patient can be a donor of adult stem cells to provide a more customized and efficient therapy against various diseases, in other words, they allow the opportunity of autologous transplantation. But in order to start clinical trials and achieve great results, we need to understand how these cells interact with the host tissue, how they can manipulate or be manipulated by the microenvironment where they will be transplanted and for how long they can maintain their multipotent state to provide a full regeneration.

**Key words:** Stem cells; Stem cell therapy; Adult stem cells; Neural stem cells; Bone marrow stem cells; Mesenchymal stem cells; Olfactory ensheathing cells

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**Core tip:** Adult stem cells are useful tools to treat various diseases, but we must first comprehend how they work to use their capacity at maximum. Ageing, inflammation, other stem cells of the host tissue and the co-transplantation with another stem cells type can change their profile and compromise the regeneration process. Having these barriers in mind, several researchers started to look more closely to adult stem cells. In this review we will show some interesting results from experimental and clinical trials at this group of stem cells.

### Abstract

Stem cells represent a promising step for the future of regenerative medicine. As they are able to differentiate into any cell type, tissue or organ, these cells are great candidates for treatments against the worst diseases



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## INTRODUCTION

Stem cells represent a promising step for the future of regenerative medicine. As they are able to differentiate into any cell type, tissue or organ, these cells are great candidates for treatments against the worst diseases that defy doctors and researchers around the world.

Stem cells can be divided into three main groups: (1) embryonic stem cells, which are cells of the inner layer of the blastocyst and form the three primary germ layers (ectoderm, mesoderm and endoderm); (2) fetal stem cells, which are found in the embryo and form the various organs of the human body; fetal stem cells include neural crest cells, hematopoietic cells, fetal mesenchymal stem cells, fetal neural stem cells and pancreatic islet progenitors; and (3) adult stem cells, which are found in various regions of the adult organism, such as bone marrow, skin, eyes, viscera and brain (adult stem cells are also known as somatic stem cells).

In terms of their capacity for proliferation, stem cells are also classified as totipotent, pluripotent or multipotent. Totipotent stem cells have a high capacity for differentiation and can form a complete individual, but they lack the capacity for self-renewal. Pluripotent cells, on the other hand, are capable of differentiating into any of the 200 or more kinds of cells that make up the human body. Finally, multipotent cells give rise to cells of a specific tissue, *e.g.*, hematopoietic stem cells give rise to blood cells, while neural stem cells give rise to neurons and glia<sup>[1,2]</sup>. Another group of stem cells that has been extensively studied in recent years includes induced pluripotent stem cells (IPS). IPS cells are adult differentiated cells that return to their pluripotent state by genetic engineering mechanisms. Some researchers believe these cells have great potential, but there have been numerous reports of extensive epigenetic and transcriptome aberrations with these cells, as well as tumor formation<sup>[3,4]</sup>.

## ADULT STEM CELLS

Adult stem cells are multipotent cells that can differentiate into unipotent cells of the residing tissue, generally for the purpose of repair. These cells can be obtained from several different regions of the adult organism, they can be used for autologous transplantation and their great advantage resides in the fact that embryos do not have to be destroyed in order for these cells to be obtained<sup>[5,6]</sup>.

Regardless of the cell type chosen, there is a

relative degree of difficulty in extracting viable stem cells from their *in vivo* niche. There is approximately one hematopoietic stem cell for every 10000 bone marrow cells, and one mesenchymal stem cell for every 10000-100000 cells<sup>[7]</sup>. For this reason, researchers use substances that can stimulate the growth of these cell populations as well as facilitate the process by which they are obtained. For example, G-CSF (granulocyte stimulating factor) stimulates the production of CD34<sup>+</sup> hematopoietic stem cells that can be extracted by simple puncture of peripheral blood, which is less invasive than bone marrow aspiration. Other substances that enhance the isolation of adult stem cells are currently under investigation<sup>[8]</sup>.

Another issue of concern is the cell senescence related to the expansion time *in vitro*. Cell proliferation is decreased mainly due to telomerase activity, and several researchers have focused on telomerase activation in an attempt to increase and maintain population growth *in vitro*. However, it focusing only on correcting the shortened telomeres is not sufficient, and studies should focus on the role of other factors that participate in telomere maintenance, which may also play an important role in cell differentiation<sup>[9]</sup>.

Senescence of adult organisms has to be considered, given that cell function, potency and replication decrease with time, while mutations may increase. In the case of the nervous system, mutations may result in neuronal loss, abnormal cell function, increased neuronal longevity (resulting in an even greater accumulation of mutations) and decreased regenerative capacity after an injury. These findings have been reported in studies investigating cell proliferation during learning: while young animals showed better performance than older animals, pharmacological stimulation in the older animals led to improved performance, with the newborn neurons integrating into the existent neural circuits<sup>[10-12]</sup>. Moreover, the aging of stem cell niches may contribute to the aging of stem cells, changing the organism's regulatory microenvironment, which in turn influences the aging process of other cells. To summarize can be quoted the influence of protein p53 expression levels: when expressed at low levels, it contributes to stem cell metabolic maintenance, but when expressed at high levels, it decreases the number of stem cells by inducing cell death, and the role of energetic metabolism and the production of reactive oxygen species that acts directly in stem cell niches<sup>[13]</sup>.

Another important feature of stem cells is their immunomodulatory power, which allows them to "feel" the environment and change their patterns of migration, interaction and survival. When transplanted into the lesion site, some stem cells interact with the proinflammatory environment, migrate to the site of injury and differentiate into the required cell type in order to effect repair. Moreover, some stem cells play an important role in the proliferation and migration of new cells to the site of injury. This helper profile has



been observed in neural stem cells and mesenchymal stem cells. In the case of mesenchymal stem cells, this function can play an important role in changing the macrophage proinflammatory profile into a proregenerating role, once there is no longer an urgent need for a robust immune response<sup>[14-16]</sup>.

Understanding the biology of stem cells and their niches, as well as the mechanisms involved in their multipotent state, are basic criteria to achieve great experimental results and to take the next step towards clinical trials.

## MESENCHYMAL STEM CELLS/BONE MARROW STEM CELLS

The bone marrow is the only organ in which two different types of stem cells coexist: the bone marrow stem cell (BMC, also called hematopoietic stem cell) and the mesenchymal stem cell (MSC, also called mesenchymal stromal cell). Bone marrow cells are responsible for generating blood cells, while mesenchymal stromal cells are responsible for supporting hematopoietic progenitors by regulating the niche microenvironment and by facilitating the maturation of blood cells. Mesenchymal cells can also be extracted from adipose tissue, umbilical cord, skin and placenta<sup>[17,18]</sup>.

Experimental studies have shown that MSCs can survive and migrate to the lesion site, prevent astrogliosis and microglial activation, and delay the loss of motoneurons. A comparative study between BMCs and MSCs showed that BMCs are relatively more effective in motor function recovery and have a higher survival rate<sup>[18,19]</sup>. In clinical trials with BMCs, authors reported motor and sensory improvement, as well as improvement in patients' quality of life, with some being able to sit again and even get dressed with partial assistance<sup>[19-23]</sup>. MSCs also showed great results when transplanted with T cells, controlling inflammatory activity in order to create the proper microenvironment for cell transplantation<sup>[24]</sup>. The immunomodulating effect of MSCs has also been reported in other clinical trials and MSCs have been shown to play an important role as immunomodulators and angiogenic agents in drug resistant patients as well as in patients with cerebral palsy, critical limb ischemia and kidney transplants<sup>[25-32]</sup>. In another study conducted with 40 patients, bone regeneration was achieved when a great number of CD34<sup>+</sup> cells were transplanted to the lesion site, 20 patients achieved mature bone regeneration, even in the group that received a low number of cells 4 patients achieved bone regeneration. However, when transplanted together with MSCs, these cells were able to use paracrine and autocrine cross-talk up-regulation to achieve bone regeneration<sup>[33]</sup>.

Despite these positive results, several side effects were reported when MSCs from adipose tissue were

administered *via* the cephalic vein, including chest pain and tightness, mild fever, furuncle on the upper thigh, musculoskeletal pain, painful neck and shoulder, increased sputum, upper respiratory infection, urinary incontinence, urinary tract infection, aggravation of spasticity, neuropathic pain, pain exacerbation, headache, low thyroid stimulating hormone and somnolence<sup>[34]</sup>.

## OLFACTORY ENSHEATHING CELLS

Neurogenesis in the olfactory system continues to take place even in the adult. Stem cells proliferate in the subventricular zone of the forebrain, generating neural progenitors that migrate to the olfactory bulb to create new interneurons. If an injury occurs, these neurons are immediately replaced through a surge in neurogenesis. Olfactory ensheathing cells (OECs) surround the axons of the sensory neurons in the olfactory epithelium and form synapses in the olfactory bulb in the brain. Due to their ability to guide the connections between the peripheral nervous system and the central nervous system, as well as their ability to differentiate into non-olfactory cell types, these cells are excellent candidates for cell transplantation<sup>[35]</sup>. These multipotent cells have been extensively studied in cases of spinal cord injury, and authors have reported that transplants were safe and patients experienced motor and sensory improvement, as well as recovered bladder function and activity of several muscles below the injury level<sup>[36,37]</sup>. In an amyotrophic lateral sclerosis (ALS) clinical trial conducted with OECs in China, researchers reported that patients experienced no benefits, two patients had severe side effects and one even died following transplantation<sup>[38]</sup>. One theory contends that OECs should be transplanted together with neural stem cells in order to potentiate the growth of neural processes. OECs have been shown to stimulate axon regeneration by secreting growth factors, axon guidance molecules and basement membrane components. They also aid in tissue repair by effecting structural remodeling and support, modulating the immune system, enhancing neurotrophic and antigenic stimuli and metabolizing toxic macromolecules. Finally, OECs may be transplanted together with growth factor (*e.g.*, bFGF, or basic fibroblast growth factor) to sustain cell survival and proliferation<sup>[39-41]</sup>.

## NEURAL STEM CELLS

Neural stem cells were first described by Altman in 1960 and have the potential to differentiate into any cell type in the central nervous system<sup>[42-45]</sup>. It is known that adult neurogenesis occurs in two brain regions, the subventricular and subgranular zones of the dentate gyrus, and the spinal cord<sup>[45]</sup>.

Neural stem cells have been used to treat several neurologic conditions such as spinal cord injury, ALS, Parkinson's disease, traumatic brain injury, Huntington's



**Table 1 Adult stem cells transplantations: Published clinical trials from the past 4 years**

Ref.	Year	No. of patients	Age range	Type of cell grafted	Adjuvant treatments	Follow-up
Dai <i>et al</i> <sup>[53]</sup>	2013	20	22-54 yr	MSCs	-	6 mo
García-Santos <i>et al</i> <sup>[54]</sup>	2013	11	33-61 yr	MSCs	-	12 mo
Tian <i>et al</i> <sup>[55]</sup>	2013	97	21.1-38.2 <sup>1</sup> yr	MSCs	-	14 d
Frolov <i>et al</i> <sup>[56]</sup>	2012	20	18-55 yr	HSCs	-	48 mo
Karamouzian <i>et al</i> <sup>[20]</sup>	2012	31	10-50 yr	BMCs	-	33 mo
Martínez <i>et al</i> <sup>[57]</sup>	2012	67	49.2 ± 10.3 <sup>1</sup> yr	CD133+	-	12 mo
Mazzini <i>et al</i> <sup>[28]</sup>	2012	19	20-75 yr	MSCs	-	108 mo
Moviglia <i>et al</i> <sup>[47]</sup>	2012	7	33-78 yr	NSCs	T-cell vaccine	12 mo
Prasad <i>et al</i> <sup>[58]</sup>	2012	11	30-70 yr	MNCs	-	52 wk
Brazzini <i>et al</i> <sup>[59]</sup>	2010	53	38-81 yr	BMCs	-	1-18 mo
Karussis <i>et al</i> <sup>[26]</sup>	2010	19	53.0 <sup>1</sup> yr	MSCs	-	25 mo
Lee <i>et al</i> <sup>[60]</sup>	2010	16	64.0 ± 11.6 <sup>1</sup> yr	MSCs	-	5 yr
Venkataramana <i>et al</i> <sup>[61]</sup>	2010	7	22-62 yr	MSCs	-	12-36 mo

<sup>1</sup>The authors only provide mean values. MSCs: Mesenchymal stem cells; HSCs: Hematopoietic stem cells; BMCs: Bone marrow stem cells; NSCs: Neural stem cells; MNCs: Mononuclear cells from bone marrow.

s disease and several demyelinating diseases. The main issues to consider when using neural stem cells are how the transplanted cells will interact with the host microenvironment, how the local immunological response will interfere with and prevent neurorestoration, and how the transplanted neural stem cells will modulate the microenvironment *via* paracrine and autocrine effects. These mechanisms need to be clarified before moving on to clinical trials. Several investigators have questioned the behavior of transplanted cells and several transplantation strategies have been tested, including co-transplantation with other stem cell types, T cells or neurospheres. Nevertheless, much work needs to be done in order to better comprehend how neural stem cells interact with the host tissue<sup>[45-52]</sup>. Clinical trials have not shown statistically significant results, Moviglia *et al*<sup>[47]</sup> transplanted neural stem cells in seven patients with ALS and only in five patients were observed motor improvements. In their combined protocol the local immunological response were controlled by a T-cell vaccination before the transplants of NSCs, but authors agree that the transplantation of neural stem cells is safe and feasible<sup>[46-49]</sup> (Table 1).

## CONCLUSION

Adult stem cell transplantation represents a promising choice of treatment for the field of regenerative medicine, but several aspects must still be clarified before proceeding with clinical trials. More studies are needed to establish how to obtain a large population of adult stem cells and to ensure the safety and viability of the transplants. We must also understand these cells' mechanisms of interaction and how we can use these mechanisms to achieve full regeneration.

Based on the studies cited here, it is possible to affirm that in the near future we will have effective therapies against various diseases that affect and challenge the medical community and the population

at large.

## REFERENCES

- 1 **Gage FH.** Mammalian neural stem cells. *Science* 2000; **287**: 1433-1438 [PMID: 10688783 DOI: 10.1126/science.287.5457.1433]
- 2 **Hosseinkhani M, Shirazi R, Rajaei F, Mahmoudi M, Mohammadi N, Abbasi M.** Engineering of the embryonic and adult stem cell niches. *Iran Red Crescent Med J* 2013; **15**: 83-92 [PMID: 23682319 DOI: 10.5812/ircmj.7541]
- 3 **Ma H, Morey R, O'Neil RC, He Y, Daughtry B, Schultz MD, Hariharan M, Nery JR, Castanon R, Sabatini K, Thiagarajan RD, Tachibana M, Kang E, Tippner-Hedges R, Ahmed R, Gutierrez NM, Van Dyken C, Polat A, Sugawara A, Sparman M, Gokhale S, Amato P, Wolf DP, Ecker JR, Laurent LC, Mitalipov S.** Abnormalities in human pluripotent cells due to reprogramming mechanisms. *Nature* 2014; **511**: 177-183 [PMID: 25008523 DOI: 10.1038/nature13551]
- 4 **Nishimori M, Yakushiji H, Mori M, Miyamoto T, Yaguchi T, Ohno S, Miyake Y, Sakaguchi T, Ueda M, Ohno E.** Tumorigenesis in cells derived from induced pluripotent stem cells. *Hum Cell* 2014; **27**: 29-35 [PMID: 24122447 DOI: 10.1007/s13577-013-0078-3]
- 5 **Bongso A, Richards M.** History and perspective of stem cell research. *Best Pract Res Clin Obstet Gynaecol* 2004; **18**: 827-842 [PMID: 15582541 DOI: 10.1016/j.bpobgyn.2004.09.002]
- 6 **Larijani B, Esfahani EN, Amini P, Nikbin B, Alimoghaddam K, Amiri S, Malekzadeh R, Yazdi NM, Ghodsi M, Dowlati Y, Sahraian MA, Ghavamzadeh A.** Stem cell therapy in treatment of different diseases. *Acta Med Iran* 2012; **50**: 79-96 [PMID: 22359076]
- 7 **Spradling A, Drummond-Barbosa D, Kai T.** Stem cells find their niche. *Nature* 2001; **414**: 98-104 [PMID: 11689954 DOI: 10.1038/35102160]
- 8 **Bonig H, Becker PS, Schwebig A, Turner M.** Biosimilar granulocyte-colony-stimulating factor for healthy donor stem cell mobilization: need we be afraid? *Transfusion* 2014; **55**: 430-439 [PMID: 24965197 DOI: 10.1111/trf.12770]
- 9 **Zimmermann S, Glaser S, Ketteler R, Waller CF, Klingmüller U, Martens UM.** Effects of telomerase modulation in human hematopoietic progenitor cells. *Stem Cells* 2004; **22**: 741-749 [PMID: 15342938 DOI: 10.1634/stemcells.22-5-741]
- 10 **Brann JH, Firestein SJ.** A lifetime of neurogenesis in the olfactory system. *Front Neurosci* 2014; **8**: 182 [PMID: 25018692 DOI: 10.3389/fnins.2014.00182]
- 11 **Mandairon N, Sultan S, Nouvian M, Sacquet J, Didier A.** Involvement of newborn neurons in olfactory associative learning? The operant or non-operant component of the task makes all the difference. *J Neurosci* 2011; **31**: 12455-12460 [PMID: 21880907 DOI: 10.1523/JNEUROSCI.2919-11.2011]



- 12 **Moreno M**, Richard M, Landrein B, Sacquet J, Didier A, Mandairon N. Alteration of olfactory perceptual learning and its cellular basis in aged mice. *Neurobiol Aging* 2014; **35**: 680-691 [PMID: 24112795 DOI: 10.1016/j.neurobiolaging.2013.08.034]
- 13 **Signer RA**, Morrison SJ. Mechanisms that regulate stem cell aging and life span. *Cell Stem Cell* 2013; **12**: 152-165 [PMID: 23395443 DOI: 10.1016/j.stem.2013.01.001]
- 14 **Giusto E**, Donegà M, Cossetti C, Pluchino S. Neuro-immune interactions of neural stem cell transplants: from animal disease models to human trials. *Exp Neurol* 2014; **260**: 19-32 [PMID: 23507035 DOI: 10.1016/j.expneurol.2013.03.009]
- 15 **Ulivi V**, Tasso R, Cancedda R, Descalzi F. Mesenchymal stem cell paracrine activity is modulated by platelet lysate: induction of an inflammatory response and secretion of factors maintaining macrophages in a proinflammatory phenotype. *Stem Cells Dev* 2014; **23**: 1858-1869 [PMID: 24720766 DOI: 10.1089/scd.2013.0567]
- 16 **Xing J**, Hou T, Jin H, Luo F, Change Z, Li Z, Xie Z, Xu J. Inflammatory microenvironment changes the secretory profile of mesenchymal stem cells to recruit mesenchymal stem cells. *Cell Physiol Biochem* 2014; **33**: 905-919 [PMID: 24713626 DOI: 10.1159/000358663]
- 17 **Chen Z**, Wang Y, Shi C. Therapeutic implications of newly identified stem cell populations from the skin dermis. *Cell Transplant* 2014 Jun 26; Epub ahead of print [PMID: 24972091 DOI: 10.3727/096368914X682431]
- 18 **Pastor D**, Viso-León MC, Jones J, Jaramillo-Merchán J, Toledo-Aral JJ, Moraleda JM, Martínez S. Comparative effects between bone marrow and mesenchymal stem cell transplantation in GDNF expression and motor function recovery in a motoneuron degenerative mouse model. *Stem Cell Rev* 2012; **8**: 445-458 [PMID: 21717132 DOI: 10.1007/s12015-011-9295-x]
- 19 **Vercelli A**, Mereuta OM, Garbossa D, Muraca G, Mareschi K, Rustichelli D, Ferrero I, Mazzini L, Madon E, Fagioli F. Human mesenchymal stem cell transplantation extends survival, improves motor performance and decreases neuroinflammation in mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis* 2008; **31**: 395-405 [PMID: 18586098 DOI: 10.1016/j.nbd.2008.05.016]
- 20 **Karamouzian S**, Nematollahi-Mahani SN, Nakhaee N, Eskandary H. Clinical safety and primary efficacy of bone marrow mesenchymal cell transplantation in subacute spinal cord injured patients. *Clin Neurol Neurosurg* 2012; **114**: 935-939 [PMID: 22464434 DOI: 10.1016/j.clineuro.2012.02.003]
- 21 **Pal R**, Venkataramana NK, Bansal A, Balaraju S, Jan M, Chandra R, Dixit A, Rauthan A, Murgod U, Totey S. Ex vivo-expanded autologous bone marrow-derived mesenchymal stromal cells in human spinal cord injury/paraplegia: a pilot clinical study. *Cytotherapy* 2009; **11**: 897-911 [PMID: 19903102 DOI: 10.3109/14653240903253857]
- 22 **Saito F**, Nakatani T, Iwase M, Maeda Y, Hirakawa A, Murao Y, Suzuki Y, Onodera R, Fukushima M, Ide C. Spinal cord injury treatment with intrathecal autologous bone marrow stromal cell transplantation: the first clinical trial case report. *J Trauma* 2008; **64**: 53-59 [PMID: 18188099 DOI: 10.1097/TA.0b013e31815b847d]
- 23 **Saito F**, Nakatani T, Iwase M, Maeda Y, Murao Y, Suzuki Y, Fukushima M, Ide C. Administration of cultured autologous bone marrow stromal cells into cerebrospinal fluid in spinal injury patients: a pilot study. *Restor Neurol Neurosci* 2012; **30**: 127-136 [PMID: 22232031 DOI: 10.3233/RNN-2011-0629]
- 24 **Moviglia GA**, Fernandez Viña R, Brizuela JA, Saslavsky J, Vrsalovic F, Varela G, Bastos F, Farina P, Etchegaray G, Barbieri M, Martinez G, Picasso F, Schmidt Y, Brizuela P, Gaeta CA, Costanzo H, Moviglia Brandolino MT, Merino S, Pes ME, Veloso MJ, Rugilo C, Tamer I, Shuster GS. Combined protocol of cell therapy for chronic spinal cord injury. Report on the electrical and functional recovery of two patients. *Cytotherapy* 2006; **8**: 202-209 [PMID: 16793729 DOI: 10.1080/14653240600736048]
- 25 **Gupta PK**, Chullikana A, Parakh R, Desai S, Das A, Gottipamula S, Krishnamurthy S, Anthony N, Pherwani A, Majumdar AS. A double blind randomized placebo controlled phase I/II study assessing the safety and efficacy of allogeneic bone marrow derived mesenchymal stem cell in critical limb ischemia. *J Transl Med* 2013; **11**: 143 [PMID: 23758736 DOI: 10.1186/1479-5876-11-143]
- 26 **Karussis D**, Karageorgiou C, Vaknin-Dembinsky A, Gowda-Kurkalli B, Gomori JM, Kassis I, Bulte JW, Petrou P, Ben-Hur T, Abramsky O, Slavin S. Safety and immunological effects of mesenchymal stem cell transplantation in patients with multiple sclerosis and amyotrophic lateral sclerosis. *Arch Neurol* 2010; **67**: 1187-1194 [PMID: 20937945 DOI: 10.1001/archneurol.2010.248]
- 27 **Mazzini L**, Ferrero I, Luparello V, Rustichelli D, Gunetti M, Mareschi K, Testa L, Stecco A, Tarletti R, Miglioretti M, Fava E, Nasuelli N, Cisari C, Massara M, Vercelli R, Oggioni GD, Carriero A, Cantello R, Monaco F, Fagioli F. Mesenchymal stem cell transplantation in amyotrophic lateral sclerosis: A Phase I clinical trial. *Exp Neurol* 2010; **223**: 229-237 [PMID: 19682989 DOI: 10.1016/j.expneurol.2009.08.007]
- 28 **Mazzini L**, Mareschi K, Ferrero I, Miglioretti M, Stecco A, Servo S, Carriero A, Monaco F, Fagioli F. Mesenchymal stromal cell transplantation in amyotrophic lateral sclerosis: a long-term safety study. *Cytotherapy* 2012; **14**: 56-60 [PMID: 21954839 DOI: 10.3109/14653249.2011.613929]
- 29 **Mazzini L**, Mareschi K, Ferrero I, Vassallo E, Oliveri G, Nasuelli N, Oggioni GD, Testa L, Fagioli F. Stem cell treatment in Amyotrophic Lateral Sclerosis. *J Neurol Sci* 2008; **265**: 78-83 [PMID: 17582439 DOI: 10.1016/j.jns.2007.05.016]
- 30 **Perico N**, Casiraghi F, Gotti E, Introna M, Todeschini M, Cavinato RA, Capelli C, Rambaldi A, Cassis P, Rizzo P, Cortinovis M, Noris M, Remuzzi G. Mesenchymal stromal cells and kidney transplantation: pretransplant infusion protects from graft dysfunction while fostering immunoregulation. *Transpl Int* 2013; **26**: 867-878 [PMID: 23738760 DOI: 10.1111/tri.12132]
- 31 **Skrachin A**, Ahmed RK, Ferrara G, Rane L, Poiret T, Isaikina Y, Skrahina A, Zumla A, Maeurer MJ. Autologous mesenchymal stromal cell infusion as adjunct treatment in patients with multidrug and extensively drug-resistant tuberculosis: an open-label phase I safety trial. *Lancet Respir Med* 2014; **2**: 108-122 [PMID: 24503266 DOI: 10.1016/S2213-2600(13)70234-0]
- 32 **Wang X**, Cheng H, Hua R, Yang J, Dai G, Zhang Z, Wang R, Qin C, An Y. Effects of bone marrow mesenchymal stromal cells on gross motor function measure scores of children with cerebral palsy: a preliminary clinical study. *Cytotherapy* 2013; **15**: 1549-1562 [PMID: 24100132 DOI: 10.1016/j.jcyt.2013.06.001]
- 33 **Marx RE**, Harrell DB. Translational research: The CD34+ cell is crucial for large-volume bone regeneration from the milieu of bone marrow progenitor cells in craniomandibular reconstruction. *Int J Oral Maxillofac Implants* 2014; **29**: e201-e209 [PMID: 24683583 DOI: 10.11607/jomi.te56]
- 34 **Ra JC**, Shin IS, Kim SH, Kang SK, Kang BC, Lee HY, Kim YJ, Jo JY, Yoon EJ, Choi HJ, Kwon E. Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev* 2011; **20**: 1297-1308 [PMID: 21303266 DOI: 10.1089/scd.2010.0466]
- 35 **Mackay-Sim A**, St John JA. Olfactory ensheathing cells from the nose: clinical application in human spinal cord injuries. *Exp Neurol* 2011; **229**: 174-180 [PMID: 20832402 DOI: 10.1016/j.expneurol.2010.08.025]
- 36 **Rao Y**, Zhu W, Liu H, Jia C, Zhao Q, Wang Y. Clinical application of olfactory ensheathing cells in the treatment of spinal cord injury. *J Int Med Res* 2013; **41**: 473-481 [PMID: 23569013 DOI: 10.1177/0300060513476426]
- 37 **Tabakow P**, Jarmundowicz W, Czapiaga B, Fortuna W, Miedzybrodzki R, Czyz M, Huber J, Szarek D, Okurowski S, Szewczyk P, Gorski A, Raisman G. Transplantation of autologous olfactory ensheathing cells in complete human spinal cord injury. *Cell Transplant* 2013; **22**: 1591-1612 [PMID: 24007776 DOI: 10.3727/096368912X663532]
- 38 **Piepers S**, van den Berg LH. No benefits from experimental treatment with olfactory ensheathing cells in patients with ALS. *Amyotroph Lateral Scler* 2010; **11**: 328-330 [PMID: 20433414 DOI: 10.3109/17482961003663555]
- 39 **Pellitteri R**, Catania MV, Bonaccorso CM, Ranno E, Dell'Albani



- P, Zaccheo D. Viability of olfactory ensheathing cells after hypoxia and serum deprivation: Implication for therapeutic transplantation. *J Neurosci Res* 2014; **92**: 1757-1766 [PMID: 24975631 DOI: 10.1002/jnr.23442]
- 40 Roet KC, Verhaagen J. Understanding the neural repair-promoting properties of olfactory ensheathing cells. *Exp Neurol* 2014; **261C**: 594-609 [PMID: 24842489 DOI: 10.1016/j.expneurol.2014.05.007]
- 41 Sethi R, Redmond A, Lavik E. Olfactory Ensheathing Cells Promote Differentiation of Neural Stem Cells and Robust Neurite Extension. *Stem Cell Rev* 2014; **10**: 772-785 [PMID: 24996386 DOI: 10.1007/s12015-014-9539-7]
- 42 Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol* 1965; **124**: 319-335 [PMID: 5861717 DOI: 10.1002/cne.901240303]
- 43 Altman J, Das GD. Post-natal origin of microneurons in the rat brain. *Nature* 1965; **207**: 953-956 [PMID: 5886931 DOI: 10.1038/207953a0]
- 44 Andressen C. Neural stem cells: from neurobiology to clinical applications. *Curr Pharm Biotechnol* 2013; **14**: 20-28 [PMID: 23092257 DOI: 10.2174/1389201011314010005]
- 45 Batista CE, Mariano ED, Marie SK, Teixeira MJ, Morgalla M, Tatagiba M, Li J, Lepski G. Stem cells in neurology--current perspectives. *Arq Neuropsiquiatr* 2014; **72**: 457-465 [PMID: 24964114 DOI: 10.1590/0004-282X20140045]
- 46 Glass JD, Boulis NM, Johe K, Rutkove SB, Federici T, Polak M, Kelly C, Feldman EL. Lumbar intraspinal injection of neural stem cells in patients with amyotrophic lateral sclerosis: results of a phase I trial in 12 patients. *Stem Cells* 2012; **30**: 1144-1151 [PMID: 22415942 DOI: 10.1002/stem.1079]
- 47 Moviglia GA, Moviglia-Brandolino MT, Varela GS, Albanese G, Piccone S, Echegaray G, Martinez G, Blassetti N, Farias J, Farina P, Perusso A, Gaeta CA. Feasibility, safety, and preliminary proof of principles of autologous neural stem cell treatment combined with T-cell vaccination for ALS patients. *Cell Transplant* 2012; **21** Suppl 1: S57-S63 [PMID: 22507681 DOI: 10.3727/096368912X633770]
- 48 Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G, Martinello M, Cattalini A, Bergami A, Furlan R, Comi G, Constantin G, Martino G. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. *Nature* 2005; **436**: 266-271 [PMID: 16015332 DOI: 10.1038/nature03889]
- 49 Riley J, Federici T, Polak M, Kelly C, Glass J, Raore B, Taub J, Kesner V, Feldman EL, Boulis NM. Intraspinal stem cell transplantation in amyotrophic lateral sclerosis: a phase I safety trial, technical note, and lumbar safety outcomes. *Neurosurgery* 2012; **71**: 405-416; discussion 416 [PMID: 22565043 DOI: 10.1227/NEU.0b013e31825ca05f]
- 50 Zhu J, Wu X, Zhang HL. Adult neural stem cell therapy: expansion in vitro, tracking in vivo and clinical transplantation. *Curr Drug Targets* 2005; **6**: 97-110 [PMID: 15720217 DOI: 10.2174/1389450053345055]
- 51 Zhu J, Zhou L, Xingwu F. Tracking neural stem cells in patients with brain trauma. *N Engl J Med* 2006; **355**: 2376-2378 [PMID: 17135597 DOI: 10.1056/NEJMc055304]
- 52 Zhu W, Mao Y, Zhou LF. Reduction of neural and vascular damage by transplantation of VEGF-secreting neural stem cells after cerebral ischemia. *Acta Neurochir Suppl* 2005; **95**: 393-397 [PMID: 16463888 DOI: 10.1007/3-211-32318-X\_80]
- 53 Dai G, Liu X, Zhang Z, Yang Z, Dai Y, Xu R. Transplantation of autologous bone marrow mesenchymal stem cells in the treatment of complete and chronic cervical spinal cord injury. *Brain Res* 2013; **1533**: 73-79 [PMID: 23948102 DOI: 10.1016/j.brainres.2013.08.016]
- 54 García Santos JM, Blanquer M, Torres del Río S, Iniesta F, Espuch JG, Pérez-Espejo MÁ, Martínez S, Moraleda JM. Acute and chronic MRI changes in the spine and spinal cord after surgical stem cell grafting in patients with definite amyotrophic lateral sclerosis: post-infusion injuries are unrelated with clinical impairment. *Magn Reson Imaging* 2013; **31**: 1298-1308 [PMID: 23810205 DOI: 10.1016/j.mri.2013.05.006]
- 55 Tian C, Wang X, Wang X, Wang L, Wang X, Wu S, Wan Z. Autologous bone marrow mesenchymal stem cell therapy in the subacute stage of traumatic brain injury by lumbar puncture. *Exp Clin Transplant* 2013; **11**: 176-181 [PMID: 22891928 DOI: 10.6002/ect.2012.0053]
- 56 Frolov AA, Bryukhovetskiy AS. Effects of hematopoietic autologous stem cell transplantation to the chronically injured human spinal cord evaluated by motor and somatosensory evoked potentials methods. *Cell Transplant* 2012; **21** Suppl 1: S49-S55 [PMID: 22507680 DOI: 10.3727/096368912X633761]
- 57 Martínez HR, Molina-Lopez JF, González-Garza MT, Moreno-Cuevas JE, Caro-Orsorio E, Gil-Valadez A, Gutierrez-Jimenez E, Zazueta-Fierro OE, Meza JA, Couret-Alcaraz P, Hernandez-Torre M. Stem cell transplantation in amyotrophic lateral sclerosis patients: methodological approach, safety, and feasibility. *Cell Transplant* 2012; **21**: 1899-1907 [PMID: 23356668 DOI: 10.3727/096368911X582769]
- 58 Prasad K, Mohanty S, Bhatia R, Srivastava MV, Garg A, Srivastava A, Goyal V, Tripathi M, Kumar A, Bal C, Vij A, Mishra NK. Autologous intravenous bone marrow mononuclear cell therapy for patients with subacute ischaemic stroke: a pilot study. *Indian J Med Res* 2012; **136**: 221-228 [PMID: 22960888]
- 59 Brazzini A, Cantella R, De la Cruz A, Yupanqui J, León C, Jorquiera T, Brazzini M, Ortega M, Saenz LN. Intraarterial autologous implantation of adult stem cells for patients with Parkinson disease. *J Vasc Interv Radiol* 2010; **21**: 443-451 [PMID: 20346882 DOI: 10.1016/j.jvir.2010.01.008]
- 60 Lee JS, Hong JM, Moon GJ, Lee PH, Ahn YH, Bang OY. A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells* 2010; **28**: 1099-1106 [PMID: 20506226 DOI: 10.1002/stem.430]
- 61 Venkataramana NK, Kumar SK, Balaraju S, Radhakrishnan RC, Bansal A, Dixit A, Rao DK, Das M, Jan M, Gupta PK, Totey SM. Open-labeled study of unilateral autologous bone-marrow-derived mesenchymal stem cell transplantation in Parkinson's disease. *Transl Res* 2010; **155**: 62-70 [PMID: 20129486 DOI: 10.1016/j.trsl.2009.07.006]

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## Development of cancer-initiating cells and immortalized cells with genomic instability

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epigenetic aberrations. The development of genomic instability is associated with mutations that contribute to cellular immortalization and transformation. Cancer occurs when cancer-initiating cells (CICs), also called cancer stem cells, develop as a result of these mutations. In this paper, we explore how CICs develop as a result of genomic instability, including looking at which cancer suppression mechanisms are abrogated. A recent *in vitro* study revealed the existence of a CIC induction pathway in differentiating stem cells. Under aberrant differentiation conditions, cells become senescent and develop genomic instabilities that lead to the development of CICs. The resulting CICs contain a mutation in the alternative reading frame of *CDKN2A* (ARF)/p53 module, *i.e.*, in either ARF or p53. We summarize recently established knowledge of CIC development and cellular immortality, explore the role of the ARF/p53 module in protecting cells from transformation, and describe a risk factor for genomic destabilization that increases during the process of normal cell growth and differentiation and is associated with the downregulation of histone H2AX to levels representative of growth arrest in normal cells.

**Key words:** ARF/p53 module; Cancer stem cells; Cancer-initiating cells; Differentiation; Genomic instability; H2AX

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**Core tip:** Cancer usually develops in conjunction with genomic instability and multiple genetic mutations. Only a small number of cells, called cancer-initiating cells (CICs), are the progenitors of cancerous tissue; but how genomic instability and genetic mutations prompt CICs to develop is still unclear. Recent investigations have uncovered the existence of a pathway that could be responsible. This review explores how that pathway might induce the development of CICs, the tumor suppression mechanisms that must be abrogated in order for malignancies to occur, and the role of the alternative reading frame of *CDKN2A* (ARF)/p53 module/p53 module in protecting

### Abstract

Cancers that develop after middle age usually exhibit genomic instability and multiple mutations. This is in direct contrast to pediatric tumors that usually develop as a result of specific chromosomal translocations and



normal cells from oncologic transformation.

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## INTRODUCTION

Somatic stem cells are responsible for the development and homeostasis of organs and other bodily tissues. Cancer-initiating cells (CICs), or cancer stem cells, are responsible for the development<sup>[1,2]</sup>, metastasis, and drug resistance<sup>[3-6]</sup> of tumors. Although CIC characteristics are increasingly being well defined, how CICs develop remains unclear.

Unlike pediatric tumors, which usually develop as a result of very specific chromosomal translocations<sup>[7,8]</sup> and epigenetic aberrations<sup>[9,10]</sup>, cancers that become more common around the age of 40 years exhibit extreme chromosomal instability (CIN) or microsatellite instability (MSI)<sup>[11-14]</sup>. MSI occurs in cells that do not have adequate mismatch repair systems<sup>[15-17]</sup>, and CIN occurs in the presence of other types of repair deficiencies. The hereditary versions of myelodysplastic syndrome<sup>[18]</sup>, breast and ovarian cancers<sup>[19-22]</sup>, and skin cancers<sup>[23-25]</sup> are examples of CIN. CIN can even occur in normal senescent cells<sup>[26,27]</sup>.

MSI and CIN usually do not occur together and are considered mutually exclusive. For example, 15% of colorectal cancers exhibit MSI, but most of the remainder exhibit CIN. There are also tumors that do not exhibit either MSI or CIN and result from DNA polymerase  $\epsilon$  mutations, which interfere with proofreading functions and produce hypermutations<sup>[28,29]</sup>. Massive genomic rearrangements occur in a wide variety of cancers<sup>[30-32]</sup>, and the unstable structures that result vary widely<sup>[33,34]</sup>. Since only a very small number of CICs are necessary to produce a malignancy, what causes CICs to develop in the first place is an important question<sup>[35,36]</sup>.

In malignant cells, mutations in either alternative reading frame of *CDKN2A* (ARF) or p53 are common. The ARF/p53 module plays a major role in keeping normal cells from transforming into malignancies, so these mutations that interfere with ARF/p53 functioning show us how the barrier reactions performed by the ARF/p53 module normally work and the way genomic instability promotes the development of CICs.

## EFFECTS OF GENOMIC INSTABILITY

Like cancer cells that develop because of genomic instability *in vivo*<sup>[37-40]</sup>, cells cultured *in vitro* can also be transformed and/or immortalized in association with either CIN- or MSI-type genomic instabilities<sup>[26]</sup> and

mutations in the ARF/p53 module<sup>[41]</sup>. Following serial proliferation, normal mouse embryonic fibroblast cells (MEFs) stop reproducing after a growth arrest command issued by the ARF/p53 module<sup>[41]</sup>. This command prevents cells from immortalizing<sup>[27,42]</sup>, but immortality can develop if the genome is destabilized<sup>[43]</sup>, clearly demonstrating that genomic instability is a triggering event in cellular immortalization.

Genomic instability probably contributes to the induction of mutations in the ARF/p53 module<sup>[27,42]</sup>. In MEFs, immortalization can result if genomic instability is induced and/or mutations occur in either ARF or p53 (Figure 1), but is blocked in cells with stable genomes that are under the continuous regulation of the ARF/p53 module<sup>[27]</sup>. Healthy ARF/p53 regulation is essential for the prevention of cellular transformation and immortalization, but which functions of ARF and p53 are responsible for tumor suppression is controversial.

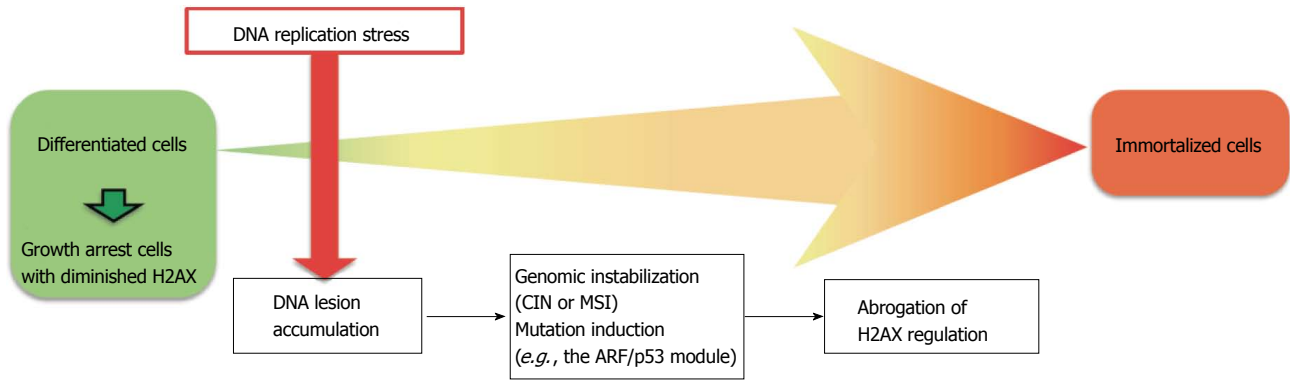
It was generally believed that p53 suppresses tumors by inducing apoptosis and senescence<sup>[44-47]</sup>, but recent studies have cast doubt on this hypothesis. For example, in multiple transgenic mouse models in which p53 cannot induce cell-cycle arrest and apoptosis after DNA damage, the number of malignancies that form as a result is not significantly higher than the rate of malignancies in normal mice<sup>[48-50]</sup>. These observations raise questions regarding the exact role of p53 in tumor suppression. One hypothesis is that p53 regulates metabolism by inhibiting glycolysis<sup>[51-53]</sup> and/or activating the mammalian target of rapamycin pathway<sup>[52,54,55]</sup>.

## EFFECT OF MUTATIONS IN THE ARF/P53 MODULE

Because ARF and p53 are mutated in cancer cells in a mutually exclusive manner, it is likely that the p53 functions that are essential for cancer suppression are expressed under the control of ARF<sup>[56]</sup>. Intriguingly, transgenic mice with an extra copy of *Arf* and *p53* (super-*Arf/p53* mice) exhibit both reduced rates of cancer and slower overall aging<sup>[41]</sup>. Furthermore, MEFs derived from these mice rarely immortalize spontaneously. Therefore, it is likely that the roles of ARF and p53 in cancer suppression are involved in the maintenance of homeostasis *in vivo* and are primarily regulated at the level of individual cells. This cellular-level protection against transformation is generally associated with growth arrest and low levels of the histone H2A variant H2AX<sup>[27,57]</sup>, which is required for active cell growth<sup>[58]</sup>.

The downregulation of H2AX is governed by the ARF/p53 module<sup>[27,42]</sup>. Growth-arrested cells in the liver, spleen, and other organs of healthy adult mice generally exhibit downregulated levels of H2AX, whereas immortalized/transformed cells, which have mutations in the ARF/p53 module, have normal H2AX levels and exhibit active growth<sup>[27,57]</sup>.





**Figure 1 Model of cellular immortalization in association with genomic instability.** As illustrated in mouse embryonic fibroblasts, immortality is induced by genomic instability and mutation of either Arf or p53. Growth arrest and the downregulation of H2AX protect mouse embryonic fibroblasts against immortalization. Because H2AX downregulation is dependent on the ARF/p53 module, cells with mutations in this module recover H2AX expression and growth activity. CIN: Chromosomal instability; MSI: Microsatellite instability.

As described above, cellular transformation is suppressed primarily by the induction of growth arrest and the downregulation of H2AX under the control of the ARF/p53 module. It is promoted by genomic instability and the mutations it produces. Unfortunately, when H2AX is downregulated, the mechanisms that repair lesions fail and this is a risk factor for the genomic instability and tumorigenesis seen in many hereditary cancers<sup>[15-25]</sup>. Despite the aforementioned protective effect of H2AX downregulation, the risk of sporadic cancer development is probably due, in large part, to a reduction in H2AX levels and the associated repair deficiencies<sup>[27]</sup>. In fact, cells without H2AX exhibit faulty homologous recombination and non-homologous end-joining during DNA repair<sup>[59,60]</sup>, which results in elevated genomic instability<sup>[58]</sup>.

## CIC DEVELOPMENT IS DISTINCT FROM IMMORTALIZATION

Although immortalized MEFs develop in association with genomic instability and mutations in the ARF/p53 module<sup>[26,41]</sup>, the resultant cells do not exhibit robust tumor-forming ability unless they are pre-transformed by oncogenes<sup>[61,62]</sup>. This observation illustrates the difference between immortalized MEFs and CICs. MEFs are differentiated cells and do not exhibit stem cell characteristics, but CICs act like stem cells in cancer-tissue development. In fact, CICs share a number of common characteristics with normal stem cells, including embryonic stem (ES) cells and induced pluripotent stem cells, but do not resemble immortalized MEFs with their high dependence on normal glycolysis<sup>[63,64]</sup>, sphere-forming ability, and expressed stem cell marker genes<sup>[65-67]</sup>. Cancer tissues generally exhibit the "Warburg Effect" seen in normal stem cells that produce elevated glycolysis.

### Transformation of normal stem cells into CICs after genomic destabilization

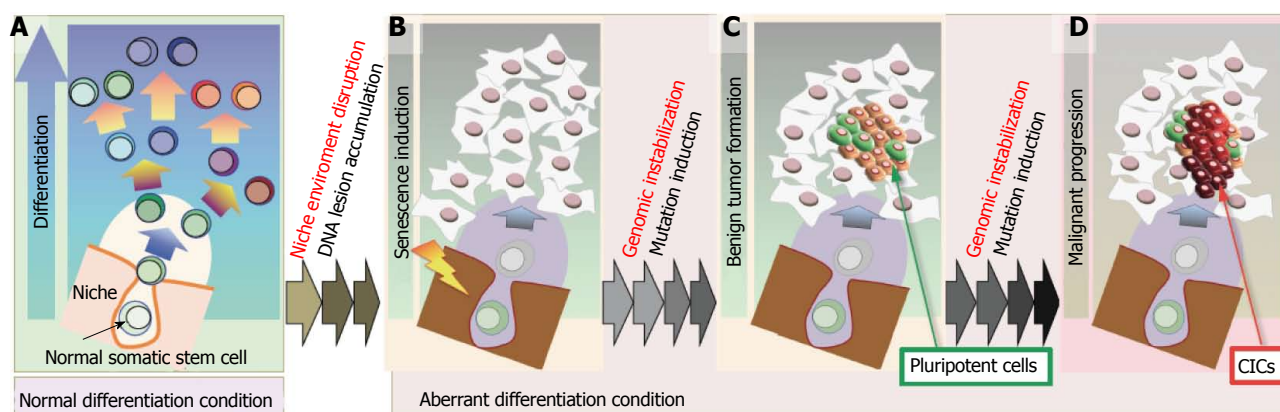
To truly understand cancer, we must discover how

genomic instability promotes the development of CICs. CICs may develop *via* multiple pathways. A recent study using normal murine ES cells as a model showed that one of these possible pathways might be created when normal stem cells are developing under unstable genomic conditions. Under aberrant differentiation conditions, ES cells with the ability to form normal mice develop high levels of DNA lesions. These lesions induce cellular senescence and genomic instability, ultimately leading to renewed growth in cells that harbor mutations in the ARF/p53 module<sup>[68]</sup>. These cells exhibit a number of stem cell characteristics, including sphere formation and the expression of undifferentiated marker genes such as *Nanog*, *Klf4*, *Oct3/4*, and *Sox2*, even in a growth factor-poor medium containing 10% newborn bovine serum with no leukemia inhibitory factor. Furthermore, these cells possess tumor-forming ability and express c-Myc and CIC markers such as CD133, CD33, and CD34.

The fact that oncogenesis can be triggered by niche disruption supports this theory. For example, both leukemia and myelodysplasia develop after dysregulation of the stem cell niche<sup>[69]</sup>, and cancers often develop from stem cells that are injected at heterotopic sites<sup>[70]</sup>. Stem cells growing in such an aberrant environment do not get what they need to maintain themselves properly and often transform into CICs<sup>[70]</sup>. Another example is embryonal carcinomas that can develop from cells transplanted from the inside of blastocysts and from primordial germ cells derailed from the migration track<sup>[71,72]</sup>. These *in vivo* and *in vitro* findings suggest that the genomic instability that leads to the development of CICs can be triggered by aberrations in the environment when stem cells are differentiating.

Because cancer development is initiated by CICs, these cells are considered promising targets for chemotherapy aimed at either killing them directly or getting them to differentiate<sup>[73-75]</sup>. However, this strategy is not always successful, partly because CICs are so adaptable and plastic<sup>[76,77]</sup>. In one study in





**Figure 2 Model of cancer-initiating cell development.** A: In normal tissues, stem cells are maintained in a specific niche and the niche environment is important both for stem cell maintenance and organ homeostasis; B: Niche disruption deranges the differentiation environment of stem cells, leading to the accumulation of DNA lesions and the induction of cellular senescence. Such DNA lesions trigger genomic destabilization, leading to the development of benign tumors that contain a small number of C: Pluripotent cells that eventually develop into; D: Cancer-initiating cells (CICs) that cause malignancies.

which CICs developed from ES cells, transformation was coupled with the acquisition of plasticity. ES cells start to differentiate in response to leukemia inhibitory factor withdrawal and become senescent when differentiation conditions are aberrant; but the CICs that result re-acquire stem cell characteristics as they develop genomic instability, even under conditions in which stem cells ordinarily do not thrive and cannot be cultivated<sup>[68]</sup>. CICs that re-acquire stemness under these conditions maintain stem cell characteristics with great persistence, and it is difficult to get them to differentiate completely.

## RELEVANCE OF *IN VITRO* MODELS TO CANCER DEVELOPMENT

Unlike *in vitro* malignancy transformation, *in vivo* cancer development has multiple steps that include abrogating organic regulation, which results in the development of benign tumors such as adenomatous polyposis coli mutations in the colon<sup>[78,79]</sup>, and losing the ARF/p53 module that leads to uncontrolled growth and the development of malignancies<sup>[80,81]</sup>. Each organ in a complex animal is regulated by its own specific mechanisms that keep the organ in homeostasis, which can malfunction and allow tissues to become precancerous or malignant when the genome becomes unstable or the mutations affect the ARF/p53 module<sup>[40,56]</sup>.

## CONCLUSION

Genomic instability contributes to the development of CICs by directly transforming somatic stem cells, reprogramming differentiated cancer cells, and a number of other mechanisms. Several lines of evidence suggest that one of these pathways is triggered by the disruption of the niche environment (Figure 2). Maintenance of the niche environment is essential for

somatic stem cell maintenance and differentiation and homeostasis (Figure 2A). The stem cell niche can be damaged by exogenous stresses that cause irregular stem cell differentiation, the accumulation of DNA damage, and the induction of senescence (Figure 2B). DNA damage often triggers genomic destabilization, which can promote the development of precancerous (Figure 2C) and cancerous (Figure 2D) lesions through the maintenance of small pockets of pluripotent stem cells that eventually become CICs.

If stem cells start to differentiate under unfavorable differentiation conditions, they become senescent, but can start developing again and reacquire stem cell characteristics when conditions change. Stemness recovered under these circumstances is associated with robust plasticity and the ability of these cells to self-renew, even under conditions in which normal stem cells do not thrive. Unfortunately, such cells do not differentiate completely and remain permanently in less developed states that often lead to malignancies.

## REFERENCES

- 1 **Perez-Losada J**, Balmain A. Stem-cell hierarchy in skin cancer. *Nat Rev Cancer* 2003; **3**: 434-443 [PMID: 12778133 DOI: 10.1038/nrc1095]
- 2 **Ratajczak MZ**. Cancer stem cells--normal stem cells "Jedi" that went over to the "dark side". *Folia Histochem Cytobiol* 2005; **43**: 175-181 [PMID: 16382880 DOI: 10.5603/FHC2005]
- 3 **Li F**, Tiede B, Massagué J, Kang Y. Beyond tumorigenesis: cancer stem cells in metastasis. *Cell Res* 2007; **17**: 3-14 [PMID: 17179981 DOI: 10.1038/sj.cr.7310118]
- 4 **Lawson DA**, Witte ON. Stem cells in prostate cancer initiation and progression. *J Clin Invest* 2007; **117**: 2044-2050 [PMID: 17671638 DOI: 10.1172/JCI32810]
- 5 **Bleau AM**, Agliano A, Larzabal L, de Aberasturi AL, Calvo A. Metastatic dormancy: a complex network between cancer stem cells and their microenvironment. *Histol Histopathol* 2014; **29**: 1499-510 [PMID: 24887025]
- 6 **Ni J**, Cozzi PJ, Hao JL, Beretov J, Chang L, Duan W, Shigdar S, Delprado WJ, Graham PH, Bucci J, Kearsley JH, Li Y. CD44 variant 6 is associated with prostate cancer metastasis and chemo-/radioresistance. *Prostate* 2014; **74**: 602-617 [PMID: 24615685]



- DOI: 10.1002/pros.22775]
- 7 **Carroll WL**, Bhojwani D, Min DJ, Raetz E, Relling M, Davies S, Downing JR, Willman CL, Reed JC. Pediatric acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program* 2003; 102-131 [PMID: 14633779 DOI: 10.1182/asheducation-2003.1.102]
  - 8 **Greaves M**. Childhood leukaemia. *BMJ* 2002; **324**: 283-287 [PMID: 11823363 DOI: 10.1136/bmj.324.7332.283]
  - 9 **Huether R**, Dong L, Chen X, Wu G, Parker M, Wei L, Ma J, Edmonson MN, Hedlund EK, Rusch MC, Shurtleff SA, Mulder HL, Boggs K, Vadordaria B, Cheng J, Yergeau D, Song G, Becksfort J, Lemmon G, Weber C, Cai Z, Dang J, Walsh M, Gedman AL, Faber Z, Easton J, Gruber T, Kriwacki RW, Partridge JF, Ding L, Wilson RK, Mardis ER, Mullighan CG, Gilbertson RJ, Baker SJ, Zambetti G, Ellison DW, Zhang J, Downing JR. The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. *Nat Commun* 2014; **5**: 3630 [PMID: 24710217 DOI: 10.1038/ncomms4630]
  - 10 **Garcia-Manero G**, Jeha S, Daniel J, Williamson J, Albitar M, Kantarjian HM, Issa JP. Aberrant DNA methylation in pediatric patients with acute lymphocytic leukemia. *Cancer* 2003; **97**: 695-702 [PMID: 12548613 DOI: 10.1002/cncr.11090]
  - 11 **Lengauer C**, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. *Nature* 1997; **386**: 623-627 [PMID: 9121588 DOI: 10.1038/386623a0]
  - 12 **Lengauer C**, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998; **396**: 643-649 [PMID: 9872311 DOI: 10.1038/25292]
  - 13 **Shih IM**, Zhou W, Goodman SN, Lengauer C, Kinzler KW, Vogelstein B. Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. *Cancer Res* 2001; **61**: 818-822 [PMID: 11221861]
  - 14 **Hveem TS**, Merok MA, Pretorius ME, Novelli M, Bævre MS, Sjo OH, Clinch N, Liestøl K, Svindland A, Lothe RA, Nesbakken A, Danielsen HE. Prognostic impact of genomic instability in colorectal cancer. *Br J Cancer* 2014; **110**: 2159-2164 [PMID: 24642618 DOI: 10.1038/bjc.2014.133]
  - 15 **Poulogiannis G**, Frayling IM, Arends MJ. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. *Histopathology* 2010; **56**: 167-179 [PMID: 20102395 DOI: 10.1111/j.1365-2559.2009.03392.x]
  - 16 **Saletti P**, Edwin ID, Pack K, Cavalli F, Atkin WS. Microsatellite instability: application in hereditary non-polyposis colorectal cancer. *Ann Oncol* 2001; **12**: 151-160 [PMID: 11300317]
  - 17 **Yacoub G**, Nagalla S, Aklilu M. Oncologic management of hereditary colorectal cancer. *Clin Colon Rectal Surg* 2012; **25**: 118-122 [PMID: 23730227 DOI: 10.1055/s-0032-1313783]
  - 18 **Zhou T**, Hasty P, Walter CA, Bishop AJ, Scott LM, Rebel VI. Myelodysplastic syndrome: an inability to appropriately respond to damaged DNA? *Exp Hematol* 2013; **41**: 665-674 [PMID: 23643835 DOI: 10.1016/j.exphem.2013.04.008]
  - 19 **Burgess M**, Puhalla S. BRCA 1/2-Mutation Related and Sporadic Breast and Ovarian Cancers: More Alike than Different. *Front Oncol* 2014; **4**: 19 [PMID: 24579064]
  - 20 **Ewald IP**, Ribeiro PL, Palmero EI, Cossio SL, Giugliani R, Ashton-Prolla P. Genomic rearrangements in BRCA1 and BRCA2: A literature review. *Genet Mol Biol* 2009; **32**: 437-446 [PMID: 21637503 DOI: 10.1590/S1415-47572009005000049]
  - 21 **Liu Y**, West SC. Distinct functions of BRCA1 and BRCA2 in double-strand break repair. *Breast Cancer Res* 2002; **4**: 9-13 [PMID: 11879553 DOI: 10.1186/bcr417]
  - 22 **Metcalfe KA**, Finch A, Poll A, Horsman D, Kim-Sing C, Scott J, Royer R, Sun P, Narod SA. Breast cancer risks in women with a family history of breast or ovarian cancer who have tested negative for a BRCA1 or BRCA2 mutation. *Br J Cancer* 2009; **100**: 421-425 [PMID: 19088722 DOI: 10.1038/sj.bjc.6604830]
  - 23 **Cleaver JE**. Common pathways for ultraviolet skin carcinogenesis in the repair and replication defective groups of xeroderma pigmentosum. *J Dermatol Sci* 2000; **23**: 1-11 [PMID: 10699759 DOI: 10.1016/S0923-1811(99)00088-2]
  - 24 **Gratchev A**, Strein P, Utikal J, Sergij G. Molecular genetics of Xeroderma pigmentosum variant. *Exp Dermatol* 2003; **12**: 529-536 [PMID: 14705792 DOI: 10.1034/j.1600-0625.2003.00124.x]
  - 25 **Friedberg EC**. How nucleotide excision repair protects against cancer. *Nat Rev Cancer* 2001; **1**: 22-33 [PMID: 11900249 DOI: 10.1038/35094000]
  - 26 **Ichijima Y**, Yoshioka K, Yoshioka Y, Shinohe K, Fujimori H, Unno J, Takagi M, Goto H, Inagaki M, Mizutani S, Teraoka H. DNA lesions induced by replication stress trigger mitotic aberration and tetraploidy development. *PLoS One* 2010; **5**: e8821 [PMID: 20098673 DOI: 10.1371/journal.pone.0008821]
  - 27 **Atsumi Y**, Fujimori H, Fukuda H, Inase A, Shinohe K, Yoshioka Y, Shikanai M, Ichijima Y, Unno J, Mizutani S, Tsuchiya N, Hippo Y, Nakagama H, Masutani M, Teraoka H, Yoshioka K. Onset of quiescence following p53 mediated down-regulation of H2AX in normal cells. *PLoS One* 2011; **6**: e23432 [PMID: 21858116 DOI: 10.1371/journal.pone.0023432]
  - 28 **Yoshida R**, Miyashita K, Inoue M, Shimamoto A, Yan Z, Egashira A, Oki E, Kakeji Y, Oda S, Maehara Y. Concurrent genetic alterations in DNA polymerase proofreading and mismatch repair in human colorectal cancer. *Eur J Hum Genet* 2011; **19**: 320-325 [PMID: 21157497 DOI: 10.1038/ejhg.2010.216]
  - 29 **The Cancer Genome Atlas Network**. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012; **487**: 330-337 [PMID: 22810696 DOI: 10.1038/nature11252]
  - 30 **Lawrence MS**, Stojanov P, Mermel CH, Robinson JT, Garraway LA, Golub TR, Meyerson M, Gabriel SB, Lander ES, Getz G. Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* 2014; **505**: 495-501 [PMID: 24390350 DOI: 10.1038/nature12912]
  - 31 **Razzak M**. Genetics: new molecular classification of gastric adenocarcinoma proposed by The Cancer Genome Atlas. *Nat Rev Clin Oncol* 2014; **11**: 499 [PMID: 25113841 DOI: 10.1038/nrclinonc.2014.138]
  - 32 **Stephens PJ**, Greenman CD, Fu B, Yang F, Bignell GR, Mudie LJ, Pleasance ED, Lau KW, Beare D, Stebbings LA, McLaren S, Lin ML, McBride DJ, Varela I, Nik-Zainal S, Leroy C, Jia M, Menzies A, Butler AP, Teague JW, Quail MA, Burton J, Swerdlow H, Carter NP, Morsberger LA, Jacobuzio-Donahue C, Follows GA, Green AR, Flanagan AM, Stratton MR, Futreal PA, Campbell PJ. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell* 2011; **144**: 27-40 [PMID: 21215367 DOI: 10.1016/j.cell.2010.11.055]
  - 33 **Pleasance ED**, Cheetham RK, Stephens PJ, McBride DJ, Humphray SJ, Greenman CD, Varela I, Lin ML, Ordóñez GR, Bignell GR, Ye K, Alipaz J, Bauer MJ, Beare D, Butler A, Carter RJ, Chen L, Cox AJ, Edkins S, Kokko-Gonzales PI, Gormley NA, Grocock RJ, Haudenschild CD, Hims MM, James T, Jia M, Kingsbury Z, Leroy C, Marshall J, Menzies A, Mudie LJ, Ning Z, Royce T, Schulz-Trieglaff OB, Spiridou A, Stebbings LA, Szajkowski L, Teague J, Williamson D, Chin L, Ross MT, Campbell PJ, Bentley DR, Futreal PA, Stratton MR. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature* 2010; **463**: 191-196 [PMID: 20016485 DOI: 10.1038/nature08658]
  - 34 **Stephens PJ**, McBride DJ, Lin ML, Varela I, Pleasance ED, Simpson JT, Stebbings LA, Leroy C, Edkins S, Mudie LJ, Greenman CD, Jia M, Latimer C, Teague JW, Lau KW, Burton J, Quail MA, Swerdlow H, Churcher C, Natrajan R, Sieuwerts AM, Martens JW, Silver DP, Langerød A, Russnes HE, Foekens JA, Reis-Filho JS, van 't Veer L, Richardson AL, Børresen-Dale AL, Campbell PJ, Futreal PA, Stratton MR. Complex landscapes of somatic rearrangement in human breast cancer genomes. *Nature* 2009; **462**: 1005-1010 [PMID: 20033038 DOI: 10.1038/nature08645]
  - 35 **Grichnik JM**. Genomic instability and tumor stem cells. *J Invest Dermatol* 2006; **126**: 1214-1216 [PMID: 16702970 DOI: 10.1038/sj.jid.5700240]
  - 36 **Li L**, Borodyansky L, Yang Y. Genomic instability en route to and from cancer stem cells. *Cell Cycle* 2009; **8**: 1000-1002 [PMID: 19270518 DOI: 10.4161/cc.8.7.8041]
  - 37 **Danes BS**. Increased in vitro tetraploidy: tissue specific within the



- heritable colorectal cancer syndromes with polyposis coli. *Cancer* 1978; **41**: 2330-2334 [PMID: 657097]
- 38 **Dutrillaux B**, Gerbault-Seureau M, Remvikos Y, Zafrani B, Prieur M. Breast cancer genetic evolution: I. Data from cytogenetics and DNA content. *Breast Cancer Res Treat* 1991; **19**: 245-255 [PMID: 1663804]
  - 39 **Heselmeyer K**, Schröck E, du Manoir S, Blegen H, Shah K, Steinbeck R, Auer G, Ried T. Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. *Proc Natl Acad Sci USA* 1996; **93**: 479-484 [PMID: 8552665]
  - 40 **Maley CC**, Galipeau PC, Li X, Sanchez CA, Paulson TG, Blount PL, Reid BJ. The combination of genetic instability and clonal expansion predicts progression to esophageal adenocarcinoma. *Cancer Res* 2004; **64**: 7629-7633 [PMID: 15492292 DOI: 10.1158/0008-5472.CAN-04-1738]
  - 41 **Matheu A**, Maraver A, Klatt P, Flores I, Garcia-Cao I, Borrás C, Flores JM, Viña J, Blasco MA, Serrano M. Delayed ageing through damage protection by the Arf/p53 pathway. *Nature* 2007; **448**: 375-379 [PMID: 17637672 DOI: 10.1038/nature05949]
  - 42 **Osawa T**, Atsumi Y, Sugihara E, Saya H, Kanno M, Tashiro F, Masutani M, Yoshioka K. Arf and p53 act as guardians of a quiescent cellular state by protecting against immortalization of cells with stable genomes. *Biochem Biophys Res Commun* 2013; **432**: 34-39 [PMID: 23376716 DOI: 10.1016/j.bbrc.2013.01.091]
  - 43 **Yoshioka K**, Atsumi Y, Fukuda H, Masutani M, Teraoka H. The Quiescent Cellular State is Arf/p53-Dependent and Associated with H2AX Downregulation and Genome Stability. *Int J Mol Sci* 2012; **13**: 6492-6506 [PMID: 22754379 DOI: 10.3390/ijms13056492]
  - 44 **Rufini A**, Tucci P, Celardo I, Melino G. Senescence and aging: the critical roles of p53. *Oncogene* 2013; **32**: 5129-5143 [PMID: 23416979 DOI: 10.1038/onc.2012.640]
  - 45 **Vigneron A**, Vousden KH. p53, ROS and senescence in the control of aging. *Aging (Albany NY)* 2010; **2**: 471-474 [PMID: 20729567]
  - 46 **Zuckerman V**, Wolyniec K, Sionov RV, Haupt S, Haupt Y. Tumour suppression by p53: the importance of apoptosis and cellular senescence. *J Pathol* 2009; **219**: 3-15 [PMID: 19562738 DOI: 10.1002/path.2584]
  - 47 **Choisy-Rossi C**, Reisdorf P, Yonish-Rouach E. Mechanisms of p53-induced apoptosis: in search of genes which are regulated during p53-mediated cell death. *Toxicol Lett* 1998; **102-103**: 491-496 [PMID: 10022301]
  - 48 **Brady CA**, Jiang D, Mello SS, Johnson TM, Jarvis LA, Kozak MM, Kenzelmann Broz D, Basak S, Park EJ, McLaughlin ME, Karnezis AN, Attardi LD. Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell* 2011; **145**: 571-583 [PMID: 21565614 DOI: 10.1016/j.cell.2011.03.035]
  - 49 **Li T**, Kon N, Jiang L, Tan M, Ludwig T, Zhao Y, Baer R, Gu W. Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. *Cell* 2012; **149**: 1269-1283 [PMID: 22682249 DOI: 10.1016/j.cell.2012.04.026]
  - 50 **Valente LJ**, Gray DH, Michalak EM, Pinon-Hofbauer J, Egle A, Scott CL, Janic A, Strasser A. p53 efficiently suppresses tumor development in the complete absence of its cell-cycle inhibitory and proapoptotic effectors p21, Puma, and Noxa. *Cell Rep* 2013; **3**: 1339-1345 [PMID: 23665218 DOI: 10.1016/j.celrep.2013.04.012]
  - 51 **Blagosklonny MV**. Tumor suppression by p53 without apoptosis and senescence: conundrum or rapalog-like gerosuppression? *Aging (Albany NY)* 2012; **4**: 450-455 [PMID: 22869016]
  - 52 **Feng Z**, Hu W, de Stanchina E, Teresky AK, Jin S, Lowe S, Levine AJ. The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. *Cancer Res* 2007; **67**: 3043-3053 [PMID: 17409411 DOI: 10.1158/0008-5472.CAN-06-4149]
  - 53 **Liu J**, Zhang C, Feng Z. Tumor suppressor p53 and its gain-of-function mutants in cancer. *Acta Biochim Biophys Sin (Shanghai)* 2014; **46**: 170-179 [PMID: 24374774 DOI: 10.1093/abbs/gmt144]
  - 54 **Budanov AV**, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. *Cell* 2008; **134**: 451-460 [PMID: 18692468 DOI: 10.1016/j.cell.2008.06.028]
  - 55 **Zhang XD**, Qin ZH, Wang J. The role of p53 in cell metabolism. *Acta Pharmacol Sin* 2010; **31**: 1208-1212 [PMID: 20729871 DOI: 10.1038/aps.2010.151]
  - 56 **Matheu A**, Maraver A, Serrano M. The Arf/p53 pathway in cancer and aging. *Cancer Res* 2008; **68**: 6031-6034 [PMID: 18676821 DOI: 10.1158/0008-5472.CAN-07-6851]
  - 57 **Atsumi Y**, Inase A, Osawa T, Sugihara E, Sakasai R, Fujimori H, Teraoka H, Saya H, Kanno M, Tashiro F, Nakagama H, Masutani M, Yoshioka K. The Arf/p53 protein module, which induces apoptosis, down-regulates histone H2AX to allow normal cells to survive in the presence of anti-cancer drugs. *J Biol Chem* 2013; **288**: 13269-13277 [PMID: 23536184 DOI: 10.1074/jbc.M112.402560]
  - 58 **Bonner WM**, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, Pommier Y. GammaH2AX and cancer. *Nat Rev Cancer* 2008; **8**: 957-967 [PMID: 19005492 DOI: 10.1038/nrc2523]
  - 59 **Bassing CH**, Alt FW. H2AX may function as an anchor to hold broken chromosomal DNA ends in close proximity. *Cell Cycle* 2004; **3**: 149-153 [PMID: 14712078 DOI: 10.4161/cc.3.2.684]
  - 60 **Celeste A**, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, Reina-San-Martin B, Coppola V, Meffre E, Difilippantonio MJ, Redon C, Pilch DR, Orlan A, Eckhaus M, Camerini-Otero RD, Tessarollo L, Livak F, Manova K, Bonner WM, Nussenzweig MC, Nussenzweig A. Genomic instability in mice lacking histone H2AX. *Science* 2002; **296**: 922-927 [PMID: 11934988 DOI: 10.1126/science.1069398]
  - 61 **Newbold RF**, Overell RW. Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. *Nature* 1983; **304**: 648-651 [PMID: 6877385]
  - 62 **Land H**, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 1983; **304**: 596-602 [PMID: 6308472]
  - 63 **Bunting KD**. ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* 2002; **20**: 11-20 [PMID: 11796918 DOI: 10.1634/stemcells.20-3-274]
  - 64 **Kondoh H**, Leonart ME, Gil J, Wang J, Degan P, Peters G, Martinez D, Carnero A, Beach D. Glycolytic enzymes can modulate cellular life span. *Cancer Res* 2005; **65**: 177-185 [PMID: 15665293]
  - 65 **Leahy A**, Xiong JW, Kuhnert F, Stuhlmann H. Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation. *J Exp Zool* 1999; **284**: 67-81 [PMID: 10368935]
  - 66 **Seaberg RM**, van der Kooy D. Stem and progenitor cells: the premature desertion of rigorous definitions. *Trends Neurosci* 2003; **26**: 125-131 [PMID: 12591214 DOI: 10.1016/S0166-2236(03)00031-6]
  - 67 **Yang YM**, Chang JW. Current status and issues in cancer stem cell study. *Cancer Invest* 2008; **26**: 741-755 [PMID: 18608212 DOI: 10.1080/0737900801901856]
  - 68 **Fujimori H**, Shikanai M, Teraoka H, Masutani M, Yoshioka K. Induction of cancerous stem cells during embryonic stem cell differentiation. *J Biol Chem* 2012; **287**: 36777-36791 [PMID: 22961983 DOI: 10.1074/jbc.M112.372557]
  - 69 **Raaijmakers MH**, Mukherjee S, Guo S, Zhang S, Kobayashi T, Schoonmaker JA, Ebert BL, Al-Shahrour F, Hasserjian RP, Scadden EO, Aung Z, Matza M, Merkenschlager M, Lin C, Rommens JM, Scadden DT. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* 2010; **464**: 852-857 [PMID: 20305640 DOI: 10.1038/nature08851]
  - 70 **Knoepfler PS**. Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. *Stem Cells* 2009; **27**: 1050-1056 [PMID: 19415771 DOI: 10.1002/stem.37]
  - 71 **Morange M**. What history tells us VII. Twenty-five years ago: the production of mouse embryonic stem cells. *J Biosci* 2006; **31**: 537-541 [PMID: 17301490]
  - 72 **Wylie C**. Germ cells. *Cell* 1999; **96**: 165-174 [PMID: 9988212 DOI: 10.1016/S0092-8674(00)80557-7]
  - 73 **Sell S**. Stem cell origin of cancer and differentiation therapy. *Crit Rev Oncol Hematol* 2004; **51**: 1-28 [PMID: 15207251 DOI: 10.1016/j.critrevonc.2004.04.007]



- 74 **Duggal R**, Minev B, Geissinger U, Wang H, Chen NG, Koka PS, Szalay AA. Biotherapeutic approaches to target cancer stem cells. *J Stem Cells* 2013; **8**: 135-149 [PMID: 24699023]
- 75 **Rajan P**, Srinivasan R. Targeting cancer stem cells in cancer prevention and therapy. *Stem Cell Rev* 2008; **4**: 211-216 [PMID: 18663609 DOI: 10.1007/s12015-008-9037-x]
- 76 **Pinto CA**, Widodo E, Waltham M, Thompson EW. Breast cancer stem cells and epithelial mesenchymal plasticity - Implications for chemoresistance. *Cancer Lett* 2013; **341**: 56-62 [PMID: 23830804 DOI: 10.1016/j.canlet.2013.06.003]
- 77 **Tang DG**. Understanding cancer stem cell heterogeneity and plasticity. *Cell Res* 2012; **22**: 457-472 [PMID: 22357481 DOI: 10.1038/cr.2012.13]
- 78 **Boman BM**, Fields JZ. An APC: WNT Counter-Current-Like Mechanism Regulates Cell Division Along the Human Colonic Crypt Axis: A Mechanism That Explains How APC Mutations Induce Proliferative Abnormalities That Drive Colon Cancer Development. *Front Oncol* 2013; **3**: 244 [PMID: 24224156 DOI: 10.3389/fonc.2013.00244]
- 79 **Najdi R**, Holcombe RF, Waterman ML. Wnt signaling and colon carcinogenesis: beyond APC. *J Carcinog* 2011; **10**: 5 [PMID: 21483657 DOI: 10.4103/1477-3163.78111]
- 80 **Farber E**. The multistep nature of cancer development. *Cancer Res* 1984; **44**: 4217-4223 [PMID: 6467183]
- 81 **Vogelstein B**, Kinzler KW. The multistep nature of cancer. *Trends Genet* 1993; **9**: 138-141 [PMID: 8516849]

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## Adult stem-like cells in kidney

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cell or system is reportedly presents suggesting that adult stem-like cells in kidney can be practical clinical targets for kidney diseases. However, it is still unclear if kidney stem cells or stem-like cells exist or not. In general, stemness is defined by several factors such as self-renewal capacity, multi-lineage potency and characteristic gene expression profiles. The definite use of stemness may be obstacle to understand kidney regeneration, and here we describe the recent broad findings of kidney regeneration and the cells that contribute regeneration.

**Key words:** Stem cell; Label-retaining cells; rKS56; SP cells; CD24; CD133; Sca-1; Induced pluripotent stem; ES cell

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**Core tip:** Controversies still persist whether kidney stem cells exist or not, but renal progenitor cell or system is reportedly presents suggesting that adult stem-like cells in kidney can be practical clinical targets for kidney diseases. In this mini-review, we describe the recent broad findings of kidney regeneration and the cells that contribute regeneration.

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### Abstract

Human pluripotent cells are promising for treatment for kidney diseases, but the protocols for derivation of kidney cell types are still controversial. Kidney tissue regeneration is well confirmed in several lower vertebrates such as fish, and the repair of nephrons after tubular damages is commonly observed after renal injury. Even in adult mammal kidney, renal progenitor

### INTRODUCTION

Recent developments in human pluripotent cells including both embryonic stem cells and induced pluripotent stem (iPS) cells<sup>[1]</sup> are promising for clinical development of cell therapies and tissue



engineering<sup>[2-4]</sup>. There is an urgent need for stem cell and regenerative medicine approaches to kidney diseases, because patients with end-stage kidney disease require lifelong dialysis treatment or transplantation that incurs a significant cost. Several established protocols for derivation of cardiomyocytes and neurons were already reported, but that for kidney cell types are still controversial. Recently human iPS cells were successfully differentiated into kidney lineage *via* OSR1 (+) cells<sup>[5,6]</sup>. However metanephric progenitors were not induced from OSR1(+) cells<sup>[7]</sup>, and efficient differentiation of human pluripotent cells into intermediate mesoderm was reported by treatment the cells with glycogen synthase kinase-3 $\beta$  inhibitor<sup>[8]</sup>. Different from mammals, the capacity to regenerate kidney tissue is well confirmed in several lower vertebrates such as fish<sup>[9]</sup>. Kidney regeneration, such as the repair of nephrons after tubular damages, is commonly observed after renal injury even in humans<sup>[10,11]</sup>. Concerning adult mammal kidney, there are lots of evidences that suggest existence of renal progenitor system<sup>[12-19]</sup>. Collectively, these results suggest the potential role of adult stem-like cells in kidney for practical clinical treatment of kidney diseases. In this review, we describe the localization and recent functional findings of kidney regeneration and the cells that contribute regeneration.

### **TUBULAR CELLS (LRC, RKS56, NFATC-1<sup>+</sup>, ALDH<sup>HIGH</sup>, CD24<sup>+</sup>/CD133<sup>+</sup>, MRPC)**

To identify the stem cells, BrdU-DNA labeling is commonly used to find slow-cycling cells because stem cells have a slow cell cycle. Slow cycling cells were also called label-retaining cells (LRC), and LRC in tubules were confirmed by several groups<sup>[20-22]</sup> (Table 1). In rat kidneys, LRC were distributed among renal epithelial tubular cells<sup>[20]</sup>. In 3 dimensional culture system, LRC formed tubule-like structure. Moreover, injected LRC into cultured metanephros formed nephrons and collecting ducts<sup>[23]</sup>. rKS56 cells were established from proximal tubules (S3) by using microdissection of a single nephron<sup>[24]</sup>. rKS56 cells expressed markers of an immature progenitor state such as c-Kit and Sca-1. When rKS56 cells were transplanted into acute kidney disease models, the cells differentiated into epithelium. A resident progenitor cells in proximal tubular (PTC) cell was identified by Nfatc1-P2-Cre reporter system<sup>[25]</sup>. Nfatc-1-labeled PTC cells were apoptosis-resistant and proliferated to repair the damaged proximal tubule segment. ALDH activity was used to isolate cells with progenitor-like characteristics from the tubular fraction of the renal cortex<sup>[26-29]</sup>. ALDH<sup>high</sup> cells displayed typical stem cell properties such as sphere

formation and anchorage-independent growth. CD24/CD133 double-positive cells were localized in the tubular epithelium, and demonstrated clonogenic multipotency and self-renewal ability<sup>[28]</sup>. Bombelli *et al.*<sup>[30]</sup> recently proposed existence of CD133<sup>+</sup>/CD24<sup>-</sup> renal stem cells but Romagnani reported that CD133<sup>+</sup> renal stem definitely co-express CD24 in human kidney<sup>[31]</sup>. Gupta *et al.*<sup>[32]</sup> reported localization of stem-like cells around the tubules. They named the cells multi-potent renal progenitor cells (MRPC). By using similar culture condition used for culture of bone marrow-derived multi-potent adult progenitor cells, MRPS were isolated from rat kidney. The plasticity of MRPC was confirmed by expression of endothelial, hepatocyte, and neural markers by RT-PCR and protein expression. When MRPC were injected under the capsule of an uninjured kidney or arterially into acute kidney injury model, the cells differentiated into renal tubules. However, differentiation/induction of matured kidney cells from MRPC *in vitro* was not confirmed yet.

### **RENAL PAPILLA (LRC, CD133<sup>+</sup>)**

To identify the stem-like cells in kidney, Oliver *et al.*<sup>[33,34]</sup> performed pulse label of rat and mouse pups by BrdU, and confirmed the existence of LRC cells in the kidney. LRC cells was very sparse in the kidney, but they found numerous LRC cells in renal papilla<sup>[33]</sup>. In 3 dimensional culture, LRC cells in renal papilla spontaneously formed spheroids, and clones from single cell of LRC cells expressed both mesenchymal and epithelial markers. The papillary cells also differentiated to myofibroblasts and neuronal cells. In acute kidney injury model such as ischemic injury, LRC cells in papilla migrated to the upper papilla and formed a compartment of rapidly proliferating cells suggesting that the cells contributed to repair of kidney tissue<sup>[34]</sup>. Papillary cells that expressed CD133 were also expressed nestin and embryonic cell markers (Oct3/4, Nanog, SOX2 and SSEA-4)<sup>[35]</sup>.

### **INTERSTITIAL SPACE (SCA-1<sup>+</sup>CD45<sup>-</sup>, SP, CD133<sup>+</sup>)**

Several groups have found adult stem-like cells in murine kidney interstitial space using different approaches. Stem cell antigen-1 (Sca-1)-positive and CD45-negative cells were isolated from whole kidney tissue by magnetic assisted cell sorting and fluorescence activated cell sorting (FACS) sorting<sup>[36]</sup>. The cells were negative for hematopoietic stem cell and lineage markers and located in the renal interstitial spaces. The differentiation of the cells into multi-lineage (myogenic, osteogenic, adipogenic and neural) was also confirmed. In acute kidney injury model, injected Sca-1<sup>+</sup>CD45<sup>-</sup> cells contributed



**Table 1** Localization and characteristics of adult stem-like cells in kidney

Localization	Characteristics	Species	Ref.
Tubular cells	LRC	Rat	[20-23]
	rKS56	Rat	[24]
	Nfactc-1 <sup>+</sup>	Mice	[25]
	ALDH <sup>high</sup> /CD24 <sup>+</sup> /CD133 <sup>+</sup>	Human	[26-29]
	MRPC	Rat	[32]
Renal papilla	LRC	Rat, mice	[31,32]
	CD133 <sup>+</sup>	Human	[33]
Interstitial space	Sca-1 <sup>+</sup> /CD45 <sup>+</sup>	Mice	[34]
	SP cell	Rat, mice, human	[38-41]
Bowman's capsule	CD133 <sup>+</sup>	Human	[27]
	CD24 <sup>+</sup> /CD133 <sup>+</sup> /PDX <sup>+</sup>	Human	[44-48]
	CD24 <sup>+</sup> /CD133 <sup>+</sup> /PDX <sup>-</sup>	Human	[44-48]

LRC: Label-retaining cells; MRPC: Multi-potent renal progenitor cell; PDX: Podocyte marker.

kidney repair. To isolate hematopoietic stem cell-rich population in a single step, Goodell *et al.*<sup>[37]</sup> stained cells with Hoechst 33342 dye and isolated the cells by FACS. The cells isolated by this method were named side population (SP) cells. This method was also used to purify a stem cell-rich population in various kinds of tissue. SP cells isolated from adult kidney demonstrated ability of self-renewal and differentiation into multiple lineages<sup>[38]</sup>. SP cells isolated from adult kidney located in interstitial spaces, and secreted reno-regenerative/protective factors (HGF, VEGF, and BMP-7)<sup>[39-42]</sup>. The injection of SP cells isolated from adult kidney cells into a model of acute kidney injury demonstrated the recovery of renal function<sup>[39,40]</sup>. Interestingly, Inowa *et al.*<sup>[43]</sup> confirmed the existence of kidney SP cells in human. Concerning CD133 positive cells, Bussolati *et al.*<sup>[27]</sup> reported that the cells were localized to the interstitium, but not in glomeruli. Kidney CD133 positive cells lacked expression of hematopoietic markers and expressed Pax-2, an embryonic renal marker. Intravenous injection of kidney CD133 positive cells in SCID mice with glycerol-induced tubulonecrosis, the cells homed into the injured kidney and integrated in tubules.

## BOWMAN'S CAPSULE

### (CD24<sup>+</sup>CD133<sup>+</sup>PDX<sup>+</sup>, CD24<sup>+</sup>CD133<sup>+</sup>PDX<sup>-</sup>)

Some parietal epithelial cells (PEC) are reported to be adult stem-like cells<sup>[44-47]</sup>. Sagrinati *et al.*<sup>[45]</sup> confirmed the existence of PEC that expressed CD24, CD133 Oct-4 and Bmi-1 in the Bowman's capsule. CD24<sup>+</sup>CD133<sup>+</sup> PEC were isolated by culture of capsulated glomeruli plated on fibronectin-coated dishes. CD24<sup>+</sup>CD133<sup>+</sup> PEC showed potential of self-renewal and a high cloning efficiency. Transplantation of CD24<sup>+</sup>CD133<sup>+</sup> PEC into acute kidney injury model significantly improved not only morphologic but functional kidney damage. Further characterization

using podocyte marker (PDX) of CD24<sup>+</sup>CD133<sup>+</sup> PEC revealed a hierarchical population of the cells in a precise sequence with Bowman's capsule and exhibited heterogeneous potential such as differentiation and regeneration<sup>[48]</sup>. CD24<sup>+</sup>CD133<sup>+</sup>PDX<sup>-</sup> cells localized to the urinary pole could differentiate into both tubular cells and podocytes, but CD24<sup>+</sup>CD133<sup>+</sup>PDX<sup>+</sup> cells localized between the urinary pole and vascular pole could differentiate into only podocytes. Transplantation of CD24<sup>+</sup>CD133<sup>+</sup>PDX<sup>-</sup> cells reduced proteinuria and improved chronic glomerular damage in adriamycin-induced nephropathy models.

## FUNCTIONAL REGULATION OF ADULT STEM-LIKE CELLS IN KIDNEY

As mentioned above, different kinds of adult stem-like cells in kidney have been reported, but their functional regulations were poorly understood. If it is possible to regulate multi-potent adult stem like-cell *in situ*, this can be a good regenerative treatment. Recently MyoR was reported to regulate regenerative function of kidney SP cells<sup>[49]</sup>, and such a molecule can be a good target for pharmacological treatment for kidney disease.

## CONCLUSION

Several adult stem-like cells in kidney reportedly demonstrated multi-potency. However, it is impossible to get enough cells for cell therapy from the patient. The adult stem-like cell in kidney is expected to play key role to preserve kidney function, and the cells may be the good targets for pharmacological treatment. For cell therapy, iPS or ES cells might be applicable as in the case with neural and cardiac regeneration.

## REFERENCES

1. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]
2. Song JJ, Guyette JP, Gilpin SE, Gonzalez G, Vacanti JP, Ott HC. Regeneration and experimental orthotopic transplantation of a bioengineered kidney. *Nat Med* 2013; **19**: 646-651 [PMID: 23584091 DOI: 10.1038/nm.3154]
3. Humes HD, Weitzel WF, Bartlett RH, Swaniker FC, Paganini EP, Luderer JR, Sobota J. Initial clinical results of the bioartificial kidney containing human cells in ICU patients with acute renal failure. *Kidney Int* 2004; **66**: 1578-1588 [PMID: 15458454 DOI: 10.1111/j.1523-1755.2004.00923]
4. Tumlin J, Wali R, Williams W, Murray P, Tolwani AJ, Vinnikova AK, Szerlip HM, Ye J, Paganini EP, Dworkin L, Finkel KW, Kraus MA, Humes HD. Efficacy and safety of renal tubule cell therapy for acute renal failure. *J Am Soc Nephrol* 2008; **19**: 1034-1040 [PMID: 18272842 DOI: 10.1681/ASN.2007080895]
5. Mae S, Shono A, Shiota F, Yasuno T, Kajiwarra M, Gotoda-Nishimura N, Arai S, Sato-Otubo A, Toyoda T, Takahashi K, Nakayama N, Cowan CA, Aoi T, Ogawa S, McMahon AP, Yamanaka S, Osafune K. Monitoring and robust induction of



- nephrogenic intermediate mesoderm from human pluripotent stem cells. *Nat Commun* 2013; **4**: 1367 [PMID: 23340407 DOI: 10.1038/ncomms2378]
- 6 **Araoka T**, Mae S, Kurose Y, Uesugi M, Ohta A, Yamanaka S, Osafune K. Efficient and rapid induction of human iPSCs/ESCs into nephrogenic intermediate mesoderm using small molecule-based differentiation methods. *PLoS One* 2014; **9**: e84881 [PMID: 24454758 DOI: 10.1371/journal.pone.0084881]
  - 7 **Taguchi A**, Kaku Y, Ohmori T, Sharmin S, Ogawa M, Sasaki H, Nishinakamura R. Redefining the in vivo origin of metanephric nephron progenitors enables generation of complex kidney structures from pluripotent stem cells. *Cell Stem Cell* 2014; **14**: 53-67 [PMID: 24332837 DOI: 10.1016/j.stem.2013.11.010]
  - 8 **Lam AQ**, Freedman BS, Morizane R, Lerou PH, Valerius MT, Bonventre JV. Rapid and efficient differentiation of human pluripotent stem cells into intermediate mesoderm that forms tubules expressing kidney proximal tubular markers. *J Am Soc Nephrol* 2014; **25**: 1211-1225 [PMID: 24357672 DOI: 10.1681/ASN.2013080831]
  - 9 **Diep CQ**, Ma D, Deo RC, Holm TM, Naylor RW, Arora N, Wingert RA, Bollig F, Djordjevic G, Lichman B, Zhu H, Ikenaga T, Ono F, Englert C, Cowan CA, Hukriede NA, Handin RI, Davidson AJ. Identification of adult nephron progenitors capable of kidney regeneration in zebrafish. *Nature* 2011; **470**: 95-100 [PMID: 21270795 DOI: 10.1038/nature09669]
  - 10 **Remuzzi G**, Benigni A, Remuzzi A. Mechanisms of progression and regression of renal lesions of chronic nephropathies and diabetes. *J Clin Invest* 2006; **116**: 288-296 [PMID: 16453013 DOI: 10.1172/JCI27699]
  - 11 **Ma LJ**, Nakamura S, Aldigier JC, Rossini M, Yang H, Liang X, Nakamura I, Marcantoni C, Fogo AB. Regression of glomerulosclerosis with high-dose angiotensin inhibition is linked to decreased plasminogen activator inhibitor-1. *J Am Soc Nephrol* 2005; **16**: 966-976 [PMID: 15728787 DOI: 10.1681/ASN.2004060492]
  - 12 **Blanpain C**, Horsley V, Fuchs E. Epithelial stem cells: turning over new leaves. *Cell* 2007; **128**: 445-458 [PMID: 17289566 DOI: 10.1016/j.cell.2007.01.014]
  - 13 **Romagnani P**. Toward the identification of a “renopietic system”? *Stem Cells* 2009; **27**: 2247-2253 [PMID: 19739254 DOI: 10.1002/stem.140]
  - 14 **Davidson AJ**. Uncharted waters: nephrogenesis and renal regeneration in fish and mammals. *Pediatr Nephrol* 2011; **26**: 1435-1443 [PMID: 21336813 DOI: 10.1007/s00467-011-1795-z]
  - 15 **Lazzeri E**, Mazzeinghi B, Romagnani P. Regeneration and the kidney. *Curr Opin Nephrol Hypertens* 2010; **19**: 248-253 [PMID: 20061947 DOI: 10.1097/MNH.0b013e32833680dc]
  - 16 **Biancone L**, Camussi G. Stem cells in 2013: Potential use of stem or progenitor cells for kidney regeneration. *Nat Rev Nephrol* 2014; **10**: 67-68 [PMID: 24296627 DOI: 10.1038/nrneph.2013.257]
  - 17 **Dziedzic K**, Pleniceanu O, Dekel B. Kidney stem cells in development, regeneration and cancer. *Semin Cell Dev Biol* 2014; **36**: 57-65 [PMID: 25128731 DOI: 10.1016/j.semcdb.2014.08.003]
  - 18 **Aggarwal S**, Moggio A, Bussolati B. Concise review: stem/progenitor cells for renal tissue repair: current knowledge and perspectives. *Stem Cells Transl Med* 2013; **2**: 1011-1019 [PMID: 24167320 DOI: 10.5966/sctm.2013-0097]
  - 19 **Zhu XY**, Lerman A, Lerman LO. Concise review: mesenchymal stem cell treatment for ischemic kidney disease. *Stem Cells* 2013; **31**: 1731-1736 [PMID: 23766020 DOI: 10.1002/stem.1449]
  - 20 **Maeshima A**, Yamashita S, Nojima Y. Identification of renal progenitor-like tubular cells that participate in the regeneration processes of the kidney. *J Am Soc Nephrol* 2003; **14**: 3138-3146 [PMID: 14638912]
  - 21 **Vogetseder A**, Karadeniz A, Kaissling B, Le Hir M. Tubular cell proliferation in the healthy rat kidney. *Histochem Cell Biol* 2005; **124**: 97-104 [PMID: 16133123 DOI: 10.1007/s00418-005-0023-y]
  - 22 **Fujigaki Y**, Goto T, Sakakima M, Fukasawa H, Miyaji T, Yamamoto T, Hishida A. Kinetics and characterization of initially regenerating proximal tubules in S3 segment in response to various degrees of acute tubular injury. *Nephrol Dial Transplant* 2006; **21**: 41-50 [PMID: 16077144 DOI: 10.1093/ndt/gfi035]
  - 23 **Maeshima A**, Sakurai H, Nigam SK. Adult kidney tubular cell population showing phenotypic plasticity, tubulogenic capacity, and integration capability into developing kidney. *J Am Soc Nephrol* 2006; **17**: 188-198 [PMID: 16338966 DOI: 10.1681/ASN.2005040370]
  - 24 **Kitamura S**, Yamasaki Y, Kinomura M, Sugaya T, Sugiyama H, Maeshima Y, Makino H. Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney. *FASEB J* 2005; **19**: 1789-1797 [PMID: 16260649 DOI: 10.1096/fj.05-3942com]
  - 25 **Langworthy M**, Zhou B, de Caestecker M, Moeckel G, Baldwin HS. NFATc1 identifies a population of proximal tubule cell progenitors. *J Am Soc Nephrol* 2009; **20**: 311-321 [PMID: 19118153 DOI: 10.1681/ASN.2008010094]
  - 26 **Ma I**, Allan AL. The role of human aldehyde dehydrogenase in normal and cancer stem cells. *Stem Cell Rev* 2011; **7**: 292-306 [PMID: 21103958 DOI: 10.1007/s12015-010-9208-4]
  - 27 **Bussolati B**, Bruno S, Grange C, Buttiglieri S, Deregibus MC, Cantino D, Camussi G. Isolation of renal progenitor cells from adult human kidney. *Am J Pathol* 2005; **166**: 545-555 [PMID: 15681837 DOI: 10.1016/S0002-9440(10)62276-6]
  - 28 **Sallustio F**, De Benedictis L, Castellano G, Zaza G, Loverre A, Costantino V, Grandaliano G, Schena FP. TLR2 plays a role in the activation of human resident renal stem/progenitor cells. *FASEB J* 2010; **24**: 514-525 [PMID: 19843711 DOI: 10.1096/fj.09-136481]
  - 29 **Lindgren D**, Boström AK, Nilsson K, Hansson J, Sjölund J, Möller C, Jirstrom K, Nilsson E, Landberg G, Axelsson H, Johansson ME. Isolation and characterization of progenitor-like cells from human renal proximal tubules. *Am J Pathol* 2011; **178**: 828-837 [PMID: 21281815 DOI: 10.1016/j.ajpath.2010.10.026]
  - 30 **Bombelli S**, Zipeto MA, Torsello B, Bovo G, Di Stefano V, Bugarin C, Zordan P, Viganò P, Cattoretto G, Strada G, Bianchi C, Perego RA. PKH(high) cells within clonal human nephrospheres provide a purified adult renal stem cell population. *Stem Cell Res* 2013; **11**: 1163-1177 [PMID: 24012544 DOI: 10.1016/j.scr.2013.08.004]
  - 31 **Romagnani P**, Remuzzi G. CD133+ renal stem cells always co-express CD24 in adult human kidney tissue. *Stem Cell Res* 2014; **12**: 828-829 [PMID: 24467938 DOI: 10.1016/j.scr.2013.12.011]
  - 32 **Gupta S**, Verfaillie C, Chmielewski D, Kren S, Eidman K, Connaire J, Heremans Y, Lund T, Blackstad M, Jiang Y, Luttun A, Rosenberg ME. Isolation and characterization of kidney-derived stem cells. *J Am Soc Nephrol* 2006; **17**: 3028-3040 [PMID: 16988061 DOI: 10.1681/ASN.2006030275]
  - 33 **Oliver JA**, Maarouf O, Cheema FH, Martens TP, Al-Awqati Q. The renal papilla is a niche for adult kidney stem cells. *J Clin Invest* 2004; **114**: 795-804 [PMID: 15372103 DOI: 10.1172/JCI20921]
  - 34 **Oliver JA**, Klinakis A, Cheema FH, Friedlander J, Sampogna RV, Martens TP, Liu C, Efstratiadis A, Al-Awqati Q. Proliferation and migration of label-retaining cells of the kidney papilla. *J Am Soc Nephrol* 2009; **20**: 2315-2327 [PMID: 19762493 DOI: 10.1681/ASN.2008111203]
  - 35 **Ward HH**, Romero E, Welford A, Pickett G, Bacallao R, Gattone VH, Ness SA, Wandinger-Ness A, Roitbak T. Adult human CD133/1(+) kidney cells isolated from papilla integrate into developing kidney tubules. *Biochim Biophys Acta* 2011; **1812**: 1344-1357 [PMID: 21255643 DOI: 10.1016/j.bbdis.2011.01.010]
  - 36 **Dekel B**, Zangi L, Shezen E, Reich-Zeliger S, Eventov-Friedman S, Katchman H, Jacob-Hirsch J, Amariglio N, Rechavi G, Margalit R, Reisner Y. Isolation and characterization of nontubular sca-1+lin-multipotent stem/progenitor cells from adult mouse kidney. *J Am Soc Nephrol* 2006; **17**: 3300-3314 [PMID: 17093069 DOI: 10.1681/ASN.2005020195]
  - 37 **Goodell MA**, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996; **183**: 1797-1806 [PMID: 8666936]
  - 38 **Hishikawa K**, Marumo T, Miura S, Nakanishi A, Matsuzaki Y, Shibata K, Kohike H, Komori T, Hayashi M, Nakaki T, Nakauchi



- H, Okano H, Fujita T. Leukemia inhibitory factor induces multi-lineage differentiation of adult stem-like cells in kidney *via* kidney-specific cadherin 16. *Biochem Biophys Res Commun* 2005; **328**: 288-291 [PMID: 15670782 DOI: 10.1016/j.bbrc.2004.12.167]
- 39 **Hishikawa K**, Marumo T, Miura S, Nakanishi A, Matsuzaki Y, Shibata K, Ichiyonagi T, Kohike H, Komori T, Takahashi I, Takase O, Imai N, Yoshikawa M, Inowa T, Hayashi M, Nakaki T, Nakauchi H, Okano H, Fujita T. Musculin/MyoR is expressed in kidney side population cells and can regulate their function. *J Cell Biol* 2005; **169**: 921-928 [PMID: 15967813 DOI: 10.1083/jcb.200412167]
- 40 **Challen GA**, Bertonecello I, Deane JA, Ricardo SD, Little MH. Kidney side population reveals multilineage potential and renal functional capacity but also cellular heterogeneity. *J Am Soc Nephrol* 2006; **17**: 1896-1912 [PMID: 16707564 DOI: 10.1681/ASN.2005111228]
- 41 **Marumo T**, Hishikawa K, Matsuzaki Y, Imai N, Takase O, Shimomura T, Okano H, Fujita T. Angiotensin II type 1 receptor blockade prevents decrease in adult stem-like cells in kidney after ureteral obstruction. *Eur J Pharmacol* 2007; **573**: 216-220 [PMID: 17692840 DOI: 10.1016/j.ejphar.2007.07.032]
- 42 **Nishinakamura R**, Sakaguchi M. BMP signaling and its modifiers in kidney development. *Pediatr Nephrol* 2014; **29**: 681-686 [PMID: 24217785 DOI: 10.1007/s00467-013-2671-9]
- 43 **Inowa T**, Hishikawa K, Takeuchi T, Kitamura T, Fujita T. Isolation and potential existence of side population cells in adult human kidney. *Int J Urol* 2008; **15**: 272-274 [PMID: 18304230 DOI: 10.1111/j.1442-2042.2007.01984.x]
- 44 **Lasagni L**, Romagnani P. Glomerular epithelial stem cells: the good, the bad, and the ugly. *J Am Soc Nephrol* 2010; **21**: 1612-1619 [PMID: 20829409 DOI: 10.1681/ASN.2010010048]
- 45 **Sagrinati C**, Netti GS, Mazzinghi B, Lazzeri E, Liotta F, Frosali F, Ronconi E, Meini C, Gacci M, Squecco R, Carini M, Gesualdo L, Francini F, Maggi E, Annunziato F, Lasagni L, Serio M, Romagnani S, Romagnani P. Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys. *J Am Soc Nephrol* 2006; **17**: 2443-2456 [PMID: 16885410 DOI: 10.1681/ASN.2006010089]
- 46 **Lazzeri E**, Crescioli C, Ronconi E, Mazzinghi B, Sagrinati C, Netti GS, Angelotti ML, Parente E, Ballerini L, Cosmi L, Maggi L, Gesualdo L, Rotondi M, Annunziato F, Maggi E, Lasagni L, Serio M, Romagnani S, Vannelli GB, Romagnani P. Regenerative potential of embryonic renal multipotent progenitors in acute renal failure. *J Am Soc Nephrol* 2007; **18**: 3128-3138 [PMID: 17978305 DOI: 10.1681/ASN.2007020210]
- 47 **Park HC**, Yasuda K, Kuo MC, Ni J, Ratliff B, Chander P, Goligorsky MS. Renal capsule as a stem cell niche. *Am J Physiol Renal Physiol* 2010; **298**: F1254-F1262 [PMID: 20200095 DOI: 10.1152/ajprenal.00406.2009]
- 48 **Ronconi E**, Sagrinati C, Angelotti ML, Lazzeri E, Mazzinghi B, Ballerini L, Parente E, Becherucci F, Gacci M, Carini M, Maggi E, Serio M, Vannelli GB, Lasagni L, Romagnani S, Romagnani P. Regeneration of glomerular podocytes by human renal progenitors. *J Am Soc Nephrol* 2009; **20**: 322-332 [PMID: 19092120 DOI: 10.1681/ASN.2008070709]
- 49 **Kamiura N**, Hirahashi J, Matsuzaki Y, Idei M, Takase O, Fujita T, Takato T, Hishikawa K. Basic helix-loop-helix transcriptional factor MyoR regulates BMP-7 in acute kidney injury. *Am J Physiol Renal Physiol* 2013; **304**: F1159-F1166 [PMID: 23515721 DOI: 10.1152/ajprenal.00510.2012]

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## Niche interactions in epidermal stem cells

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skin diseases. Thus, strategies for manipulating cell-ECM interactions are critical for treating wounds and a variety of skin diseases. Many of these strategies focus on epidermal stem cells, which reside in a unique niche in which the ECM is the most important component; interactions between the ECM and epidermal stem cells play a major role in regulating stem cell fate. As they constitute a major portion of the ECM, it is likely that integrins and type IV collagens are important in stem cell regulation and maintenance. In this review, we highlight recent research-including our previous work-exploring the role that the ECM and its associated components play in shaping the epidermal stem cell niche.

**Key words:** Stem cell niche; Epidermal stem cells; Integrins; Type IV collagen; MiR135b

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**Core tip:** Epidermal stem cells reside in a unique niche within the skin, which is shaped by interactions between stem cell-associated integrins and components of the extracellular matrix. Here, we review literature evaluating the role that integrins play in epidermal stem cell maintenance and proliferation, and highlight methods that have been used to enrich for epidermal stem cells. We stress that by understanding the epidermal stem cell niche, new regenerative medicine applications can be developed.

### Abstract

Within the epidermis and dermis of the skin, cells secrete and are surrounded by the extracellular matrix (ECM), which provides structural and biochemical support. The ECM of the epidermis is the basement membrane, and collagen and other dermal components constitute the ECM of the dermis. There is significant variation in the composition of the ECM of the epidermis and dermis, which can affect "cell to cell" and "cell to ECM" interactions. These interactions, in turn, can influence biological responses, aging, and wound healing; abnormal ECM signaling likely contributes to

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### INTRODUCTION

The skin has epithelial and mesenchymal components<sup>[1]</sup>.



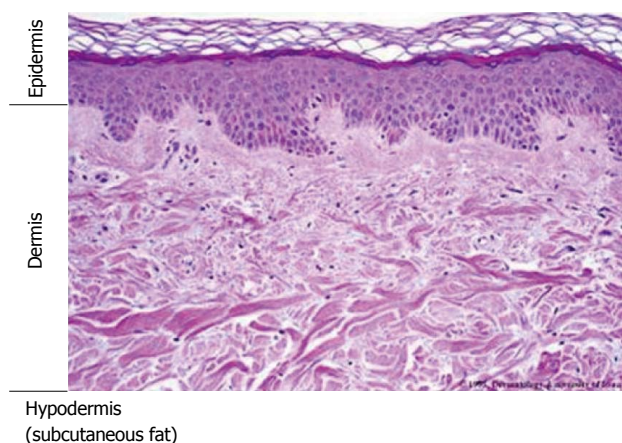


Figure 1 Skin structure involves the epidermis, dermis and hypodermis.

Normally, tissues consist of differentiated cells and a small number of stem cells localized to specific niches<sup>[2]</sup>. Stem cell niches are specific microenvironments that determine stem cell number and affect stem cell mobility<sup>[3]</sup>. Furthermore, the complex nature of stem cell niches allows for the formation of distinct structures specialized for different stem cell types<sup>[4]</sup>. Epidermis is multilayered and is continually renewed and replaced by new cells, a process that results from the action of stem cells. It is known that the extracellular matrix (ECM) is a key component of the stem cell niche in the skin, and biophysical properties of the ECM (such as stiffness) also can affect cell fate determination<sup>[4]</sup>. As epidermal stem cells express high levels of integrins, these cells are able to interact directly with the ECM<sup>[1,4]</sup>. Thus, the interaction between stem cell-associated integrins and the ECM may be important in shaping the epidermal stem cell niche. In addition to the ECM and integrins, growth factors or mechanobiological factors can also affect stem cell fate<sup>[4,5]</sup>. Furthermore, low oxygen tensions (hypoxia) are necessary to maintain undifferentiated stem cell phenotypes, and also influence proliferation and stem cell fate<sup>[6]</sup>. However, among all of these factors, the ECM seems to play the largest role in shaping stem cell niches and stem cell behavior, and may thus have a major underlying role in physiological and pathological conditions.

## SKIN STRUCTURE: A BRIEF OVERVIEW

Skin is composed of the epidermis, dermis, and hypodermis (Figure 1). Multilayered epidermis serves as the outer layer of the skin. Additional structures such as hair follicles and sebaceous glands are also found within the skin. Stem cell proliferation and differentiation are vital for the maintenance of the epidermis, and different pools of skin stem cells are located in the hair follicle (the bulge) and the interfollicular epidermis<sup>[7,8]</sup>. Histologically, the lower layer of the epidermis is attached to the basement

membrane (BM), which separates the epidermis from the underlying connective tissue layer known as the dermis.

## THE BASEMENT MEMBRANE AND THE EPIDERMAL STEM CELL NICHE

The BM of the epidermis contains distinct subtypes of laminin, type IV collagen, nidogen, and perlecan (a heparan sulfate proteoglycan); immunohistochemical staining and gene expression studies have demonstrated that there is considerable regional variation in the BM<sup>[1]</sup>. The mechanical support provided by the BM is determined primarily by its type IV collagen scaffold; however, laminin is essential for the initial assembly of the BM *in vivo*<sup>[9]</sup>. Integrins also demonstrate variable expression in different regions of the epidermis<sup>[10]</sup>, and these transmembrane proteins themselves have different kinds of combination (*i.e.*,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ , and  $\alpha 6\beta 4$ ). Integrins are the major receptors for ECM proteins, and interact with growth factors and cytokine receptors<sup>[4]</sup>. Growth factors and morphogens can also directly bind to the ECM; this suggests that the ECM might integrate different types of signals in biological microenvironments. As a result, clinical applications of stem cells have emphasized the relationship between epidermal stem cells and the ECM<sup>[4]</sup>. Recent research has sought to understand how the ECM and integrins cooperate with one another to form the architecture of the epidermal stem cell niche, and how they maintain the balance between stem cell renewal and differentiation<sup>[4]</sup>. It is possible that variations within the ECM may create distinct environments that can potentially affect the properties of different stem cell populations<sup>[1]</sup>.

## EPIDERMAL STEM CELLS: IDENTIFICATION, REGULATION, AND APPLICATIONS

### Markers of epidermal stem cells and the epidermal stem cell niche

Stem cells are localized to “niches”—specific sites that regulate how stem cells participate in tissue maintenance and repair. In a niche, stem cell proliferation is regulated and these cells are protected from depletion<sup>[11]</sup>. Interestingly, evidence suggests that cancer—a common cause of death—is also a stem cell-based disease. Cancer stem cells (CSCs) are a subset of cancer cells that demonstrate stem cell-like abilities—abilities that may induce tumor growth and tumor recurrence. CSCs also require a special “CSC niche” to regulate their stemness and proliferation, and as with endogenous stem cells, this niche prevents CSCs from being depleted<sup>[12]</sup>. Thus, the stem cell niche is a key factor in the maintenance of stem cells.



In the skin, an increased level of integrins expression is considered to be a marker of epidermal stem cells<sup>[13]</sup>. Although the expression levels of integrins in stem cells are only 2-3-fold higher than in stem cell progeny, this is sufficient to make stem cells more adhesive to ECM proteins<sup>[14]</sup>. Based on these findings, we developed a simple method to isolate epidermal stem cells based on integrin expression. As epidermal stem cells express high levels of integrins, type IV collagen is a good candidate to bind epidermal stem cells<sup>[14]</sup>. To purify epidermal stem cells, three keratinocyte populations were first characterized by their ability to bind type IV collagen: rapidly adhering (RA), slowly adhering (SA), and non-adhering (NA) cells. The expression levels of *p63* and *c-Myc* were also compared between cells in these three populations. It was concluded that RA cells could be easily isolated from the epidermis, and that these cells were likely epidermal stem cells<sup>[15]</sup>. This research suggests that integrins may play an important role in the regulation of epidermal stem cells. Type IV collagen has not only been used to isolate epidermal stem cells, but has also been suggested to be directly involved in maintaining epidermal stem cells. Very recently, we reported that inhibition of miR135b could increase the proliferative potential of normal human keratinocytes<sup>[16]</sup>. Our data demonstrated that type IV collagen is a target of miR135b, and that the inhibition of miR135b may improve the epidermal stem cell microenvironment and increase the proliferative potential of basal cells (Figure 2). These findings suggest that restoration of type IV collagen to the basement membrane is important for stem cell maintenance in skin. Microarray analysis also revealed differences in the expression of ECM proteins between hair follicle stem cells and other epidermal stem cells<sup>[17]</sup>. These results suggest that integrins and type IV collagen are not the only proteins involved in determining the epidermal stem cell niche; ECM proteins are also important in shaping the niche within the interfollicular epidermis. Although no specific markers are available for the identification of epidermal stem cells, *p63* is commonly regarded as a potential epidermal stem cell marker, as *p63*<sup>-/-</sup> mice fail to develop stratified squamous epithelia<sup>[18]</sup>.

### **The stem cell niche in regenerative medicine**

An effective response of the body to injury depends on regenerative processes that maintain proper cell numbers and replace damaged cells. This "reparative" potential is determined by the presence of stem and progenitor cells, which respond to exogenous cues such as tissue damage. Therapeutic applications of stem cells address the biological constraints of these cells, the stem cell niche, and the location to which these cells are summoned to respond. Understanding the role of the stem cell niche is essential for the application of stem cells in regenerative medicine<sup>[19]</sup>.

A major concern in regenerative medicine is obtaining an adequate number of stem cells to treat

an injury or disease. For practical applications, one of the most important problems is the small number of stem cells. In order to overcome this challenge, researchers often expand the number of stem cells by *in vitro* culture; however, most stem cells begin to lose their stem cell character during this process. In many cases, replicating the niche environment in culture is critical to maintain stem cell characteristics<sup>[20]</sup>. Similarly, "mimicking the native niche" has been shown to be important in tissue engineering of human corneal limbal crypts<sup>[21]</sup>. As wound healing is a critical process that re-establishes the epithelial barrier following disease or injury, poor wound healing increases infection risk, causes patient morbidity, and may lead to the scar formation. If there is a defect in the stem cell niche, wound repair could be delayed by the abnormal response of stem cells to injury<sup>[5]</sup>. Thus, understanding how to recreate the stem cell niche is key to regenerative medicine applications.

### **Integrins and niche interactions**

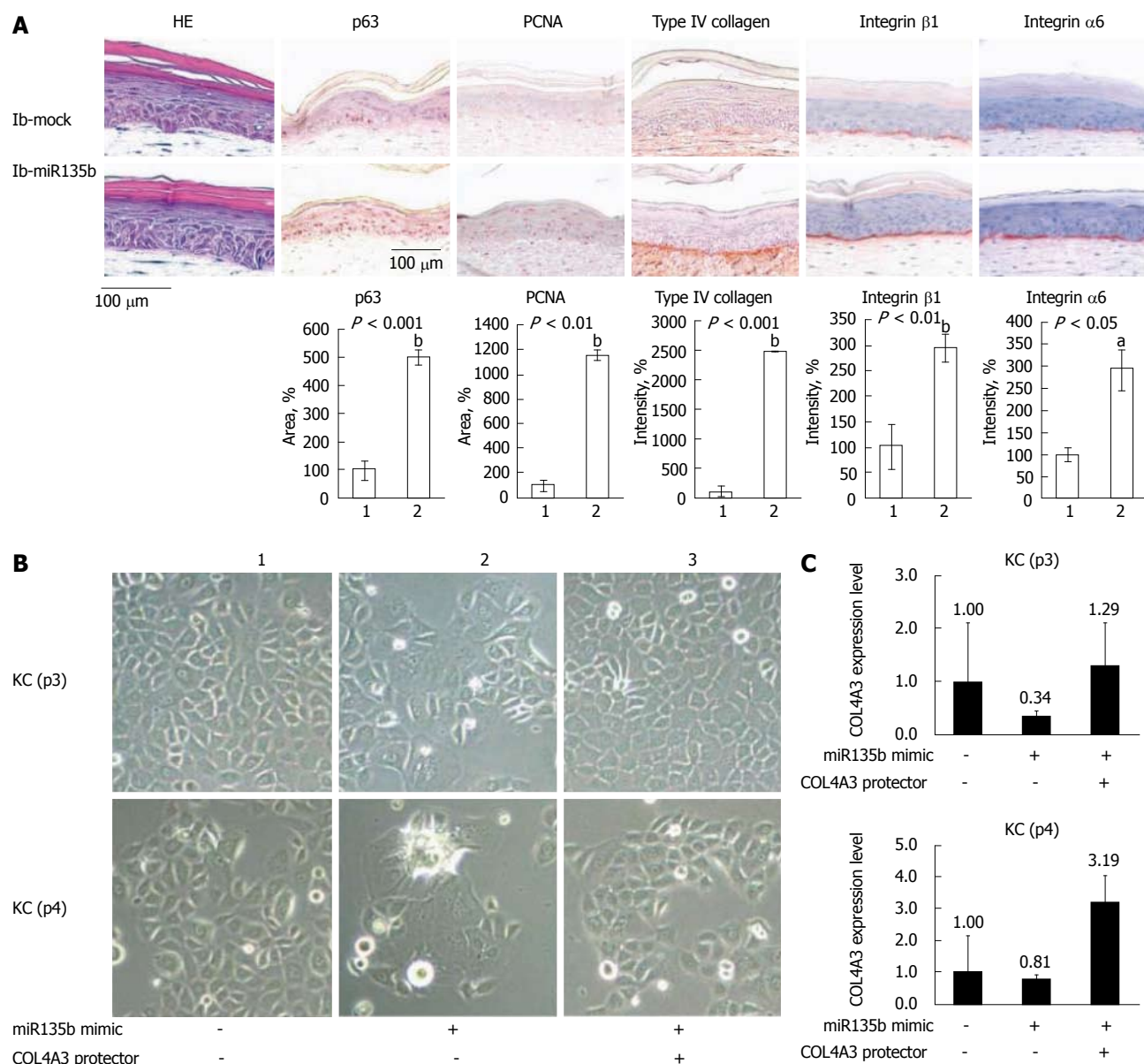
Integrins are a complicated group of proteins that function as transmembrane receptors; these proteins either connect cells to one another, or to the ECM. Such connections trigger signal transduction pathways, which result in biological responses such as cell proliferation or cell motility. Fibronectin, vitronectin, collagen, and laminin are all ligands for integrins. However, other proteins can mediate cell-cell or cell-ECM interactions; these include cadherins, the immunoglobulin superfamily of cell adhesion molecules, namely, selectins, and syndecans.

Stem cells, whether multipotent or tissue-specific, embryonic or adult, are controlled by intrinsic transcriptional regulation and extrinsic signals<sup>[22]</sup>. The extrinsic signals originate from the local microenvironment in which stem cells reside. It is well known that the ECM is an important component of the epidermal stem cell niche (see above). In the skin, high integrin levels can distinguish epidermal stem cells<sup>[14]</sup>, and now integrin expression can be used to enrich for stem cells in mixed cell populations<sup>[23,24]</sup>. In our reports, on skin equivalents (SEs), addition of IGFBP-2 to culture media increased the expression levels of both  $\alpha 6$  integrins and  $\beta 1$  integrins in cultured keratinocytes, which are associated with an increased number of epidermal stem cells (*p63* positive cells)<sup>[25]</sup> (Figure 3). Furthermore, it has been reported that activation of  $\beta 1$  integrin decreases terminal differentiation<sup>[26]</sup>. Signaling studies have demonstrated that the Erk/MAP kinase pathway, which acts downstream of  $\beta 1$  integrins, provides a signal which inhibits differentiation<sup>[26,27]</sup>. Collectively, these findings are consistent with previous reports that integrins are related to stem cell maintenance.

### **Integrin regulation and stem cells**

Few studies have dissected how epidermal integrin expression and *ECM* gene expression are regulated. In our previous study, we reported that when hair



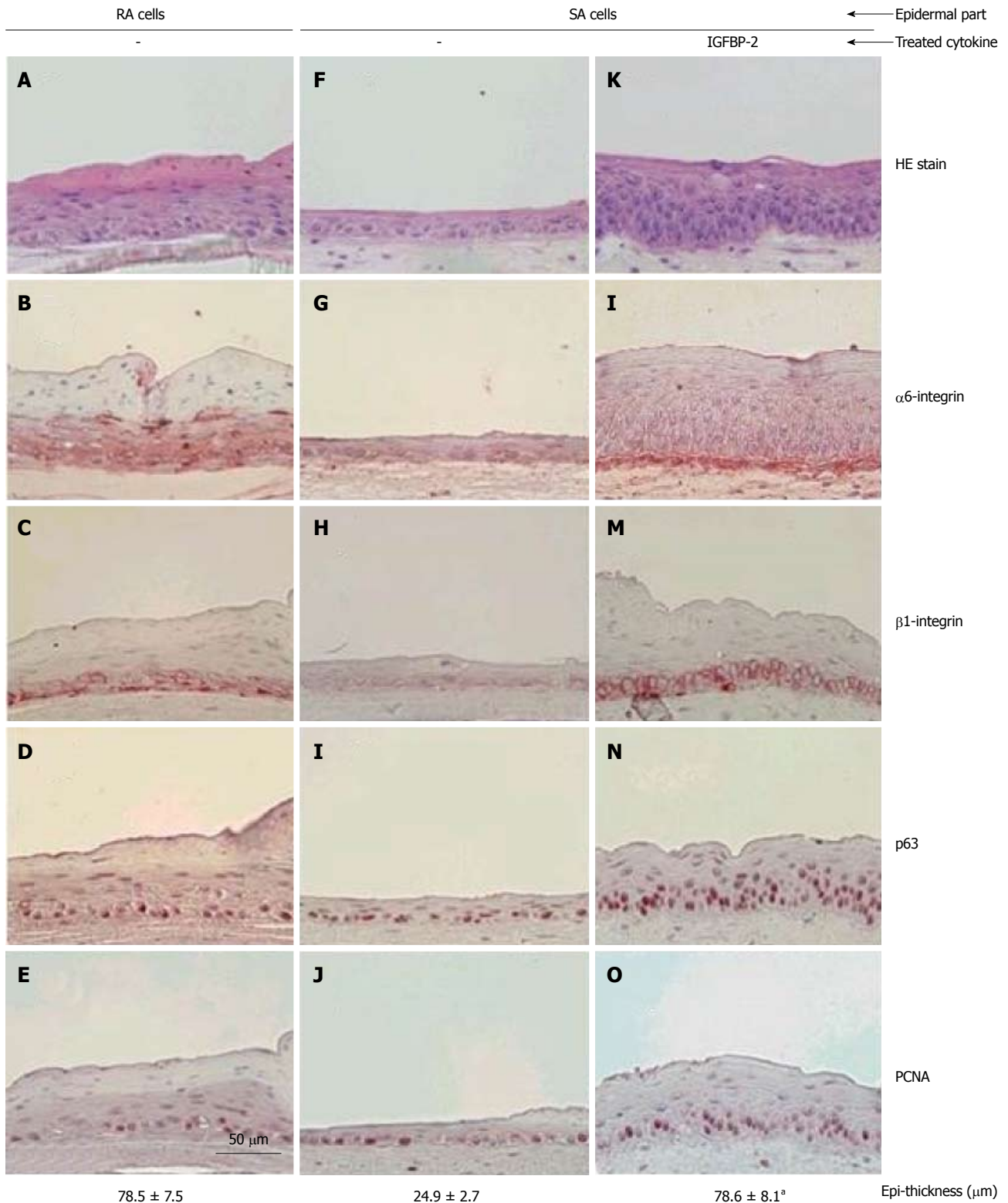


**Figure 2** The inhibition of miR135b prolongs the stemness and proliferative potential of epidermal keratinocytes in skin equivalents models. **A:** Histology of skin equivalent (SE) models constructed with ib-mock-transfected or ib-miR135b-transfected keratinocytes, and immunohistochemical staining of these models for p63, proliferating cell nuclear antigen, type IV collagen, and integrin  $\beta 1$ , and integrin  $\alpha 6$ . Expression levels of p63, PCNA, type IV collagen, integrin  $\beta 1$ , and integrin  $\alpha 6$  were increased in SEs generated from ib-miR135b-transfected cells; 1: ib-mock; 2: ib-miR135b; **B:** Fast-forward co-transfection of keratinocytes with “miRNA mimic” and “target protector”. 1: Negative control of miRNA mimic (10 nmol/L) and negative control of target protector (500 nmol/L); 2: miRNA mimic (10 nmol/L) and negative control of target protector (500 nmol/L); 3: MiRNA mimic and target protector (500 nmol/L). The inhibition of miR135b increases proliferation of keratinocyte via targeting type IV collagen; **C:** Real-time PCR analysis of gene expression in co-transfected keratinocytes. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control. The result was same as (B). HE: Hematoxylin and eosin.

follicle dermal sheath cells were used to construct SEs, this resulted in increased epidermal thickness and increased expression of  $\alpha 6$  integrins, compared to SEs generated from fibroblasts<sup>[28]</sup> (Figure 4). Building upon this study, it was found that IGFBP-2 is a major factor produced by dermal sheath cells, which regulates the regenerative capacity of the skin; IGFBP-2 plays an important role in maintaining stem cell characteristics in human epidermal keratinocytes<sup>[25]</sup>. These findings suggest that levels of integrins, which can be regulated by IGFBP-2, are vital for the maintenance of epidermal stem cell characteristics in the skin.

As it is well known that fetal wounds heal rapidly, we also evaluated the regenerative potential of and expression of integrins in neonatal keratinocytes and fibroblasts. When neonatal keratinocytes were used for epidermis reconstruction, expression of p63 was increased compared to adult keratinocytes; levels of integrins were also increased in this model<sup>[28]</sup>. Recently, we also demonstrated that when neonatal fibroblasts were used to make a dermal substitute, both integrin and p63 expression increased (personal communication). Collectively, these results demonstrate that neonatal cells maintain a higher



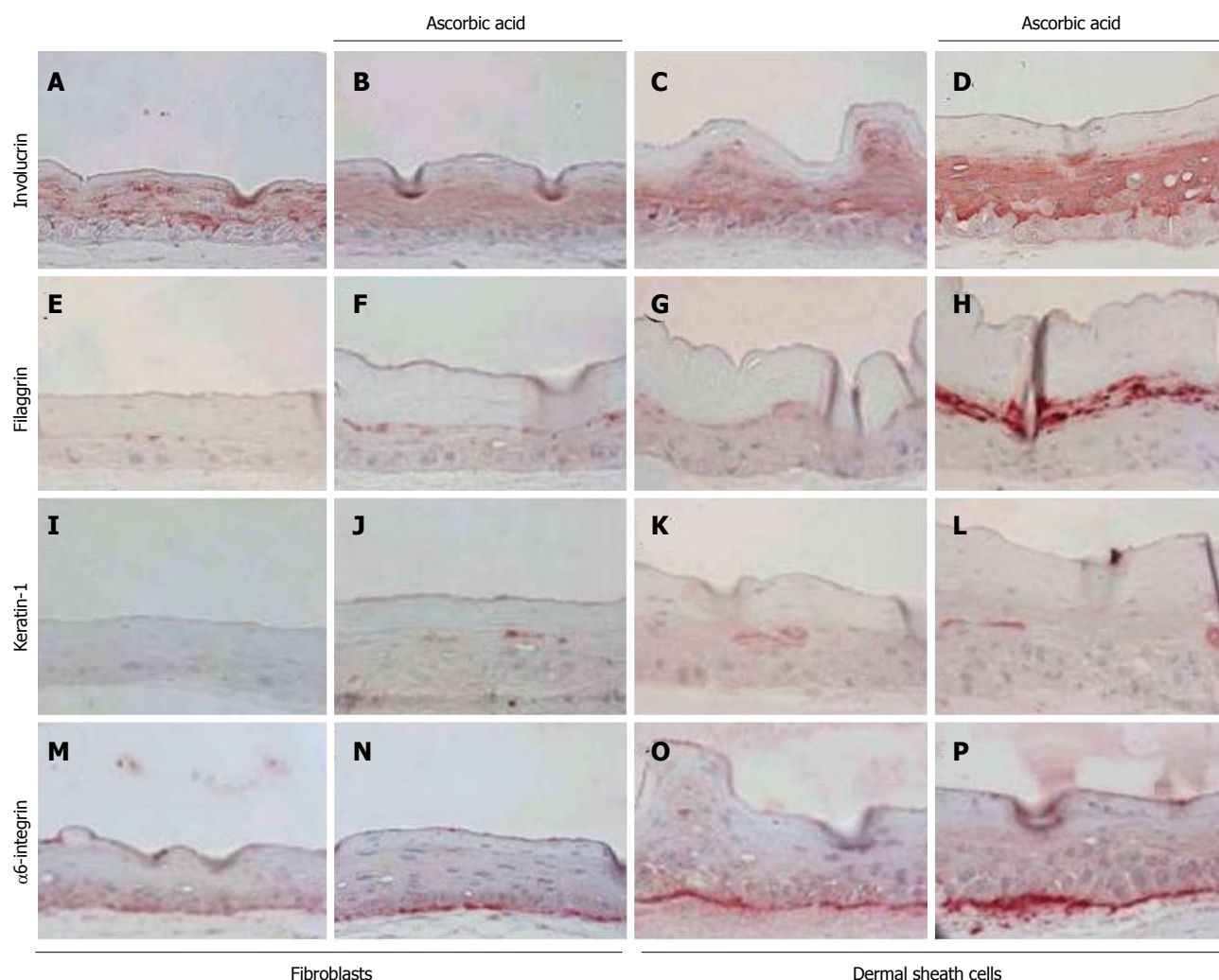


**Figure 3** Effects of IGFBP-2 on skin equivalents reconstruction. Skin equivalents (SEs) were cultured with rapidly adhering (RA) cells (A-E) or slowly adhering (SA) cells (F-O). Sections of SEs cultured under different treatments [media only (A-J) and 200 ng/mL IGFBP-2 (K-O)] and stained for hematoxylin and eosin (HE) (A, F, K),  $\alpha 6$  integrin (B, G, I),  $\beta 1$  integrin (C, H, M), p63 (D, I, N), and proliferating cell nuclear antigen or proliferating cell nuclear antigen (PCNA) (E, J, O). Addition of IGFBP-2 to culture media increased the expression levels of  $\alpha 6$  integrins,  $\beta 1$  integrins, p63, and PCNA in cultured keratinocytes. Original magnification 400  $\times$ . Epidermal thickness was measured at 10 randomly selected locations using an Olysia Soft Imaging System (Olympus). Statistical analysis was performed using the Student's *t*-test. <sup>a</sup>*P* < 0.01 vs SA + no cytokine model.

regenerative potential compared to adult cells. This may result from the fact that neonatal cells have the

potential to express more integrins, which favors the environment of the epidermal stem cell niche.





**Figure 4** Immunohistochemical staining for involucrin, filaggrin, keratin 1, and  $\alpha 6$  integrin in skin equivalents generated from fibroblasts or dermal sheath cells. Sections of skin equivalents (SEs) produced using fibroblasts (A, B, E, F, I, J, M, and N) or dermal sheath cells (C, D, G, H, K, L, O, and P) were stained for involucrin (A-D), filaggrin (E-H), keratin 1 (I-L), and  $\alpha 6$  integrin (M-P). SEs were cultured in complete medium in the presence (B, D, F, H, J, L, N, and P) or absence (A, C, E, G, I, K, M, and O) of ascorbic acid. Expression of involucrin, filaggrin, keratin 1, and  $\alpha 6$  integrin was up-regulated in SEs of dermal sheath cells compared with SEs of fibroblasts. Original magnification: 400  $\times$  (A-P).

## CONCLUSION

Many ECM genes are up regulated in epidermal stem cells. These proteins act as extrinsic signals that may affect stem cell fate. In this review, we have presented a general overview of ECM interactions, and discussed our previous reports demonstrating the important role that integrins and type IV collagen play in shaping the epidermal stem cell niche.

## REFERENCES

- 1 **Watt FM**, Fujiwara H. Cell-extracellular matrix interactions in normal and diseased skin. *Cold Spring Harb Perspect Biol* 2011; **3** [PMID: 21441589]
- 2 **Pajonk F**, Vlashi E. Characterization of the stem cell niche and its importance in radiobiological response. *Semin Radiat Oncol* 2013; **23**: 237-241 [PMID: 24012337 DOI: 10.1016/j.semradonc.2013.05.007]
- 3 **Schofield R**. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* 1978; **4**: 7-25 [PMID: 747780]
- 4 **Brizzi MF**, Tarone G, Defilippi P. Extracellular matrix, integrins, and growth factors as tailors of the stem cell niche. *Curr Opin Cell Biol* 2012; **24**: 645-651 [PMID: 22898530 DOI: 10.1016/j.ccb.2012.07.001]
- 5 **Evans ND**, Oreffo RO, Healy E, Thurner PJ, Man YH. Epithelial mechanobiology, skin wound healing, and the stem cell niche. *J Mech Behav Biomed Mater* 2013; **28**: 397-409 [PMID: 23746929 DOI: 10.1016/j.jmbbm.2013.04.023]
- 6 **Mohyeldin A**, Garzón-Muvdi T, Quiñones-Hinojosa A. Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell* 2010; **7**: 150-161 [PMID: 20682444 DOI: 10.1016/j.stem.2010.07.007]
- 7 **Fuchs E**. Skin stem cells: rising to the surface. *J Cell Biol* 2008; **180**: 273-284 [PMID: 18209104 DOI: 10.1083/jcb.200708185]
- 8 **Watt FM**, Jensen KB. Epidermal stem cell diversity and quiescence. *EMBO Mol Med* 2009; **1**: 260-267 [PMID: 20049729 DOI: 10.1002/emmm.200900033]
- 9 **Breitkreutz D**, Koholt I, Thiemann K, Nischt R. Skin basement membrane: the foundation of epidermal integrity--BM functions and diverse roles of bridging molecules nidogen and perlecan. *Biomed Res Int* 2013; **2013**: 179784 [PMID: 23586018 DOI: 10.1155/2013/179784]
- 10 **Watt FM**. Role of integrins in regulating epidermal adhesion, growth and differentiation. *EMBO J* 2002; **21**: 3919-3926 [PMID: 12145193 DOI: 10.1093/emboj/cdf399]



- 11 **Scadden DT.** The stem-cell niche as an entity of action. *Nature* 2006; **441**: 1075-1079 [PMID: 16810242 DOI: 10.1038/nature04957]
- 12 **Ye J, Wu D, Wu P, Chen Z, Huang J.** The cancer stem cell niche: cross talk between cancer stem cells and their microenvironment. *Tumour Biol* 2014; **35**: 3945-3951 [PMID: 24420150 DOI: 10.1007/s13277-013-1561-x]
- 13 **Watt FM, Jones PH.** Expression and function of the keratinocyte integrins. *Dev Suppl* 1993: 185-192 [PMID: 8049472]
- 14 **Jones PH, Harper S, Watt FM.** Stem cell patterning and fate in human epidermis. *Cell* 1995; **80**: 83-93 [PMID: 7813021 DOI: 10.1016/0092-8674(95)90453-0]
- 15 **Kim DS, Cho HJ, Choi HR, Kwon SB, Park KC.** Isolation of human epidermal stem cells by adherence and the reconstruction of skin equivalents. *Cell Mol Life Sci* 2004; **61**: 2774-2781 [PMID: 15549181 DOI: 10.1007/s00018-004-4288-4]
- 16 **Choi HR, Nam KM, Park SJ, Kim DS, Huh CH, Park WY, Park KC.** Suppression of miR135b increases the proliferative potential of normal human keratinocytes. *J Invest Dermatol* 2014; **134**: 1161-1164 [PMID: 24129066 DOI: 10.1038/jid.2013.427]
- 17 **Morris RJ, Liu Y, Marles L, Yang Z, Trempus C, Li S, Lin JS, Sawicki JA, Cotsarelis G.** Capturing and profiling adult hair follicle stem cells. *Nat Biotechnol* 2004; **22**: 411-417 [PMID: 15024388 DOI: 10.1038/nbt950]
- 18 **Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C, McKeon F.** p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 1999; **398**: 714-718 [PMID: 10227294 DOI: 10.1038/19539]
- 19 **Wagers AJ.** The stem cell niche in regenerative medicine. *Cell Stem Cell* 2012; **10**: 362-369 [PMID: 22482502 DOI: 10.1016/j.stem.2012.02.018]
- 20 **Katano T, Ootani A, Mizoshita T, Tanida S, Tsukamoto H, Ozeki K, Ebi M, Mori Y, Kataoka H, Kamiya T, Toda S, Joh T.** Establishment of a long-term three-dimensional primary culture of mouse glandular stomach epithelial cells within the stem cell niche. *Biochem Biophys Res Commun* 2013; **432**: 558-563 [PMID: 23485463 DOI: 10.1016/j.bbrc.2013.02.051]
- 21 **Levis HJ, Massie I, Dziasko MA, Kaasi A, Daniels JT.** Rapid tissue engineering of biomimetic human corneal limbal crypts with 3D niche architecture. *Biomaterials* 2013; **34**: 8860-8868 [PMID: 23968855 DOI: 10.1016/j.biomaterials.2013.08.002]
- 22 **Watt FM, Driskell RR.** The therapeutic potential of stem cells. *Philos Trans R Soc Lond B Biol Sci* 2010; **365**: 155-163 [PMID: 20008393 DOI: 10.1098/rstb.2009.0149]
- 23 **Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li H, Eaves CJ.** Purification and unique properties of mammary epithelial stem cells. *Nature* 2006; **439**: 993-997 [PMID: 16395311]
- 24 **Wagers AJ, Weissman IL.** Differential expression of alpha2 integrin separates long-term and short-term reconstituting Lin-/loThy1.1(lo)c-kit+ Sca-1+ hematopoietic stem cells. *Stem Cells* 2006; **24**: 1087-1094 [PMID: 16373693 DOI: 10.1634/stemcells.2005-0396]
- 25 **Kim DS, Cho HJ, Yang SK, Shin JW, Huh CH, Park KC.** Insulin-like growth factor-binding protein contributes to the proliferation of less proliferative cells in forming skin equivalents. *Tissue Eng Part A* 2009; **15**: 1075-1080 [PMID: 18803482 DOI: 10.1089/ten.tea.2008.0236]
- 26 **Evans RD, Perkins VC, Henry A, Stephens PE, Robinson MK, Watt FM.** A tumor-associated beta 1 integrin mutation that abrogates epithelial differentiation control. *J Cell Biol* 2003; **160**: 589-596 [PMID: 12578911 DOI: 10.1083/jcb.200209016]
- 27 **Zhu AJ, Haase I, Watt FM.** Signaling via beta1 integrins and mitogen-activated protein kinase determines human epidermal stem cell fate in vitro. *Proc Natl Acad Sci USA* 1999; **96**: 6728-6733 [PMID: 10359780 DOI: 10.1073/pnas.96.12.6728]
- 28 **Cho HJ, Bae IH, Chung HJ, Kim DS, Kwon SB, Cho YJ, Youn SW, Park KC.** Effects of hair follicle dermal sheath cells in the reconstruction of skin equivalents. *J Dermatol Sci* 2004; **35**: 74-77 [PMID: 15194152 DOI: 10.1016/j.jdermsci.2004.03.004]

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## Neural stem cells could serve as a therapeutic material for age-related neurodegenerative diseases

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### Abstract

Progressively loss of neural and glial cells is the key event that leads to nervous system dysfunctions and diseases. Several neurodegenerative diseases, for instance Alzheimer's disease, Parkinson's disease, and Huntington's disease, are associated to aging and suggested to be a consequence of deficiency of neural stem cell pool in the affected brain regions. Endogenous neural stem cells exist throughout life and are found in

specific niches of human brain. These neural stem cells are responsible for the regeneration of new neurons to restore, in the normal circumstance, the functions of the brain. Endogenous neural stem cells can be isolated, propagated, and, notably, differentiated to most cell types of the brain. On the other hand, other types of stem cells, such as mesenchymal stem cells, embryonic stem cells, and induced pluripotent stem cells can also serve as a source for neural stem cell production, that hold a great promise for regeneration of the brain. The replacement of neural stem cells, either endogenous or stem cell-derived neural stem cells, into impaired brain is highly expected as a possible therapeutic mean for neurodegenerative diseases. In this review, clinical features and current routinely treatments of age-related neurodegenerative diseases are documented. Noteworthy, we presented the promising evidence of neural stem cells and their derivatives in curing such diseases, together with the remaining challenges to achieve the best outcome for patients.

**Key words:** Alzheimer's disease; Huntington's disease; Neural stem cells; Parkinson's disease; Cell therapy; Neurodegenerative diseases

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**Core tip:** Neural stem cells present throughout life of human. The cells can be distinguished from differentiated progeny by acknowledging a few key features, including proliferation, self-renewal, multipotency and molecular markers. These features make neural stem cells as an essential component in the development of nervous system and in maintaining cell number of adult nervous tissues following injury and diseases. Besides conventional treatments, neural stem cells have been proposed as a promising approach to cure patients with neurodegenerative diseases. Several animal studies showed the efficiency of neural stem cells in treating age-related neurodegenerative diseases, in particular



## Alzheimer's, Parkinson's and Huntington's diseases.

Suksuphew S, Noisa P. Neural stem cells could serve as a therapeutic material for age-related neurodegenerative diseases. *World J Stem Cells* 2015; 7(2): 502-511 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i2/502.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i2.502>

## INTRODUCTION

Neurodegenerative diseases are a global burden and often affect elderly population worldwide. The central and shared characteristics of neurodegenerative diseases are the loss of neuronal cells in either the central or peripheral nervous systems. Aging can cause chronic neurodegeneration, which results in the loss of particular neuronal subtypes over time. Parkinson's, Alzheimer's and Huntington's diseases are the three most dominant neurodegenerative diseases, found worldwide. In the brain, Alzheimer's and Huntington's diseases result in widespread loss of neurons, while Parkinson's disease involves the specific loss of midbrain dopaminergic neurons. In spite of an extensive effort to develop treatment against these neurodegenerative diseases, effective treatments still do not exist. Recently, cell therapy has been proposed as an attractive option, and the application of stem cell technology to regenerative medicine is rapidly progressed. Stem cell-based approaches are applicable for therapeutic purposes, for instance it might be possible to replace lost neurons or glia by transplantation of stem cell-derived nervous cells. Cell replacement might also be succeeded by activating endogenous neural stem cells in patients own brain to form new neurons and glia. In this review, we summarized clinical manifestations of the major age-related neurodegenerative diseases, in particular Parkinson's, Alzheimer's, and Huntington's diseases, together with their currently available treatments. The properties and characteristics of neural stem cells were presented, highlighting key features that enable neural stem cells to be a promising therapeutic material. Lastly, we documented evidence of neural stem cells and other stem cells to cure neurodegenerative diseases, especially Parkinson's, Alzheimer's, and Huntington's diseases.

## CLINICAL MANIFESTATIONS OF AGE-RELATED NEURODEGENERATIVE DISEASES: ALZHEIMER'S, PARKINSON'S, AND HUNTINGTON'S DISEASES

Parkinson's disease (PD) is one of the most common movement malfunctions and affects nearly 1% in elderly population age over 60. The average age

of onset at 55 years, and the frequency increases markedly with age<sup>[1,2]</sup>. There are several lines of studies proposing that genetic mutations or polymorphisms are correlated and can contribute to an increased risk of PD in senior population<sup>[3]</sup>. Although genetics are only influenced a minor group of PD, the studies of genetic factors improve an understand of the pathophysiology of PD. An increasing number of genetic risks appear to be connected with PD, and many of them followed Mendelian inheritance rules, including alpha-synuclein gene (*SNCA*, PARK1/4), parkin gene (*Parkin*, PARK2), P-TEN-induced putative kinase 1 gene (*PINK1*, PARK6), Daisuke-Junko 1 gene (*DJ-1*, PARK7), and leucine-rich repeat kinase 2 gene (*LRRK2*, PARK8). These genes have been conclusively proven as a monogenic etiology for familial parkinsonism<sup>[4]</sup>. To characterize PD, patients can be diagnosed by several key manifestations, including bradykinesia (slowness of establishing the voluntary movement and declination in speed of repetitive manners), resting tremor (4-6 Hz), and rigidity<sup>[5]</sup>. A group of clinical outcome usually presents in an asymmetrical manner. The presences of a change in handwriting with micrographia, reduced sense of smell and facial expression, and loss of arm swing on one side of the body are often noted as early clinical features<sup>[6,7]</sup>. When the disease progressively developed, gait instability, drooling of saliva, and impairment of postural reflexes will appear. Non-motor symptoms, such as neurobehavioral dysfunctions, insomnia, cognitive and sexual impairment may present in early or late stages of the disease progression. Depression and dementia are also common symptoms in non-motor presentation of PD's patients. A good response to levodopa treatment can differentiate PD from Parkinsonism due to other causes. The standard diagnosis for PD still relies on the neuropathological examination. All the cardinal signs of PD involve to motor disability in asymmetrical onset, without other causes of Parkinsonian syndrome and non-motor manifestations, including psychiatric symptoms<sup>[8]</sup>. The UK Parkinson's Disease Society Brain Bank criteria is commonly used as a reference.

Alzheimer's disease (AD) is a progressively degeneration of the brain cortex, manifested by memory and intellectual decline, and progressive deficiency of daily-living activities<sup>[9]</sup>. AD affects about 6% of the population aged over 65 years old worldwide. Age is considered the single utmost vital risk factor for AD ontogeny. Amyloid- $\beta$ , oxidative stress, inflammation, and vascular injury appear to play an essential function in the neurodegeneration of AD<sup>[10]</sup>. Multiple genetic defects have been associated to the development of AD. Initially, the molecular genetics of AD can be obtained from early-onset AD families with autosomal dominant patterns of inheritance. Highly penetrant mutations were identified in three genes: *APP*, *PSEN1*, and *PSEN2*<sup>[11-13]</sup>. Interestingly, these genes are functionally participated in the processing of amyloid precursor protein. This suggests a central role of amyloid



precursor protein in AD pathology. Clinically, AD patients gradually evolve from the mildest to the most severe clinical manifestations of illness. Primarily, AD's patients present as an inability to restore newly acquired information, which can be examined by repetitively questioning. This short term memory impairment results from the atrophy of the hippocampus, especially the temporal horn<sup>[14]</sup>. The clinical features are an amnesic type of memory dysfunction, deterioration of visuospatial, and language deficits<sup>[15]</sup>. When the disease advances, devastation of other brain domains of cognition, including abstract thinking, language, judgment, calculation, and executive function, will develop. This impairment usually coincide with the aberration of art and social interpretations. AD patients evolve their symptoms from the loss of basic activities (e.g., dressing, eating, and bathing) to higher functions of activities in daily living (e.g., shopping, business management). Psychiatric and behavioural instabilities also progress over the course of AD. Emotional disorders (e.g., depression 25%-30%, anxiety 15%-25%, delusions in 20% of cases) and loss of initiation or apathy (up to 40%) commonly develop at early stage of AD and continue during disease progression. Motor and sensory dysfunctions, gait disorders, and other abnormal movements will appear at the late stage of the disease. The diagnosis of AD is frequently referred to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) and the National Institutes of Neurologic and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria<sup>[16]</sup>. The gold standard of AD diagnosis is based on neuropathological findings of neuritic plaques (extra-neuronal  $\beta$  amyloid-containing plaques) and intra-neuronal neurofibrillary tangles in patient's brain tissues.

Huntington's disease (HD) is a rare inherited neurological disease, manifested by unwanted choreiform movements, psychiatric instabilities and cognitive deficiency<sup>[17]</sup>. Disease onset is commonly between 30 and 50 years old and the patients with HD can present disease conditions at any time points during the disease progression. HD is a disease triggered by a repeated trinucleotide, CAG (more than 36 repeats), of Huntingtin gene located on the short arm of chromosome 4<sup>[18]</sup>. Large duplication of CAG repeats are susceptible to replication error of the chromosome during meiosis, leading to extension or reduction of the CAG repeats<sup>[19]</sup>. Choreiform movement is an early symptom, which causes limb incoordination and impaired function. This movement disorder is as a consequence of a selective neural cell death and atrophy in the putamen and caudate of the brain. Another significant feature in HD patients is an incapability to sustain a voluntary muscle contraction. The pattern of this symptom tends to change over time with other abnormal movements, such as rigidity, dystonia, and bradykinesia. HD patients might confront non-motor symptoms that could be more disturbing than motor symptoms, such as personality

changes, impulsivity, abnormal perceptions, lack of insight, and disability to learn new information or knowledge<sup>[20]</sup>. Depression is also found and appears as a part of disease. Metabolic signs, for example weight loss and sleep problems, may contribute to depression of HD patients. A diagnosis of HD is relied principally on questionnaires, physical examination, family medical history, and neurological and psychiatric investigations. Brain imaging technology, in particular magnetic resonance imaging, can show structural alterations at specific locations in the brain affected by mutant Huntingtin protein. In spite of identification of the Huntingtin gene and other causative proteins, the mechanisms contributed to the pathogenesis and symptoms of HD remain largely elusive and this greatly hampers efficient development of clinical treatment.

## CURRENT THERAPEUTIC APPROACH OF AGE-RELATED NEURODEGENERATIVE DISEASE

The early success in developing treatment strategies relied on the early understanding that PD is a DA deficiency disorder. Dopamine therapy is effective for improving both motor and non-motor symptoms in initial PD stage. Levodopa (LD) is considered as the most efficient and best accepted antiparkinsonian compound<sup>[21]</sup>. Initiating levodopa as first-line therapy may achieve optimal outcomes in terms of patient function in the early years of the disease. Catechol-O-methyltransferase (COMT) inhibition increases the peripheral bioavailability of LD and reduces 3-O-methyldopa formation. The administration of COMT inhibitors with LD ensures a more stable plasma LD level and, consequently, it improves motor fluctuations. The use of COMT inhibitors improve the half-life of LD, and the triple combination carbidopa (CD)-LD-entacapone provide the most sustained LD plasma level<sup>[22]</sup>. However, in long-term, this combination treatment is accompanied with the fluctuations of motor functions, dyskinesias and neuropsychiatric manifestations<sup>[23]</sup>. Switching to alternative LD formulations, or supplementation of therapy with additional agents are general strategies for managing motor complications associated with LD. In addition, severe staged patients continually developed features that do not respond well to LD treatment, such as motor fluctuations, freezing episodes, autonomic dysfunctions, gait instability, dementia, and symptoms related to side effects of other medications<sup>[24]</sup>. Surgical treatment is considered as an option for these patients who have motor fluctuations and dyskinesias, because these symptoms cannot be adequately managed by medications. The principal surgical option is deep brain stimulation, which has largely replaced neuroablative lesion surgeries for improving motor control<sup>[25]</sup>. Next, the principal risk factor for AD is age. Several routinely-used drugs



can alleviate diseased conditions, but do not prove significant disease-improving effects<sup>[26]</sup>. A number of therapeutic approaches intended at inhibiting disease progression are now developed into clinical trials. With disease advancement, brain degeneration in areas of memory, cognition, psychiatry, and movement, occurs to varying degrees. In clinical practice, non-medical interventions should be firstly tried, especially when symptoms are not causing distress to impact patients in daily living activity. Medical treatment for improving cognition, for instance acetylcholinesterase inhibitor, offers cognitive improvement (e.g., memory deterioration, language dysfunctions, and executive dysfunction,) and psychiatric repairment (e.g., depression, hallucination, agitation and delusion) in all stages of disease progression. The treatment with N-methyl-D-aspartate antagonist presents similar result, and this molecule can be applied in combination with acetylcholinesterase inhibitors in severe stage of AD patients<sup>[27]</sup>. Many clinical trials aim to target at the generation and eradication of amyloid- $\beta$  ( $A\beta$ ) peptide, a dominant element of AD pathogenesis. Currently, the anti- $A\beta$  strategies are advancing with the increasing number of potential drugs. Direct interaction with  $A\beta$  of small molecules could diminish  $A\beta$  aggregation and deposition within the brain, thus reducing  $A\beta$ -mediated synaptic dysfunction and neuronal cell death. The primarily anti- $A\beta$  drug to accomplish a clinical study was tramiprosate. Tramiprosate is a glycosaminoglycan derivative that binds to a single molecule of  $A\beta$ . The interaction of tramiprosate and  $A\beta$  reduces the aggregation and toxicity of  $A\beta$ , while promote the clearance of  $A\beta$  from the patient's brain. Although the treatment with tramiprosate was well tolerated, it fell short of an advantageous effect on the primary outcomes, which are the improvement on cognitive functions and clinical stages of AD patients. Another strategy to reduce  $A\beta$  level within the brain is to minimize its production. Gamma secretase, a transmembrane enzyme, is reportedly required for  $A\beta$  generation in several tissues, including brain. It cleaves at one end of the amyloid precursor protein, in which  $A\beta$  is generated. Tarenflurbil, a non-steroidal anti-inflammatory drug, was found to lower the activity of gamma secretase enzyme, and, consequently reduce  $A\beta$  production<sup>[28]</sup>. In a Phase II of clinical trial in AD patients with mild-to-moderate symptoms, tarenflurbil was considerably safe and well tolerated; however, the beneficial effect of tarenflurbil on cognitive repairment was not observed<sup>[29]</sup>. One possible explanation for the disappointing outcome of tarenflurbil is that oral administration cannot elevate the concentration of tarenflurbil inside the brain to be at sufficient level to reduce  $A\beta$  production. An alternative approach focuses at modulating the abnormal aggregation of neurofibrillary tau protein, which is also another key feature of AD<sup>[30]</sup>. Tau protein is a microtubule-associated protein, and found predominantly in neurons. Tau protein functions in

promoting and stabilizing the assembly of tubulin protein into microtubule filaments. In AD patients, hyperphosphorylation of tau protein is found in the cortex and hippocampus of the brain, and this could disrupt the original function of Tau protein. The hyperphosphorylated Tau results in self-assembly into paired helical filament structures that accumulate and form intraneuronal tangles, a neurotoxic agent. There are several compounds that can inhibit Tau-self aggregation. For instance, methylene blue, an extensively used histology dye, has been tested for its interference effect on Tau-self aggregation<sup>[31]</sup>. This compound is currently experimented as a potential effective molecule for AD treatment, since a Phase II clinical study of methylene blue has been completed, and some outcomes suggested a drug benefit in a group of AD participants<sup>[32]</sup>. Nevertheless, it is noted that there are no effective treatments available for AD at the present. Thus, the current strategy of AD management is to decrease symptoms and improve patient's quality of life. It is sensible to expect that, in the coming years, an optimal synergistic combination of these therapeutic compounds will be formulated, which will be able to modify neurodegenerative cascade and, therefore, reduce the global impact on the brain of this dreadful disease.

Lastly, the treatment of HD has been focusing on relieving motor dysfunctions (chorea), cognitive decline and psychiatric manifestations<sup>[33]</sup>. Pharmacological treatment of Huntington's disease might not be necessary if symptoms are mild or not troublesome. Clinically, there are various potential drugs that show the capacity to correct the conditions of HD patients. Treating chorea is an important part of HD management, because it interferes to the quality of life of HD patients. Tetrabenazine was approved in 2008 by the United States Food and Drug Agency to control the involuntary periodic movements of the limbs and face, associated with chorea<sup>[34]</sup>. Tetrabenazine is a central monoamine extingisher that reversibly binds to the type-2 vesicular monoamine transporter<sup>[35]</sup>. Dopamine and glutamate transmission and interaction are one of the affected pathway in the HD brain. The aberrations of this can result in striatal and cortical connection dysfunctions<sup>[36]</sup>. Therefore, most treatments, investigated at chorea, have targeted to these neurotransmitters and their receptors. Antipsychotic drugs, for instance clozapine, haloperidol, olanzapine, and, chlorpromazine, which act by reducing dopamine levels, are occasionally prescribed to lower chorea symptom<sup>[37]</sup>. A side benefit of these antipsychotic drugs is that they also help controlling the HD-associated psychotic behaviors. Besides pharmacological treatment, physical and occupational therapy at the initial stages of the disease can correct motor dysfunction and locomotion, which is due to the fact that exercises can reinforce muscle strength and improve balance and posture of HD patients<sup>[38]</sup>. Psychiatric therapy can help reducing stress, anxiety



**Table 1** Characteristics and conventional treatment for Alzheimer's, Parkinson's and Huntington's diseases

Disease	Characteristics	Treatment	Ref.
Alzheimer's	Memory impairment Impaired reasoning and handling of complex tasks, poor judgment Changes in personality, behavior, or comportment	Medications: Cholinesterase inhibitors NMDA antagonist Non-medicals Psychological supports	[9,15,16,26,27,30]
Parkinson's	Motor symptoms (1) Rest tremor (2) Bradykinesia (3) Rigidity (4) Loss of postural reflexes Non-motor symptoms (1) Autonomic dysfunction (2) Cognitive/ neurobehavioral abnormalities (3) Sleep disorders (4) Sensory abnormalities such as anosmia, paresthesias and pain	1 Medications Dopaminergics: (1) Levodopa (2) Ergot dopamine agonists (3) Non-ergot dopamine agonists Non-dopaminergic: (1) COMT inhibitors (2) MAO-B inhibitors 2 Non-medicals Ablative lesions Deep brain stimulation	[1,6-8,23-25]
Huntington's	Choreiform movement Cognitive impairment Behavior and psychological disorders	Medications Symptomatic agents	[17,18,20,33,36]

NMDA: N-methyl-D-aspartate receptor; COMT: Catechol-O-methyltransferase; MAO: Monoamine oxidase.

and depression, as well as help managing with the emotional changes associated with the disease. Table 1 summarized characteristics and conventional treatment for Alzheimer's, Parkinson's and Huntington's diseases.

## NEURAL STEM CELLS: PROPERTIES AND CHARACTERISTICS

Several lines of studies suggest that stem cells exist in the central nervous system (CNS)<sup>[39]</sup>. Neural stem cells exist not only in the embryonic brain, but also in the adult nervous system of all mammals, including human. Neural stem cells can be distinguished from differentiated neurons by acknowledging a few key features, including proliferation, self-renewal, multipotency and molecular markers<sup>[40]</sup>. These features make neural stem cells as an important element in CNS development and in maintenance cell number following injury and diseases or natural cell turnover.

### Proliferation and self-renewal

To reach the correct number of differentiated cells in the CNS, neural stem cells have to be precisely controlled a balance between cell proliferation and differentiation during the embryonic CNS development. This balance is regulated by both stimulatory and inhibitory signals to converge the requirements of the tissues in which neural stem cells provide newly differentiated progenies. Proliferation potential is one of the most essential characteristics of neural stem cells and it was shown that neural stem cells in adult brain can be propagated *in vitro* for years<sup>[41]</sup>. In order to generating a satisfactory number of neural stem cells, it is assumed that cell proliferation should be prevalent

in the early developmental timing, and that more cells differentiate into a specific cell type during the latter phases. This indicates that there is a high possibility for producing two undifferentiated daughter cells at early stages of development (symmetric division), and later cell division prefers the production of differentiated neurons and glial cells (asymmetric division). Neural stem cells residing in the developing neocortex undertake both symmetrical and asymmetrical divisions throughout their life span<sup>[42]</sup>. Several pathways that interconnect to control cell proliferation have been well documented. Perhaps the best comprehensive studies are those cell signalling pathways that are triggered by growth factors. All types of neural stem cells are generally responsive to multiple family of growth factors; however, the exact set of growth factors should be exclusively required for neural stem cells at specific stages and could distinguish stage-specific neural stem cells. Early neural stem cells entirely respond to fibroblast growth factor2 (FGF2 or bFGF), and the loss of FGF ligands or FGF receptors results in a significant diminution of neural stem cell proliferation<sup>[43]</sup>. On the other hand, the late emerging neural stem cells demand either FGF2 or epidermal growth factor for their proliferation<sup>[44]</sup>. It is noted that cell self-renewal is tightly connected to this growth factor responsive potential. Self-renewal is considered as a pivotal identity of neural stem cells because it is indispensable for the cells to preserve themselves, therefore at least one of the progeny retains similar molecular characteristics to the mother stem cells. It is important to note that while a process of self-renewal occurs, neural stem cells may undergo changes in their abilities to produce different progeny during development<sup>[45]</sup>.



### Multipotency

To be characterised as a neural stem cell in the CNS, a cell must contain a differentiation potential to give rise to neurons, astrocytes and oligodendrocytes<sup>[41,46]</sup>. It is noted that neural stem cell plasticity is progressively restricted as development advances, for example early neural stem cells appear to be specified a wide range of phenotypes, from anterior to posterior parts of the brain, while late neural stem cells is only restricted to its origin<sup>[47]</sup>. It was presented that adult neural stem cells exist mainly in two areas of the brain, subventricular zone (SVZ) and sub granular zone (SGZ), can be propagated *in vitro* for years<sup>[41]</sup>. Neural stem cells in the SVZ can differentiate into olfactory neurons, while neural stem cells of SGZ differentiate into granular neurons of the dentate gyrus. However, when transplanting SVZ neural stem cells into dentate gyrus, they differentiate into calbindin-positive granular cells, while transplanting SGZ neural stem cells into the olfactory bulb, tyrosine- and calretinin hydroxylase-positive cells were observed. Furthermore, when transplanted into the developing eyes, hippocampal neural stem cells exhibited several morphological and immunological properties of retinal cells, including photoreceptors<sup>[48]</sup>. This implies that the fate of adult neural stem cells could be influenced by environmental cues<sup>[49]</sup>. In addition to the effects from environment, cell intrinsic programs also influence cell differentiation capacity. The robust intrinsic differences, with respect to distinct differentiation potential, has been shown to exist between neural stem cells isolated from different brain regions<sup>[50,51]</sup>.

### Molecular markers

Many efforts have attempted to define neural stem cells according to their biological properties and molecular markers. In addition to those biological parameters, a series of immunoreactive antigens could also distinguish neural stem cells from others. Markers that define this population are now being developed; thus, they are commonly characterised retrospectively on the foundation of their behaviours. The evaluation of self-renewal of neural stem cells can be initially accomplished by the expression of specific molecular markers that can distinguish them from postmitotically differentiated cells. The successive expression of various intermediate filament genes has offered a useful system to classify distinct cell types during the early embryonic neural stem cells. The intermediate filament Nestin and Vimentin are exclusively expressed in the mitotically active cells during neural tube formation<sup>[52]</sup>. Embryonic neural stem cells express several astrocytic markers, for instance brain lipid-binding protein, glutamate transporter, S100 $\beta$ , RC2 and 3CB2<sup>[53]</sup>. Together with their differentiation potential, these astrocytic features distinguish embryonic neural stem cells from other types of astrocytes and differentiated cells. On the

other hand, adult SVZ neural stem cells appear to be slowly proliferating and long-term BrdU-retaining cells, which express GFAP and the glycoprotein CD133 (Prominin-1)<sup>[54]</sup>. Anatomical structures and comprehensive set of immunohistochemical markers help to ensure adult SVZ neural stem cell identity. A subset of these cells is identified by the expression of the intermediate filament Nestin, GFAP, transcription factor SOX2, and the RNA binding protein Musashi1, while absence of expression of the differentiated markers CD24, NeuN, and O4<sup>[55]</sup>. In the SGZ of the dentate gyrus, a comparable subset of neural stem cells expressing GFAP, SOX2 and Nestin resembles to dormant or quiescent adult neural stem cells which can give rise to mature astrocytes and neuroblasts<sup>[56]</sup>.

An evolving regulatory networks governing neural stem cell identity is delineated by an integration of cell internal transcription factors with the cell extrinsic stimuli from an environment or culture conditions. Unrevealing how these regulatory network functions to control neural stem cells is essential to better understand of neural stem cell biology. Ultimately, it will also accelerate the progression of a novel targeted therapy by using neural stem cells for curing neurological disorders, for instance brain tumours, brain injuries, and also neurodegenerative diseases, for example HD, AD and PD.

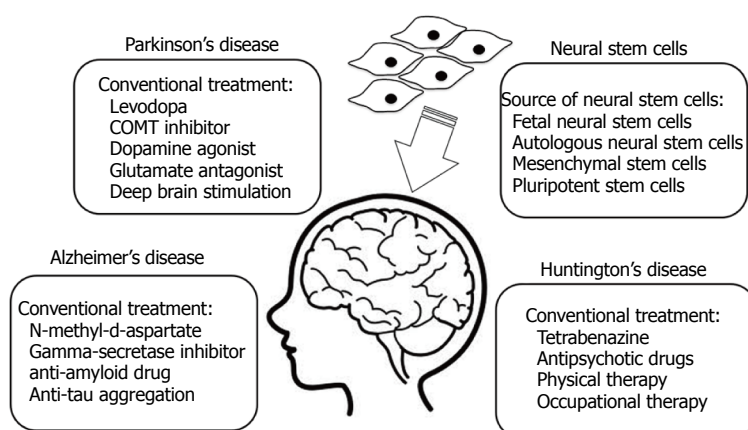
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## POTENTIAL OF NEURAL STEM CELLS FOR TREATING AGE-RELATED NEURODEGENERATIVE DISEASES

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Cell replacement by neural stem cell transplantation in the brain could alleviate pathological or functional deficits associated with diseases in the CNS. In addition to conventional clinical treatments, cell replacement therapy has offered the novel basis for the development of effective therapeutic strategies for human neurodegenerative diseases (Figure 1). It is hypothesised that the release of neurotransmitters and the production of neurotrophic factors from transplanted cells are possible mechanisms that promote neuronal regeneration in the damaged brain areas. PD is characterized by a vastly loss of midbrain dopaminergic neurons in the substantia nigra pars compacta and the striatum. It was reported that human fetal ventral mesencephalic tissues was successfully transplanted into the striatum of PD patients with advanced disease; however, this human tissue transplantation contains some limitation<sup>[57]</sup>. For example, a sufficient amount of fetal tissues was challenging to obtain and the survival and integration of transplanted cells in patient's brains was found to be minimal<sup>[58]</sup>. Besides, the development of abnormal movements was noticed in PD patients who have received embryonic nigral transplants. The potential propagation of Lewy body from host-to-graft tissues is also a serious concern<sup>[59]</sup>. The generation





**Figure 1 Neural stem cells as a therapeutic material for neurodegenerative diseases.** Schematic image presents conventional treatments for Alzheimer's, Parkinson's, and Huntington's diseases. Neural stem cells serve as a potential therapeutic material, which can be obtained from several sources.

of dopaminergic neurons from stem cells could be considered as a practical and effective alternative for PD treatment. Previous studies demonstrated that dopaminergic neurons derived from human pluripotent stem cells contained comparable molecular profiling, biochemical and electrophysiological properties to human midbrain dopaminergic neurons<sup>[60]</sup>. Importantly, the transplantation of dopaminergic neurons derived from human pluripotent stem cells can restore pathological conditions of the diseased animals, which are mice, rats and monkeys, without overgrowth of the neurons<sup>[61]</sup>. Histological analysis of the brain indicated that the transplanted cells were well survived and integrated within the experimented animals. In addition to the derivation from human pluripotent stem cells, dopaminergic neurons can be generated by the direct conversion of human fibroblasts by overexpression of five key transcription factors, including *Mash1*, *Ngn2*, *Sox2*, *Nurr1*, and *Pitx1*<sup>[62]</sup>. The direct reprogrammed dopaminergic neurons were positively stained for several markers for dopaminergic neurons, and showed characteristics of dopamine uptake and production. Moreover, dopaminergic neurons generated from fibroblasts provided symptomatic improvement when transplanted into a rat PD models<sup>[62]</sup>.

AD is classified by the degeneration and death of neurons throughout the brain, in particular in the basal forebrain, amygdale, hippocampus, and cortical areas. To date, AD therapy has been relied on small molecules designed to increase acetylcholine (ACh) concentration by inhibiting acetyl cholinesterase<sup>[63]</sup>. Since these drugs are only supportive without potential protection or regeneration against progressive brain destruction, there is a need for an effective treatment and stem cell-based therapeutic approach should satisfy this requirement. Rodent AD models that received neural stem cells grafts exhibited an increased hippocampal synaptic density and improved cognitive function<sup>[64]</sup>. Intraventricular transplantation of human neural stem cells overexpressing choline acetyltransferase (ChAT) sufficiently restored learning and memory ability of kainic-induced AD rats<sup>[65]</sup>. In addition, a recent study demonstrated that human mesenchymal stem

cells can enhance autophagy in amyloid  $\beta$ -treated neurons and mice, thus promoting amyloid  $\beta$  clearance and increasing neuronal survival against amyloid  $\beta$  toxicity<sup>[66]</sup>. Transplantation of human adipose tissue-derived mesenchymal stem cells into the brains of aged mice enhance the levels of ACh, and consequently improve the cognitive and locomotor functions of the mice<sup>[67]</sup>. Interestingly, it was studied that ChAT-positive neurons could be directly generated from mesenchymal stem cells. These cells after transplantation could significantly improve learning and memory capacity of AD animal models<sup>[68]</sup>. Nevertheless, there is still an argument raising whether mesenchymal stem cells can cross lineage boundaries and transdifferentiate into neuronal cells.

HD is an autosomal dominant neurodegenerative disorder, identified by uncontrolled choreic movement, cognitive disruption, and emotional instability. Initial cell-based therapy was examined by using human fetal striatal grafts and showed some clinical positive outcome in HD patients<sup>[69]</sup>. However, the latter study found unpleasant results; grafted striatal tissues contributed to neural overgrowth and then tumour in an HD patient, who survived 5 years post-transplantation<sup>[70]</sup>. The transplantation of neural stem cells and striatal grafts into rodent HD models demonstrated that the medium spiny neurons of transplanted cells could integrate and form neuron circuitry in the host brain<sup>[71]</sup>. Transplantation of neural stem cells has been used not only to replace degenerated neurons, but also to protect striatal neurons against excitotoxic insults. Striatal injections of human neural stem cells into HD rodents showed incorporation into host tissues as well as migration to secondary sites associated with the disease pathology<sup>[72]</sup>. In addition to primary cells, human neural stem cells derived from human embryonic stem cells could provide a viable source for cell therapy in HD. Previous works showed that neurons expressing striatal markers could be induced from human embryonic stem cells and brain transplantation of these human embryonic stem cell-derived neurons leads to behavioural recovery in the diseased animals<sup>[73]</sup>.



# FUTURE PERSPECTIVE OF NEURAL STEM CELL-BASED THERAPY

Neurodegenerative diseases affect human well-being worldwide due to their devastating nature, cost, and lack of effective therapies. Although neural stem cells offer a great promise of treating these ailments, there are still several issues needed to be solved prior to the translation of neural stem cells into clinical setting. Several research groups around the world presented supportive data of the effectiveness of neural stem cells in correcting pathological conditions of neurodegenerative diseases; nevertheless, the exact mechanisms of how transplanted cells recover host brain function is not yet elucidated. Another important concern of neural stem cells after transplantation is the ability to migrate to the correct niche and the proper differentiation and maturation into desired neuronal cell types. And, importantly, if their growth cannot be appropriately controlled, neural stem cells may form tumor, which is more devastating condition than the original disease. Recent advance in cellular reprogramming can convert skin fibroblasts toward neural progenitor cells by chemical cocktail and hypoxia condition<sup>[74]</sup>. These induced neural progenitor cells express multiple neuron-specific proteins, generate action potentials, and give rise to several neuronal subtypes. Generation of induced neural progenitor cells from non-neural lineages could have important implications for several purposes, including neurodevelopmental study, neurological disease modeling, and regenerative medicine application. In the near future, we might have a better understanding of pathogenesis of neurodegenerative diseases through patient-specific induced neural stem cells. The combination gene therapy with induced neural stem cell transplantation could formulate a new paradigm of therapeutic strategy to cure mutation-caused diseases<sup>[75]</sup>. Altogether, while number of questions for neural stem cell application remain unanswered, the concerted efforts on neural stem cell research have already made a great progress toward cell replacement therapy in order to assure the best safety for patients.

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# REFERENCES

- 1 **Davie CA.** A review of Parkinson's disease. *Br Med Bull* 2008; **86**: 109-127 [PMID: 18398010 DOI: 10.1093/bmb/ldn013]
- 2 **Dauer W, Przedborski S.** Parkinson's disease: mechanisms and models. *Neuron* 2003; **39**: 889-909 [PMID: 12971891 DOI: 10.1016/S0896-6273(03)00568-3]
- 3 **Cheon SM, Chan L, Chan DK, Kim JW.** Genetics of Parkinson's disease - a clinical perspective. *J Mov Disord* 2012; **5**: 33-41 [PMID: 24868412 DOI: 10.14802/jmd.12009]
- 4 **Lesage S, Brice A.** Parkinson's disease: from monogenic forms to

- genetic susceptibility factors. *Hum Mol Genet* 2009; **18**: R48-R59 [PMID: 19297401 DOI: 10.1093/hmg/ddp012]
- 5 **Hughes AJ, Daniel SE, Lees AJ.** Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease. *Neurology* 2001; **57**: 1497-1499 [PMID: 11673599 DOI: 10.1212/WNL.57.8.1497]
- 6 **Lang AE, Lozano AM.** Parkinson's disease. First of two parts. *N Engl J Med* 1998; **339**: 1044-1053 [PMID: 9761807 DOI: 10.1056/NEJM199810083391506]
- 7 **Lang AE, Lozano AM.** Parkinson's disease. Second of two parts. *N Engl J Med* 1998; **339**: 1130-1143 [PMID: 9770561 DOI: 10.1056/NEJM199810153391607]
- 8 **Jankovic J.** Parkinson's disease: clinical features and diagnosis. *J Neurol Neurosurg Psychiatry* 2008; **79**: 368-376 [PMID: 18344392 DOI: 10.1136/jnnp.2007.131045]
- 9 **Ballard C, Gauthier S, Corbett A, Brayne C, Aarsland D, Jones E.** Alzheimer's disease. *Lancet* 2011; **377**: 1019-1031 [PMID: 21371747 DOI: 10.1016/S0140-6736(10)61349-9]
- 10 **Finch CE, Morgan TE.** Systemic inflammation, infection, ApoE alleles, and Alzheimer disease: a position paper. *Curr Alzheimer Res* 2007; **4**: 185-189 [PMID: 17430245 DOI: 10.2174/156720507780362254]
- 11 **Levy E, Carman MD, Fernandez-Madrid IJ, Power MD, Lieberburg I, van Duinen SG, Bots GT, Luyendijk W, Frangione B.** Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science* 1990; **248**: 1124-1126 [PMID: 2111584 DOI: 10.1126/science.2111584]
- 12 **Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, Yu CE, Jondro PD, Schmidt SD, Wang K.** Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* 1995; **269**: 973-977 [PMID: 7638622 DOI: 10.1126/science.7638622]
- 13 **Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, Tsuda T, Mar L, Foncin JF, Bruni AC, Montesi MP, Sorbi S, Rainero I, Pinessi L, Nee L, Chumakov I, Pollen D, Brookes A, Sanseau P, Polinsky RJ, Wasco W, Da Silva HA, Haines JL, Pericak-Vance MA, Tanzi RE, Roses AD, Fraser PE, Rommens JM, St George-Hyslop PH.** Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 1995; **375**: 754-760 [PMID: 7596406 DOI: 10.1038/375754a0]
- 14 **Selkoe DJ.** Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 2001; **81**: 741-766 [PMID: 11274343 DOI: 10.1002/1531-8249(199912)46: 6]
- 15 **Mayeux R.** Clinical practice. Early Alzheimer's disease. *N Engl J Med* 2010; **362**: 2194-2201 [PMID: 20558370 DOI: 10.1056/NEJMc0910236]
- 16 **Querfurth HW, LaFerla FM.** Alzheimer's disease. *N Engl J Med* 2010; **362**: 329-344 [PMID: 20107219 DOI: 10.1056/NEJMr0909142]
- 17 **Ross CA, Aylward EH, Wild EJ, Langbehn DR, Long JD, Warner JH, Scahill RI, Leavitt BR, Stout JC, Paulsen JS, Reilmann R, Unschuld PG, Wexler A, Margolis RL, Tabrizi SJ.** Huntington disease: natural history, biomarkers and prospects for therapeutics. *Nat Rev Neurol* 2014; **10**: 204-216 [PMID: 24614516 DOI: 10.1038/nrneurol.2014.24]
- 18 **Walker FO.** Huntington's disease. *Lancet* 2007; **369**: 218-228 [PMID: 17240289 DOI: 10.1016/S0140-6736(07)60111-1]
- 19 **Kremer B, Almqvist E, Theilmann J, Spence N, Telenius H, Goldberg YP, Hayden MR.** Sex-dependent mechanisms for expansions and contractions of the CAG repeat on affected Huntington disease chromosomes. *Am J Hum Genet* 1995; **57**: 343-350 [PMID: 7668260]
- 20 **Marder K, Zhao H, Myers RH, Cudkowicz M, Kayson E, Kiebertz K, Orme C, Paulsen J, Penney JB, Siemers E, Shoulson I.** Rate of functional decline in Huntington's disease. Huntington Study Group. *Neurology* 2000; **54**: 452-458 [PMID: 10668713 DOI: 10.1212/WNL.54.2.452]
- 21 **Stocchi F, Rascol O, Kiebertz K, Poewe W, Jankovic J, Tolosa E, Barone P, Lang AE, Olanow CW.** Initiating levodopa/carbidopa therapy with and without entacapone in early Parkinson disease: the STRIDE-PD study. *Ann Neurol* 2010; **68**: 18-27 [PMID: 20582993]



DOI: 10.1002/ana.22060]

- 22 **Olanow CW**, Kieburtz K, Stern M, Watts R, Langston JW, Guarnieri M, Hubble J. Double-blind, placebo-controlled study of entacapone in levodopa-treated patients with stable Parkinson disease. *Arch Neurol* 2004; **61**: 1563-1568 [PMID: 15477510 DOI: 10.1001/archneur.61.10.1563]
- 23 **Horstink M**, Tolosa E, Bonuccelli U, Deuschl G, Friedman A, Kanovsky P, Larsen JP, Lees A, Oertel W, Poewe W, Rascol O, Sampaio C. Review of the therapeutic management of Parkinson's disease. Report of a joint task force of the European Federation of Neurological Societies (EFNS) and the Movement Disorder Society-European Section (MDS-ES). Part II: late (complicated) Parkinson's disease. *Eur J Neurol* 2006; **13**: 1186-1202 [PMID: 17038032 DOI: 10.1111/j.1468-1331.2006.01548.x]
- 24 **Pedrosa DJ**, Timmermann L. Review: management of Parkinson's disease. *Neuropsychiatr Dis Treat* 2013; **9**: 321-340 [PMID: 23487540 DOI: 10.2147/NDT.S32302]
- 25 **Miyasaki JM**, Shannon K, Voon V, Ravina B, Kleiner-Fisman G, Anderson K, Shulman LM, Gronseth G, Weiner WJ. Practice Parameter: evaluation and treatment of depression, psychosis, and dementia in Parkinson disease (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 2006; **66**: 996-1002 [PMID: 16606910 DOI: 10.1212/01.wnl.0000215428.46057.3d]
- 26 **Hong-Qi Y**, Zhi-Kun S, Sheng-Di C. Current advances in the treatment of Alzheimer's disease: focused on considerations targeting A $\beta$  and tau. *Transl Neurodegener* 2012; **1**: 21 [PMID: 23210837 DOI: 10.1186/2047-9158-1-21]
- 27 **Huang Y**, Mucke L. Alzheimer mechanisms and therapeutic strategies. *Cell* 2012; **148**: 1204-1222 [PMID: 22424230 DOI: 10.1016/j.cell.2012.02.040]
- 28 **Eriksen JL**, Sagi SA, Smith TE, Weggen S, Das P, McLendon DC, Ozols VV, Jessing KW, Zavitz KH, Koo EH, Golde TE. NSAIDs and enantiomers of flurbiprofen target gamma-secretase and lower Abeta 42 in vivo. *J Clin Invest* 2003; **112**: 440-449 [PMID: 12897211 DOI: 10.1172/JCI18162]
- 29 **Wilcock GK**, Black SE, Hendrix SB, Zavitz KH, Swabb EA, Laughlin MA. Efficacy and safety of tarenflurbil in mild to moderate Alzheimer's disease: a randomised phase II trial. *Lancet Neurol* 2008; **7**: 483-493 [PMID: 18450517 DOI: 10.1016/S1474-4422(08)70090-5]
- 30 **Citron M**. Alzheimer's disease: strategies for disease modification. *Nat Rev Drug Discov* 2010; **9**: 387-398 [PMID: 20431570 DOI: 10.1038/nrd2896]
- 31 **Wischik CM**, Edwards PC, Lai RY, Roth M, Harrington CR. Selective inhibition of Alzheimer disease-like tau aggregation by phenothiazines. *Proc Natl Acad Sci USA* 1996; **93**: 11213-11218 [PMID: 8855335 DOI: 10.1073/pnas.93.20.11213]
- 32 **Gura T**. Hope in Alzheimer's fight emerges from unexpected places. *Nat Med* 2008; **14**: 894 [PMID: 18776868 DOI: 10.1038/nm0908-894]
- 33 **Armstrong MJ**, Miyasaki JM. Evidence-based guideline: pharmacologic treatment of chorea in Huntington disease: report of the guideline development subcommittee of the American Academy of Neurology. *Neurology* 2012; **79**: 597-603 [PMID: 22815556 DOI: 10.1212/WNL.0b013e318263c443]
- 34 **Hayden MR**, Leavitt BR, Yasothan U, Kirkpatrick P. Tetrabenazine. *Nat Rev Drug Discov* 2009; **8**: 17-18 [PMID: 19116624 DOI: 10.1038/nrd2784]
- 35 **Frank S**. Tetrabenazine as anti-chorea therapy in Huntington disease: an open-label continuation study. Huntington Study Group/TETRA-HD Investigators. *BMC Neurol* 2009; **9**: 62 [PMID: 20021666 DOI: 10.1186/1471-2377-9-62]
- 36 **Krobitsch S**, Kazantsev AG. Huntington's disease: From molecular basis to therapeutic advances. *Int J Biochem Cell Biol* 2011; **43**: 20-24 [PMID: 21056115 DOI: 10.1016/j.biocel.2010.10.014]
- 37 **Jankovic J**. Treatment of hyperkinetic movement disorders. *Lancet Neurol* 2009; **8**: 844-856 [PMID: 19679276 DOI: 10.1016/S1474-4422(09)70183-8]
- 38 **Bilney B**, Morris ME, Perry A. Effectiveness of physiotherapy, occupational therapy, and speech pathology for people with Huntington's disease: a systematic review. *Neurorehabil Neural Repair* 2003; **17**: 12-24 [PMID: 12645441 DOI: 10.1177/0888439002250448]
- 39 **Gage FH**. Mammalian neural stem cells. *Science* 2000; **287**: 1433-1438 [PMID: 10688783 DOI: 10.1126/science.287.5457.1433]
- 40 **Weiss S**, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA. Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. *J Neurosci* 1996; **16**: 7599-7609 [PMID: 8922416]
- 41 **Reynolds BA**, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992; **255**: 1707-1710 [PMID: 1553558 DOI: 10.1126/science.1553558]
- 42 **Cai L**, Hayes NL, Nowakowski RS. Synchrony of clonal cell proliferation and contiguity of clonally related cells: production of mosaicism in the ventricular zone of developing mouse neocortex. *J Neurosci* 1997; **17**: 2088-2100 [PMID: 9045736]
- 43 **Raballo R**, Rhee J, Lyn-Cook R, Leckman JF, Schwartz ML, Vaccarino FM. Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. *J Neurosci* 2000; **20**: 5012-5023 [PMID: 10864959]
- 44 **Tropepe V**, Sibilia M, Ciruna BG, Rossant J, Wagner EF, van der Kooy D. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev Biol* 1999; **208**: 166-188 [PMID: 10075850 DOI: 10.1006/dbio.1998.9192]
- 45 **Shen Q**, Qian X, Capela A, Temple S. Stem cells in the embryonic cerebral cortex: their role in histogenesis and patterning. *J Neurobiol* 1998; **36**: 162-174 [PMID: 9712302 DOI: 10.1002/(SICI)1097-4695(199808)36:2<162::AID-NEU4>3.0.CO;2-M]
- 46 **McKay R**. Stem cells in the central nervous system. *Science* 1997; **276**: 66-71 [PMID: 9082987 DOI: 10.1126/science.276.5309.66]
- 47 **Qian X**, Shen Q, Goderie SK, He W, Capela A, Davis AA, Temple S. Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* 2000; **28**: 69-80 [PMID: 11086984 DOI: 10.1016/S0896-6273(00)00086-6]
- 48 **Takahashi M**, Palmer TD, Takahashi J, Gage FH. Widespread integration and survival of adult-derived neural progenitor cells in the developing optic retina. *Mol Cell Neurosci* 1998; **12**: 340-348 [PMID: 9888988 DOI: 10.1006/mcne.1998.0721]
- 49 **Gaiano N**, Fishell G. Transplantation as a tool to study progenitors within the vertebrate nervous system. *J Neurobiol* 1998; **36**: 152-161 [PMID: 9712301 DOI: 10.1002/(SICI)1097-4695(199808)36:2<152::AID-NEU4>3.0.CO;2-O]
- 50 **Merkle FT**, Alvarez-Buylla A. Neural stem cells in mammalian development. *Curr Opin Cell Biol* 2006; **18**: 704-709 [PMID: 17046226 DOI: 10.1016/j.ceb.2006.09.008]
- 51 **Seaberg RM**, Smukler SR, van der Kooy D. Intrinsic differences distinguish transiently neurogenic progenitors from neural stem cells in the early postnatal brain. *Dev Biol* 2005; **278**: 71-85 [PMID: 15649462 DOI: 10.1016/j.ydbio.2004.10.017]
- 52 **Lendahl U**, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. *Cell* 1990; **60**: 585-595 [PMID: 1689217 DOI: 10.1016/0092-8674(90)90662-X]
- 53 **Götz M**, Hartfuss E, Malatesta P. Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. *Brain Res Bull* 2002; **57**: 777-788 [PMID: 12031274 DOI: 10.1016/S0361-9230(01)00777-8]
- 54 **Garcia AD**, Doan NB, Imura T, Bush TG, Sofroniew MV. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. *Nat Neurosci* 2004; **7**: 1233-1241 [PMID: 15494728 DOI: 10.1038/nm1340]
- 55 **Doetsch F**, Caillé I, Lim DA, García-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 1999; **97**: 703-716 [PMID: 10380923 DOI: 10.1016/S0092-8674(00)80783-7]
- 56 **Suh H**, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH. In vivo fate analysis reveals the multipotent and self-renewal

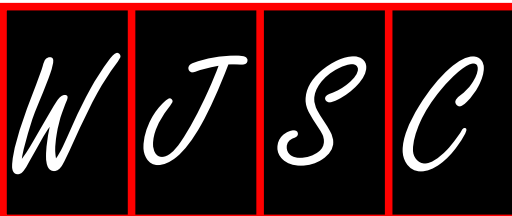


- capacities of Sox2+ neural stem cells in the adult hippocampus. *Cell Stem Cell* 2007; **1**: 515-528 [PMID: 18371391 DOI: 10.1016/j.stem.2007.09.002]
- 57 **Kordower JH**, Goetz CG, Freeman TB, Olanow CW. Dopaminergic transplants in patients with Parkinson's disease: neuroanatomical correlates of clinical recovery. *Exp Neurol* 1997; **144**: 41-46 [PMID: 9126150 DOI: 10.1006/exnr.1996.6386]
  - 58 **Hagell P**, Piccini P, Björklund A, Brundin P, Rehncrona S, Widner H, Crabb L, Pavese N, Oertel WH, Quinn N, Brooks DJ, Lindvall O. Dyskinesias following neural transplantation in Parkinson's disease. *Nat Neurosci* 2002; **5**: 627-628 [PMID: 12042822 DOI: 10.1038/nrn863]
  - 59 **Kordower JH**, Chu Y, Hauser RA, Freeman TB, Olanow CW. Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nat Med* 2008; **14**: 504-506 [PMID: 18391962 DOI: 10.1038/nm1747]
  - 60 **Doi D**, Samata B, Katsukawa M, Kikuchi T, Morizane A, Ono Y, Sekiguchi K, Nakagawa M, Parmar M, Takahashi J. Isolation of human induced pluripotent stem cell-derived dopaminergic progenitors by cell sorting for successful transplantation. *Stem Cell Reports* 2014; **2**: 337-350 [PMID: 24672756 DOI: 10.1016/j.stemcr.2014.01.013]
  - 61 **Kriks S**, Shim JW, Piao J, Ganat YM, Wakeman DR, Xie Z, Carrillo-Reid L, Auyeung G, Antonacci C, Buch A, Yang L, Beal MF, Surmeier DJ, Kordower JH, Tabar V, Studer L. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 2011; **480**: 547-551 [PMID: 22056989 DOI: 10.1038/nature10648]
  - 62 **Liu X**, Li F, Stubblefield EA, Blanchard B, Richards TL, Larson GA, He Y, Huang Q, Tan AC, Zhang D, Benke TA, Sladek JR, Zahniser NR, Li CY. Direct reprogramming of human fibroblasts into dopaminergic neuron-like cells. *Cell Res* 2012; **22**: 321-332 [PMID: 22105488 DOI: 10.1038/cr.2011.181]
  - 63 **Musial A**, Bajda M, Malawska B. Recent developments in cholinesterases inhibitors for Alzheimer's disease treatment. *Curr Med Chem* 2007; **14**: 2654-2679 [PMID: 17979717 DOI: 10.2174/092986707782023217]
  - 64 **Blurton-Jones M**, Kitazawa M, Martinez-Coria H, Castello NA, Müller FJ, Loring JF, Yamasaki TR, Poon WW, Green KN, LaFerla FM. Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proc Natl Acad Sci USA* 2009; **106**: 13594-13599 [PMID: 19633196 DOI: 10.1073/pnas.0901402106]
  - 65 **Park D**, Joo SS, Kim TK, Lee SH, Kang H, Lee HJ, Lim I, Matsuo A, Tooyama I, Kim YB, Kim SU. Human neural stem cells overexpressing choline acetyltransferase restore cognitive function of kainic acid-induced learning and memory deficit animals. *Cell Transplant* 2012; **21**: 365-371 [PMID: 21929870 DOI: 10.3727/096368911X586765]
  - 66 **Shin JY**, Park HJ, Kim HN, Oh SH, Bae JS, Ha HJ, Lee PH. Mesenchymal stem cells enhance autophagy and increase  $\beta$ -amyloid clearance in Alzheimer disease models. *Autophagy* 2014; **10**: 32-44 [PMID: 24149893 DOI: 10.4161/auto.26508]
  - 67 **Park D**, Yang G, Bae DK, Lee SH, Yang YH, Kyung J, Kim D, Choi EK, Choi KC, Kim SU, Kang SK, Ra JC, Kim YB. Human adipose tissue-derived mesenchymal stem cells improve cognitive function and physical activity in ageing mice. *J Neurosci Res* 2013; **91**: 660-670 [PMID: 23404260 DOI: 10.1002/jnr.23182]
  - 68 **Wu QY**, Li J, Feng ZT, Wang TH. Bone marrow stromal cells of transgenic mice can improve the cognitive ability of an Alzheimer's disease rat model. *Neurosci Lett* 2007; **417**: 281-285 [PMID: 17412501 DOI: 10.1016/j.neulet.2007.02.092]
  - 69 **Bachoud-Lévi AC**, Rémy P, Nguyen JP, Brugières P, Lefaucheur JP, Bourdet C, Baudic S, Gaura V, Maison P, Haddad B, Boissé MF, Grandmougin T, Jéty R, Bartolomeo P, Dalla Barba G, Degos JD, Lisovski F, Ergis AM, Pailhoux E, Cesaro P, Hantraye P, Peschanski M. Motor and cognitive improvements in patients with Huntington's disease after neural transplantation. *Lancet* 2000; **356**: 1975-1979 [PMID: 11130527 DOI: 10.1016/S0140-6736(00)03310-9]
  - 70 **Keene CD**, Chang RC, Leverenz JB, Kopyov O, Perlman S, Hevner RF, Born DE, Bird TD, Montine TJ. A patient with Huntington's disease and long-surviving fetal neural transplants that developed mass lesions. *Acta Neuropathol* 2009; **117**: 329-338 [PMID: 19057918 DOI: 10.1007/s00401-008-0465-0]
  - 71 **Nakao N**, Ogura M, Nakai K, Itakura T. Embryonic striatal grafts restore neuronal activity of the globus pallidus in a rodent model of Huntington's disease. *Neuroscience* 1999; **88**: 469-477 [PMID: 10197767 DOI: 10.1016/S0306-4522(98)00197-3]
  - 72 **McBride JL**, Behrstock SP, Chen EY, Jakel RJ, Siegel I, Svendsen CN, Kordower JH. Human neural stem cell transplants improve motor function in a rat model of Huntington's disease. *J Comp Neurol* 2004; **475**: 211-219 [PMID: 15211462 DOI: 10.1002/cne.20176]
  - 73 **Aubry L**, Bugi A, Lefort N, Rousseau F, Peschanski M, Perrier AL. Striatal progenitors derived from human ES cells mature into DARPP32 neurons in vitro and in quinolinic acid-lesioned rats. *Proc Natl Acad Sci USA* 2008; **105**: 16707-16712 [PMID: 18922775 DOI: 10.1073/pnas.0808488105]
  - 74 **Cheng L**, Hu W, Qiu B, Zhao J, Yu Y, Guan W, Wang M, Yang W, Pei G. Generation of neural progenitor cells by chemical cocktails and hypoxia. *Cell Res* 2014; **24**: 665-679 [PMID: 24638034 DOI: 10.1038/cr.2014.32]
  - 75 **Cartier N**, Haccin-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I, Vidaud M, Abel U, Dal-Cortivo L, Caccavelli L, Mahlaoui N, Kiermer V, Mittelstaedt D, Bellesme C, Lahlou N, Lefrère F, Blanche S, Audit M, Payen E, Leboulch P, l'Homme B, Bougnères P, Von Kalle C, Fischer A, Cavazzana-Calvo M, Aubourg P. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* 2009; **326**: 818-823 [PMID: 19892975 DOI: 10.1126/science.1171242]

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## Molecular mechanism of extrinsic factors affecting anti-aging of stem cells

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hypoxic environment, and intracellular factors including genome stability, mitochondria integrity, epigenetic regulators, calorie restrictions, nutrients, and vitamin D. Secreted tumor growth factor- $\beta$  and fibroblast growth factor-2 are reported to play a role in stem cell quiescence. Extracellular matrices may interact with caveolin-1, the lipid raft on cell membrane to regulate quiescence. N-cadherin, the adhesive protein on niche cells provides support for stem cells. The hypoxic micro-environment turns on hypoxia-inducible factor-1 to prevent mesenchymal stem cells aging through p16 and p21 down-regulation. Mitochondria express glucosephosphate isomerase to undergo glycolysis and prevent cellular aging. Epigenetic regulators such as p300, protein inhibitors of activated Stats and H19 help maintain stem cell quiescence. In addition, calorie restriction may lead to secretion of paracrine cyclic ADP-ribose by intestinal niche cells, which help maintain intestinal stem cells. In conclusion, it is crucial to understand the anti-aging phenomena of stem cells at the molecular level so that the key to solving the aging mystery may be unlocked.

**Key words:** Stem cells; Anti-aging; Quiescence; Micro-environment

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**Core tip:** This review approaches anti-aging from aspect of stem cells. Stem cells may interact directly with their extracellular surroundings through caveolin-1, a lipid raft protein, to carry out and maintain stem cell functions. Mechanisms through hypoxia-inducible factor-1, glycolysis, and epigenetic regulators such as p300, protein inhibitors of activated Stats and H19 play crucial role in stem cell quiescence, and anti-aging regulations. Conversely, genomic instability such as DNA double-strand-breaks modulate cellular aging through the mammalian target of rapamycin pathway, which may lead to decreased lifespan.

### Abstract

Scientific evidence suggests that stem cells possess the anti-aging ability to self-renew and maintain differentiation potentials, and quiescent state. The objective of this review is to discuss the micro-environment where stem cells reside *in vivo*, the secreted factors to which stem cells are exposed, the



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## INTRODUCTION

### **Definition of stem cells and stem cells aging**

In 1881, Weismann *et al.*<sup>[1]</sup> discovered that organ performance was influenced by the finite cell division of tissues. Followed by Hayflick *et al.*<sup>[2]</sup> in 1961, who discovered that normal human cells have finite cell replication and lifetime which represents human aging. When cells reach maximal replicative capacity, it is a phenomenon termed cellular senescence, or cellular aging. Recent evidence supports the model that stem cells *in vivo* are retained in a quiescent state, which can be reactivated into cell cycle progression in response to extracellular stimuli even after prolonged period of quiescence. Upon stimulation, stem cells divide to yield undifferentiated progeny and also differentiated cells through subsequent rounds of proliferation<sup>[3]</sup>. With self-renewal capability, stem cells pool assures a constant supply of stem cells, and also differentiated cells throughout an organism lifespan. However, loss of stem cells number or functionality with age can lead to profound consequences on tissue viability<sup>[4]</sup>. Recent data suggests that stem cells aging is partly due to heritable intrinsic event such as DNA damage, changes in their niches and micro-environment<sup>[3]</sup>. Through systemic influences, old tissues might be rejuvenated to a young state<sup>[5]</sup>. The significant sign of aging in stem cell culture is a diminishing replicative capacity, in which the maximal population doublings of mesenchymal stem cells (MSC) are reported as 30-40<sup>[6]</sup>. *In vitro*, MSC cellular aging is associated with the age of donor<sup>[7]</sup>. Conversely, *in vitro* culture of embryonic stem cells (ESC) showed no loss of proliferative potency<sup>[8]</sup>. Human MSC cultured *in vitro* have spindle-shaped fibroblastic morphology and usually cease to proliferate no more than 40 population doublings, with the cells becoming enlarged and more flattened<sup>[9]</sup>. The increase in cell size is associated with aging *in vitro*<sup>[10]</sup>. Unravelling these distinctive features of aging stem cell phenotype is critical to the success of therapeutic application of stem cells in the field of regenerative medicine with respect to tissue injury, degenerative diseases or organ declines that accompany aging<sup>[11]</sup>.

### **Anti-aging effects of stem cells**

The involvement of MSC in cell replenishment and lifespan regulations<sup>[12]</sup> is evident since aging is defined as the sum of primary restrictions in regenerative mechanisms of multicellular organisms<sup>[13]</sup>. Recently, the best strategy suggested to "cure" aging due to

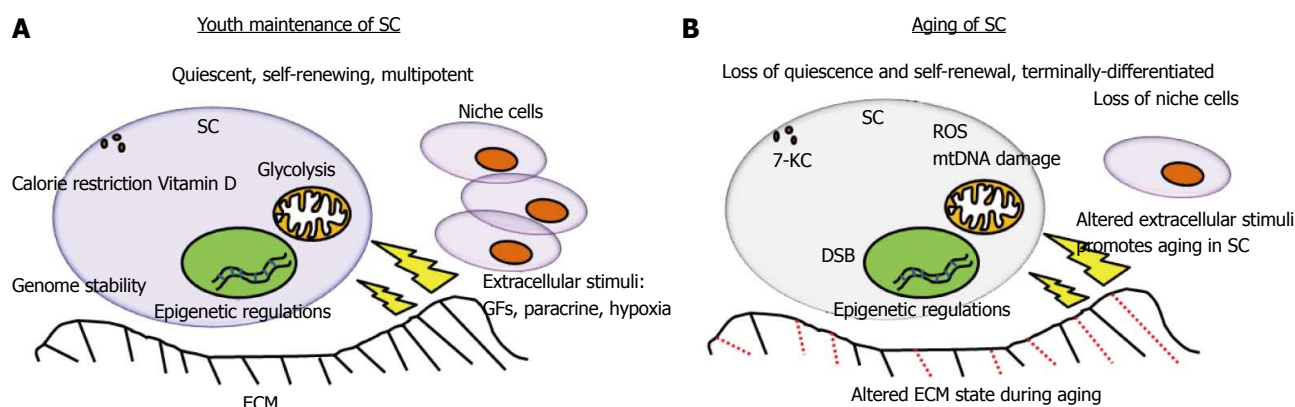
DNA damage is a rapid and effective elimination of the damaged stem cells by apoptosis<sup>[14]</sup>. This theory implies that stem cells may conceive ability to limit and repair intracellular damage. For example, stem cells possess robust cell surface transporters that exclude toxins<sup>[15]</sup>, the machinery for double-stranded DNA breaks repair<sup>[16]</sup>, and the production of telomerase to ameliorate telomere shortening<sup>[17]</sup>. Also, stem cells appear to have evolved multiple mechanisms such as senescence and apoptosis to sense damaged stem cell genome with malignant potential in order to limit replicative expansion<sup>[3,18]</sup>. These tumor-suppressor mechanisms may contribute to stem-cell irreversible growth arrest<sup>[3]</sup>. It is worthy to understand the impacts of aging on stem cells to propose valuable strategies, gather information for potential medical anti-aging interventions.

### **Definition of extrinsic factors that alter stem cells properties**

Aging can lead to changes of not only properties and number of stem cells, but also the niche cells, which gradually lead to decline in homeostasis and tissue regeneration. Aging has both quantitative and qualitative effects on stem cells. The qualitative changes affect self-renewal, developmental potential, and interactions with extrinsic signals<sup>[19]</sup>. Changes in the niche or micro-environment include amount and composition of extracellular matrix (ECM), altered expression of membrane proteins and lipids in niche cells that are in direct contact with stem cells, and changes in secretion of soluble paracrine or endocrine factors (Figure 1B). In the case of tissue injury or disease, factors released from damaged cells are altered, leading to inflammatory response<sup>[11]</sup>. Micro-environment *in vivo* is comprised of a stem cell compartment, niche cells that support stem cells, and complex ECM including fibronectin, collagen (I and IV), heparin sulphate, chondroitin sulphate, and hyaluronan<sup>[20]</sup>. The three different micro-environments: environmental enrichment, physical exercise and calorie restriction increased the number of newly regenerated neural cells in the dentate gyrus<sup>[21]</sup>. Hyaluronan preserved differentiation potentials of MSC derived from mouse<sup>[22]</sup>. Hyaluronan also maintained MSC derived from human placenta in a slow-cycling mode, which was similar to quiescent state<sup>[23]</sup>. Besides, hyaluronan is widely reviewed to regulate stem cells behavior *via* cluster determinant 44, receptor for hyaluronan-mediated motility, lymphatic vessel endothelial hyaluronan receptor, hyaluronan receptor for endocytosis, liver endothelial cell clearance receptor, or toll-like receptor 4<sup>[24]</sup>. A report implied that the activation of cells for productive tissue regeneration required a systemic micro-environment that maintains stem cells<sup>[5]</sup>.

The micro-environment where stem cells reside *in vivo* plays important role in maintaining stem cells properties, and in regulating stem cells aging. *In vivo*, the stem cells are tightly linked to their micro-





**Figure 1 Stem cell maintenance and altered state during aging.** A: Secreted growth factors, paracrine, calorie restriction, hypoxic micro-environment, ECM, and niche cells maintain stem cell functions. Quiescent, self-renewing, and multipotent status of stem cell are further maintained through intracellular hypoxia-responsive element, epigenetic and genomic regulators, and mitochondria; B: However, the status of stem cell is altered with decreased niche cells, fragmented ECM, increased genomic and mitochondria DNA damage, and increased ROS during aging. ECM: Extracellular matrix; ROS: Reactive oxygen species; SC: Stem cell.

environment such as ECM, and surrounding niche cells which promote self-renewal, maintain differentiation potential, maintain quiescence, regulate cell survival, homeostasis and also cellular lifespan (Figure 1). The adhesive protein N-cadherin on niche cells support stem cells; whereas integrins on stem cells and extrinsic regulators such as angiopoietin-1 (Ang-1), tumor growth factor- $\beta$  (TGF- $\beta$ ), bone morphogenetic protein (BMP), thrombopoietin (TPO), wntless/integrated (Wnt),  $\beta$ -catenin (CTNNB) and osteopontin (OPN) play a role in stem cells quiescence<sup>[25]</sup>. Hematopoiesis of hematopoietic stem cells (HSC) required the secreted factors, including stem cell factor, Ang-1, TPO, Wnt, NOTCH, OPN, chemokine stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ), and direct interaction with integrin<sup>[26]</sup>. Using genome-wide chromatin and transcriptional profiling of hair follicle stem cells *in vivo*, the hair follicle micro-environment triggered LIM homeobox-2 (LHX2) expression, which trans-activated genes that orchestrated cytoskeletal dynamics and adhesion<sup>[27]</sup>. The removal of LHX2 led to disastrous cellular disorganization and polarization, loss of quiescence for hair follicle stem cells, loss of normal hair anchoring, as well as progressive transformation of the niche into a sebaceous gland<sup>[27]</sup>. Since the extrinsic micro-environmental factors are required for maintaining the youth of stem cells, their interactions with stem cell membrane may also play important roles. Recently, a membrane lipid raft protein known as caveolin-1 (Cav-1), was reported to be involved in HSC quiescence and self-renewal, wherein Cav-1-deficient mice had impaired self-renewal and altered quiescent state<sup>[28]</sup>.

Furthermore, stem cells require growth factors, cytokines and mitogens for maintenance. The activation of the Wnt-Frizzled receptor with co-receptor LRP5/6 which target cytosolic CTNNB, and activation of the TGF- $\beta$  receptor kinases 1/2 that phosphorylate Smads 2/3 helped maintain differentiation potential<sup>[29]</sup>. TGF- $\beta$  is a potent inhibitor of stem cell growth and cell cycle *in vitro*, and is suggested to be a regulator of stem cell

quiescence *in vivo*<sup>[30]</sup>. Another micro-environmental factor, insulin-like growth factor-binding protein-2 (IGFBP2), used its C-terminus domain to support HSC survival and cell cycle<sup>[31]</sup>. In addition, IGFBP2 promoted hMSC osteogenesis *via* interaction with integrin  $\alpha$ -5<sup>[32]</sup>. The BMP also interacted with fibroblast growth factor-2 (FGF2) through FGF receptor, which in turn activated Smad1, Smad5 and Smad8 to inhibit differentiation, and maintain quiescence in rat neural stem cells<sup>[33]</sup>. Meanwhile, FGF2 signaling alone suppressed terminal astrocytic differentiation, and maintained rat neural stem cell potency during quiescence<sup>[33]</sup>.

## MOLECULAR MECHANISMS OF STEM CELL ANTI-AGING

### Hypoxia induced by micro-environment

Stem cells are maintained in a low oxygen environment or hypoxia in their native state. In general, 5% of pO<sub>2</sub> is the physiologically-relevant O<sub>2</sub> concentration in stem cells micro-environment. When 5% of pO<sub>2</sub> was exposed to the micro-environment, Wharton Jelly derived-MSC culture rejuvenated toward less differentiated, more primitive phenotypes<sup>[34]</sup>. It also promoted and stabilized expression of OCT4A, NANOG, SOX2, and REX1 in induced pluripotent stem cells (iPSC)<sup>[35]</sup>. Because 2% O<sub>2</sub> decreased mitochondrial DNA content<sup>[36]</sup>, this suggests that hypoxia-induced stem cell rejuvenation may be linked to energy metabolism *via* the mitochondria. Under condition of 1% O<sub>2</sub>, hypoxia-inducible factor-1 (HIF-1) activation led to decreased extracellular signal regulated kinase, followed by decreased p16 expression<sup>[37]</sup>. The decreased expression of the aging marker p16 helped MSC escaped cellular aging *in vitro*<sup>[37]</sup>. A recent study further confirmed that 1% O<sub>2</sub> down-regulated DNA damage responsive molecules, including ATM/ATR, Chk1, and Chk2, and also cellular aging markers, including senescence-associated- $\beta$ -



galactosidase, H3K9me3, heterochromatin protein 1- $\gamma$  (HP1 $\gamma$ ), p53, p21, and p16<sup>[38]</sup>. Since cellular aging is often accompanied with telomere length loss, it was observed that bone marrow MSC expanded under hypoxia (3% pO<sub>2</sub>) for 15 d demonstrated telomere length maintenance; however, telomere length decreased over time under normoxia<sup>[39]</sup>. Recently reported a major pathway that inhibits MSC senescence due to hypoxia is the HIF-1 $\alpha$ -TWIST pathway which down-regulated E2A-p21<sup>[40]</sup>. In addition, hypoxia induced an immediate and concerted down-regulation of genes involved in DNA repair and damage response pathways (including *MLH1*, *RAD51*, *BRCA1*, and *Ku80* genes)<sup>[36]</sup>, which suggests that hypoxia plays a role in genome stability. Furthermore, p53 may suppressed HIF-1 translation through targeting pro-myelocytic leukemia (PML) protein, which activated the mammalian target of rapamycin (mTOR) pathway to induce aging (Figure 2)<sup>[41,42]</sup>. Conversely, HIF-1 interacted with the aging marker p53 that led to p53 stabilization<sup>[43]</sup>, resulting to a pro-aging phenomena. Taken together, hypoxia provides stem cells with an anti-aging micro-environment and is required for healthy aging progression.

### **Genome stability linked to stress induced by micro-environment**

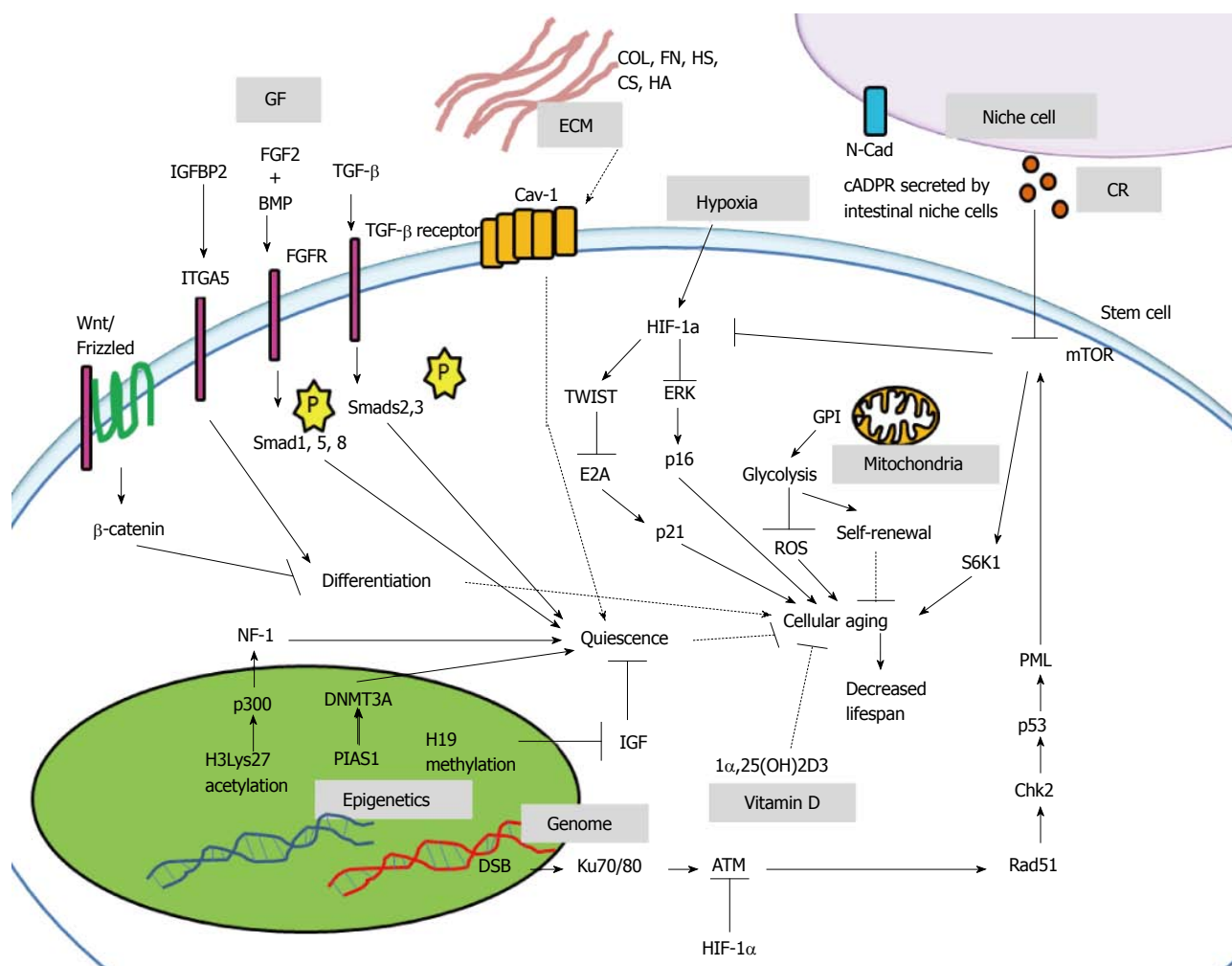
The genome is like an internal turbine engine inside a cell that works perpetually throughout the life of cell. Without it, cell survival is impossible, and youth of cells cannot be maintained. In the 1950s, researchers Mortimer and Johnston stated that aging in yeast is accompanied by genome instability<sup>[44]</sup>. In principle, there are three different modes of genome instability: mutations, mismatch repair deficiency and chromosomal instability<sup>[45]</sup>. Mechanisms of genome instability linked to mammalian aging are stress-induced reactive oxygen species (ROS), telomere loss, and germ-line genetic variations for DNA repair<sup>[46,47]</sup>. In addition, DNA damage caused by ROS was stated to elicit transient growth arrest, apoptosis and cellular senescence<sup>[48]</sup>. In adult hematopoietic stem and progenitor cells (HSPCs), the histone deacetylase Sirt1 is linked to longevity<sup>[49]</sup>, DNA damage accumulation, and loss of progenitor cells. Under stress of Sirt1 ablation, HSPCs increased *Hoxa-9* expression, leading to increased DNA damage<sup>[50]</sup>. Another sirtuin family protein, SIRT6, also regulates aging and genome stability. During DNA double-strand break repair, SIRT6 promoted DNA end resection in order to maintain genome integrity<sup>[51]</sup>. Interestingly, a family member of p53, TAp63, prevented premature aging in skin cells as observed in TAp63<sup>-/-</sup> mice by maintaining properties of epidermal and dermal precursors<sup>[52]</sup>. The most prominent evidence that links genome stability with aging is the human disease known as progeroid syndrome. Defects in the DNA repair system known

as transcription-coupled excision repair (TCER) leads to progeroid syndrome<sup>[53]</sup>, wherein lesions in transcribed strand of active genes are repaired with defective TCER<sup>[54]</sup>. In contrast to what is known about quiescence, the stem cells are prone to DNA damage accumulation while in quiescence. A previous study stated that following DNA damage, stem cells utilized non-homologous end-joining (NHEJ) repair system during quiescence; however, the NHEJ response is normally associated with genome rearrangement and abnormalities in HSC<sup>[55]</sup>. In brief, genome stability is crucial for stem cells maintenance.

### **Mitochondrial property changes upon stem cells aging**

Mitochondria play a significant role in the aging-induced signaling pathway since aging is generally characterized as a progressive decline of tissue and organ function accompanied by increased oxidative damage and mitochondrial dysfunction. Stem cells in the quiescent state appear to be less metabolically active and may be subjected to lower levels of cellular metabolism byproducts, mainly ROS. Since mitochondria are the sites for oxidative metabolism, ROS may inflict damage to its DNA and the organelle itself. Previously, functional analysis demonstrated altered mitochondrial morphology, decreased antioxidant capacities and elevated ROS levels in long-term cultivated young MSC as well as aged MSC<sup>[56]</sup>. Another previous study stated that mice expressing a mutant form of mitochondrial DNA polymerase- $\gamma$  led to HSC with compromised functions, indicating that mitochondrial integrity is crucial for stem cell maintenance<sup>[57]</sup>. In addition, long-lived post-mitotic cells with acquired stem cell damage may be inherited by progeny cells since the mitochondrial DNA damage is passed down the generations. Recent evidence indicates that loss of a negative regulator of the *Ink4a/Arf* locus, also known as *Cdkn2a* or *BMI1*, is associated with decreased mitochondrial function, decreased electron transport flux, increased ROS concentrations, and loss of stem cell functions<sup>[58]</sup>. Furthermore, hypoxic stimulation of glycolytic flux as well as inhibition of the p53 pathway<sup>[59]</sup> partly stimulated glycolysis to improve stem cell pluripotency<sup>[60]</sup> and also resulted in a younger cell phenotype. Interestingly, direct pharmacological modulation of energy metabolism or supplementation with glycolytic intermediates augmented reprogramming efficiency through glycolysis<sup>[61]</sup>. Over-expression of the glycolytic enzyme, glucosephosphate isomerase (GPI), showed enhanced glycolysis and bypassed senescence with an increased resistance to oxidative DNA damage. In addition, depletion of GPI shortened cellular lifespan. Coincidentally, mouse embryonic stem cells, which are stem cells highly resistant to oxidative stress demonstrated a high level of glycolysis<sup>[62]</sup>. Overall, these findings suggest that stem cells can utilize mitochondria to escape restrictions of cellular lifespan *via* an anti-aging mechanism (Figure 2).





**Figure 2 Signals and regulators of anti-aging in stem cell.** Up-regulation of phosphorylated Smad1, Smad5, Smad8 and Smad2, Smad3 proteins maintains stem cell quiescence. Epigenetic regulators p300, DNMT3A, and H19 also maintain stem cell quiescence. Hypoxia induces HIF-1 $\alpha$ , a key regulator of p21 and p16 to prevent cellular aging. Under hypoxic condition, mitochondria undergo glycolysis to maintain self-renewal, and lower ROS production. With genome integrity maintained, the mTOR pathway is deactivated. In addition, stem cells interact with ECM, secreted factors from niche cells, and adhesive proteins to further maintain stem cell functions. GF: Growth factors; ECM: Extracellular matrix; CR: Calorie restriction; Wnt: Wingless/integrin; IGFBP2: Insulin-growth factor binding protein 2; ITGA5: Integrin alpha-5; FGF2: Fibroblast growth factor 2; FGFR: Fibroblast growth factor receptor; BMP: Bone morphogenetic protein; Smad: Mothers against decapentaplegic; TGF- $\beta$ : Transforming growth factor- $\beta$ ; Cav-1: Caveolin-1; Col: Collagen; FN: Fibronectin; HS: Heparin sulphate; CS: Chondroitin sulphate; HA: Hyaluronan; HIF-1: Hypoxia-inducible factor-1; E2A: Transcriptional factor E2; p21: Peroxidase 21; ERK: Extracellular signal-regulated kinase; p16: Peroxidase 16; GPI: Glucosephosphate isomerase; ROS: Reactive oxygen species; N-Cad: N-cadherin; cADPR: Cyclic ADP-ribose; mTOR: Mammalian target of rapamycin; S6K1: Ribosomal S6 kinase 1; DSB: Double-strand break; ATM: Ataxia telangiectasia-mutated protein kinase; Chk2: Checkpoint kinase 2; PML: Promyelocytic leukemia protein; NF-1: Neurofibromin-1; PIAS1: Protein inhibitors of activated Stats; DNMT3A: DNA(cytosine-5)-methyltransferase 3A; IGF: Insulin-like growth factor; 1 $\alpha$ , 25(OH)2D3: 1 $\alpha$ , 25-dihydroxyvitamin D3.

### Epigenetics regulations

The process of aging is accompanied by nuclear architectural instability in the cell<sup>[63,64]</sup>; however, there is a lack of evidence to show that DNA damage reduction can extend lifespan of cells *in vitro*. As a result, scientists have suggested that aging may also be caused by epigenetic regulations<sup>[65]</sup>. Recently, protein inhibitors of activated Stats (PIAS1), a SUMO E3 ligase was shown to regulate HSCs self-renewal via GATA1 suppression by binding to GATA1 promoter region to recruit DNMT3A<sup>[66]</sup>. Using genome-wide mapping at an enhancer associated histone mark H3Lys27 acetylation and p300 binding at promoter site, the nuclear factor-1 (NF1) protein family seemed to be required for neural stem cell (NSC) entry into quiescence<sup>[67]</sup>. In addition, integrin alpha-6 was down-

regulated when the NSC entered quiescence<sup>[67]</sup>. Interestingly, the imprinting alleles H19-Igf2 helped maintain HSC quiescence, wherein the maternal H19 region is differentially methylated (H19-DMR). Deletion of the H19-DMR led to Igf-Igfr1 activation and loss of quiescence<sup>[68]</sup>. Previous findings suggested that Mym1 modulated histone modification of the Gfi1 promoter region in order to recruit Gata2 and Runx1 while maintaining HSC quiescence<sup>[69]</sup>. Moreover, the rejuvenation of muscle cells, skin cells and bone marrow cells of mice were observed through systemic connection of one old mouse to another young mouse and were suggested to be modulated by epigenetics<sup>[65]</sup>.

### Calorie intake

Apart from these, caloric restriction due to limited



calorie intake is also responsible for extrinsic signaling in stem cell maintenance and aging. In general, mechanisms involved during the stem cell quiescent state are different from those during active proliferative state. Calorie restriction led to up-regulation of cyclic ADP-ribose signaling in the intestinal niche cells known as Paneth cells, and induced proliferation of leucine rich repeat-containing G protein-coupled receptor 5-positive intestinal stem cells by inhibiting mTOR signaling<sup>[70]</sup>. From the perspective of energy metabolism for various calorie consumptions, stem cells usually undergo glycolysis rather than oxidative phosphorylation. Under normoxia, MSCs switched to oxidative phosphorylation that led to three- to fourfold increase in senescence; however, hypoxia induced glycolysis in order to prevent oxidative stress-induced senescence and preserve MSC long-term self-renewal<sup>[71]</sup>. Surprisingly, study stated that over-expression of phosphoinositide-dependent kinase may restore glycolytic metabolism in glycolytic-defective HSC<sup>[72]</sup>. In general, native stem cell metabolisms are linked to increased glycolysis, limited oxidative metabolism, and resistance to oxidative damage<sup>[73]</sup>.

### Nutrients

Nutrient-sensitive signaling pathways that are known to regulate organismal aging include the insulin-PI3K, Akt-FOXO, mTOR and AMPK pathways, which regulate the balance between quiescence and proliferation of stem cells during aging<sup>[74-78]</sup>. Decreased activity of the mTOR and its target S6K1 homologs increased life span in yeast, nematodes, and fruit flies<sup>[79]</sup>. The S6K1 is a ribosomal S6 protein kinase targeted by mTOR, and is a determinant of mammalian aging in the nutrient-sensitive signaling pathways<sup>[80]</sup>. For the AMPK pathway, deletion of the AMPK regulator LKB1 (serine/threonine protein kinase 11) may lead to loss of mouse LT-HSC quiescence<sup>[81]</sup>. In human ESCs and iPSCs, methionine deprivation resulted in rapid decrease of intracellular S-adenosylmethionine, which triggered stem cell differentiation, p53 and p38 activation, and reduced NANOG expression<sup>[82]</sup>. With regard to lipid metabolism, various lipids and fatty acids regulated proliferation and differentiation of pluripotent stem cells and adult progenitors<sup>[83]</sup>, and albumin-associated lipids promoted self-renewal of hESCs<sup>[84]</sup>. Biosynthesis of lipid such as cholesterol may influence progenitor cell differentiation, and inhibitors of cholesterol biosynthesis enhanced differentiation of mouse C2C12 myoblasts<sup>[85]</sup>. Cholesterol is easily oxidized and may be converted to various oxidation products known as oxysterols. One of the major oxysterols is 7-ketocholesterol (7-KC), wherein increased 7-KC led to gradual changes in morphology of human adipocyte MSC, beginning with the loss of unidirectional alignment at lower concentrations, and leading towards loss of actin organization and loss of intracellular contact at higher concentrations<sup>[86]</sup>.

### Vitamin D

Vitamins are important part of our diets and play some role in anti-aging of stem cells. It was reported that  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> ( $1\alpha, 25(\text{OH})_2\text{D}_3$ ) delayed replicative senescence in primary hMSC; however, its pro-aging effects due to elevated systemic phosphate levels were seen in mouse models<sup>[87]</sup>. Other findings suggested that vitamin D metabolism played a role in human osteoblastogenesis in hMSCs, in which both  $25(\text{OH})\text{D}_3$  and  $1\alpha,25(\text{OH})_2\text{D}_3$  mediated osteoblast differentiation in hMSCs through up-regulation of insulin-like growth factor-1<sup>[88]</sup>.

## CONCLUSION

The direct connection between stem cell aging and organismal aging, and lifespan is yet to be defined. The onset of aging process is often believed to influence cellular lifespan based on lowered *in vitro* replicative capability of old MSC from older compared to MSC from younger individual. Whether the influence on replicative capability is caused by systemic organismal aging, or the altered state of MSC during aging process, remains a question. Stem cells are empowered with self-renewal and differentiation potentials, and stay quiescent for long period of time while preserving their functions. The dormant state, or quiescence, seems to preserve the youth of stem cells despite the passing of time. Nevertheless, evidence suggests that stem cell population and functions may decrease in old individuals. It is likely that the youthful phenotypes of stem cells are preserved as long as it takes in order to allow healthy aging process. In times of trauma, injury, environmental stress such as ultra-violet over-exposure, and psychological stress, the process of aging may be affected, and lifespan may be shortened. Through understanding the prospect of stem cell anti-aging phenomena, the therapeutic application of stem cells can be broadened, and perhaps, a direct link between stem cell anti-aging and longevity may be established.

## REFERENCES

- 1 Weismann A. Poulton EB, editor. Collected Essays upon Heredity and Kindred Biological Problems. Oxford, UK: Clarendon Press, 1889
- 2 Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961; **25**: 585-621 [PMID: 13905658]
- 3 Sharpless NE, DePinho RA. How stem cells age and why this makes us grow old. *Nat Rev Mol Cell Biol* 2007; **8**: 703-713 [PMID: 17717515 DOI: 10.1038/nrm2241]
- 4 Pelicci PG. Do tumor-suppressive mechanisms contribute to organism aging by inducing stem cell senescence? *J Clin Invest* 2004; **113**: 4-7 [PMID: 14702099 DOI: 10.1172/jci20750]
- 5 Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* 2005; **433**: 760-764 [PMID: 15716955]
- 6 Baxter MA, Wynn RF, Jowitt SN, Wraith JE, Fairbairn LJ,



- Bellantuono I. Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. *Stem Cells* 2004; **22**: 675-682 [PMID: 15342932 DOI: 10.1634/stemcells.22-5-675]
- 7 **Rubin H.** Promise and problems in relating cellular senescence in vitro to aging in vivo. *Arch Gerontol Geriatr* 2002; **34**: 275-286 [PMID: 14764330 DOI: 10.1016/S0167-4943(01)00221-7]
  - 8 **Rosenberger RF.** The initiation of senescence and its relationship to embryonic cell differentiation. *Bioessays* 1995; **17**: 257-260 [PMID: 7748179 DOI: 10.1002/bies.950170312]
  - 9 **Bruder SP, Jaiswal N, Haynesworth SE.** Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* 1997; **64**: 278-294 [PMID: 9027588 DOI: 10.1002/(sici)1097-4644(199702)64:2<278::aid-jcb11>3.0.co;2-f]
  - 10 **Hayflick L.** The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res* 1965; **37**: 614-636 [PMID: 14315085 DOI: 10.1016/0014-4827(65)90211-9]
  - 11 **Rando TA.** Stem cells, ageing and the quest for immortality. *Nature* 2006; **441**: 1080-1086 [PMID: 16810243 DOI: 10.1038/nature04958]
  - 12 **Sethe S, Scutt A, Stolzing A.** Aging of mesenchymal stem cells. *Ageing Res Rev* 2006; **5**: 91-116 [PMID: 16310414 DOI: 10.1016/j.arr.2005.10.001]
  - 13 **James K, Sebastian S, Alexandra S, editors.** Extending the Lifespan: Biotechnical, Gerontological, and Social Problems. Hamburg: LIT Verlag Münster, 2005
  - 14 **Cairns J.** Somatic stem cells and the kinetics of mutagenesis and carcinogenesis. *Proc Natl Acad Sci USA* 2002; **99**: 10567-10570 [PMID: 12149477 DOI: 10.1073/pnas.162369899]
  - 15 **Gottesman MM, Fojo T, Bates SE.** Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002; **2**: 48-58 [PMID: 11902585 DOI: 10.1038/nrc706]
  - 16 **Park IK, He Y, Lin F, Laerum OD, Tian Q, Bumgarner R, Klug CA, Li K, Kuhr C, Doyle MJ, Xie T, Schummer M, Sun Y, Goldsmith A, Clarke MF, Weissman IL, Hood L, Li L.** Differential gene expression profiling of adult murine hematopoietic stem cells. *Blood* 2002; **99**: 488-498 [PMID: 11781229 DOI: 10.1182/blood.V99.2.488]
  - 17 **Morrison SJ, Prowse KR, Ho P, Weissman IL.** Telomerase activity in hematopoietic cells is associated with self-renewal potential. *Immunity* 1996; **5**: 207-216 [PMID: 8808676 DOI: 10.1016/S1074-7613(00)80316-7]
  - 18 **Sharpless NE, DePinho RA.** Telomeres, stem cells, senescence, and cancer. *J Clin Invest* 2004; **113**: 160-168 [PMID: 14722605 DOI: 10.1172/jci20761]
  - 19 **Roobrouck VD, Ulloa-Montoya F, Verfaillie CM.** Self-renewal and differentiation capacity of young and aged stem cells. *Exp Cell Res* 2008; **314**: 1937-1944 [PMID: 18439579 DOI: 10.1016/j.yexcr.2008.03.006]
  - 20 **Dorshkind K.** Regulation of hemopoiesis by bone marrow stromal cells and their products. *Annu Rev Immunol* 1990; **8**: 111-137 [PMID: 2188660 DOI: 10.1146/annurev.iy.08.040190.000551]
  - 21 **Rao MS, Mattson MP.** Stem cells and aging: expanding the possibilities. *Mech Ageing Dev* 2001; **122**: 713-734 [PMID: 11322994 DOI: 10.1016/S0047-6374(01)00224-X]
  - 22 **Chen PY, Huang LL, Hsieh HJ.** Hyaluronan preserves the proliferation and differentiation potentials of long-term cultured murine adipose-derived stromal cells. *Biochem Biophys Res Commun* 2007; **360**: 1-6 [PMID: 17586465 DOI: 10.1016/j.bbrc.2007.04.211]
  - 23 **Liu CM, Yu CH, Chang CH, Hsu CC, Huang LL.** Hyaluronan substratum holds mesenchymal stem cells in slow-cycling mode by prolonging G1 phase. *Cell Tissue Res* 2008; **334**: 435-443 [PMID: 18953571 DOI: 10.1007/s00441-008-0699-0]
  - 24 **Solis MA, Chen YH, Wong TY, Bittencourt VZ, Lin YC, Huang LL.** Hyaluronan regulates cell behavior: a potential niche matrix for stem cells. *Biochem Res Int* 2012; **2012**: 346972 [PMID: 22400115 DOI: 10.1155/2012/346972]
  - 25 **Li L, Bhatia R.** Molecular Pathways: Stem Cell Quiescence. *Clin Cancer Res* 2011; **17**: 4936-4941 [PMID: 21593194 DOI: 10.1158/1078-0432.CCR-10-1499]
  - 26 **Shiozawa Y, Havens AM, Pienta KJ, Taichman RS.** The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites. *Leukemia* 2008; **22**: 941-950 [PMID: 18305549 DOI: 10.1038/leu.2008.48]
  - 27 **Folgueras AR, Guo X, Pasolli HA, Stokes N, Polak L, Zheng D, Fuchs E.** Architectural niche organization by LHX2 is linked to hair follicle stem cell function. *Cell Stem Cell* 2013; **13**: 314-327 [PMID: 24012369 DOI: 10.1016/j.stem.2013.06.018]
  - 28 **Bai L, Shi G, Zhang L, Guan F, Ma Y, Li Q, Cong Y-S, Zhang L.** Cav-1 deletion impaired hematopoietic stem cell function. *Cell Death Dis* 2014; **5**: e1140 [PMID: 24675458 DOI: 10.1038/cddis.2014.105]
  - 29 **Pekovic V, Hutchison CJ.** Adult stem cell maintenance and tissue regeneration in the ageing context: the role for A-type lamins as intrinsic modulators of ageing in adult stem cells and their niches. *J Anat* 2008; **213**: 5-25 [PMID: 18638067 DOI: 10.1111/j.1469-7580.2008.00928.x]
  - 30 **Blank U, Karlsson G, Karlsson S.** Signaling pathways governing stem-cell fate. *Blood* 2008; **111**: 492-503 [PMID: 17914027 DOI: 10.1182/blood-2007-07-075168]
  - 31 **Huynh H, Zheng J, Umikawa M, Zhang C, Silvany R, Iizuka S, Holzenberger M, Zhang W, Zhang CC.** IGF binding protein 2 supports the survival and cycling of hematopoietic stem cells. *Blood* 2011; **118**: 3236-3243 [PMID: 21821709 DOI: 10.1182/blood-2011-01-331876]
  - 32 **Hamidouche Z, Fromiguet O, Ringe J, Häupl T, Marie PJ.** Crosstalks between integrin alpha 5 and IGF2/IGFBP2 signalling trigger human bone marrow-derived mesenchymal stromal osteogenic differentiation. *BMC Cell Biol* 2010; **11**: 44 [PMID: 20573191 DOI: 10.1186/1471-2121-11-44]
  - 33 **Sun Y, Hu J, Zhou L, Pollard SM, Smith A.** Interplay between FGF2 and BMP controls the self-renewal, dormancy and differentiation of rat neural stem cells. *J Cell Sci* 2011; **124**: 1867-1877 [PMID: 21558414 DOI: 10.1242/jcs.085506]
  - 34 **Drela K, Sarnowska A, Siedlecka P, Szablowska-Gadomska I, Wielgos M, Jurga M, Lukomska B, Domanska-Janik K.** Low oxygen atmosphere facilitates proliferation and maintains undifferentiated state of umbilical cord mesenchymal stem cells in an hypoxia inducible factor-dependent manner. *Cytotherapy* 2014; **16**: 881-892 [PMID: 24726658 DOI: 10.1016/j.jcyt.2014.02.009]
  - 35 **Yamanaka S.** Induced pluripotent stem cells: past, present, and future. *Cell Stem Cell* 2012; **10**: 678-684 [PMID: 22704507 DOI: 10.1016/j.stem.2012.05.005]
  - 36 **Oliveira PH, Boura JS, Abecasis MM, Gimble JM, da Silva CL, Cabral JM.** Impact of hypoxia and long-term cultivation on the genomic stability and mitochondrial performance of ex vivo expanded human stem/stromal cells. *Stem Cell Res* 2012; **9**: 225-236 [PMID: 22903042 DOI: 10.1016/j.scr.2012.07.001]
  - 37 **Jin Y, Kato T, Furu M, Nasu A, Kajita Y, Mitsui H, Ueda M, Aoyama T, Nakayama T, Nakamura T, Toguchida J.** Mesenchymal stem cells cultured under hypoxia escape from senescence via down-regulation of p16 and extracellular signal regulated kinase. *Biochem Biophys Res Commun* 2010; **391**: 1471-1476 [PMID: 20034468 DOI: 10.1016/j.bbrc.2009.12.096]
  - 38 **Kilic Eren M, Tabor V.** The Role of Hypoxia Inducible Factor-1 Alpha in Bypassing Oncogene-Induced Senescence. *PLoS One* 2014; **9**: e101064 [DOI: 10.1371/journal.pone.0101064]
  - 39 **D'Ippolito G, Diabira S, Howard GA, Roos BA, Schiller PC.** Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. *Bone* 2006; **39**: 513-522 [PMID: 16616713 DOI: 10.1016/j.bone.2006.02.061]
  - 40 **Tsai CC, Chen YJ, Yew TL, Chen LL, Wang JY, Chiu CH, Hung SC.** Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through down-regulation of E2A-p21 by HIF-TWIST. *Blood* 2011; **117**: 459-469 [PMID: 20952688 DOI: 10.1182/blood-2010-05-287508]
  - 41 **Ferbeyre G, de Stanchina E, Querido E, Baptiste N, Prives C, Lowe**



- SW. PML is induced by oncogenic ras and promotes premature senescence. *Genes Dev* 2000; **14**: 2015-2027 [PMID: 10950866 DOI: 10.1101/gad.14.16.2015]
- 42 **Bernardi R**, Guernah I, Jin D, Grisendi S, Alimonti A, Teruya-Feldstein J, Cordon-Cardo C, Simon MC, Rafii S, Pandolfi PP. PML inhibits HIF-1 $\alpha$  translation and neoangiogenesis through repression of mTOR. *Nature* 2006; **442**: 779-785 [PMID: 16915281 DOI: 10.1038/nature05029]
  - 43 **An WG**, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1 $\alpha$ . *Nature* 1998; **392**: 405-408 [PMID: 9537326 DOI: 10.1038/32925]
  - 44 **Mortimer RK**, Johnston JR. Life span of individual yeast cells. *Nature* 1959; **183**: 1751-1752 [PMID: 13666896 DOI: 10.1038/1831751a0]
  - 45 **Steinemann D**, Göhring G, Schlegelberger B. Genetic instability of modified stem cells - a first step towards malignant transformation? *Am J Stem Cells* 2013; **2**: 39-51 [PMID: 23671815]
  - 46 **Kenyon C**. The plasticity of aging: insights from long-lived mutants. *Cell* 2005; **120**: 449-460 [PMID: 15734678 DOI: 10.1016/j.cell.2005.02.002]
  - 47 **Lombard DB**. Sirtuins at the breaking point: SIRT6 in DNA repair. *Aging* (Albany NY) 2009; **1**: 12-16 [PMID: 20157593]
  - 48 **Hasty P**, Campisi J, Hoeijmakers J, van Steeg H, Vijg J. Aging and genome maintenance: lessons from the mouse? *Science* 2003; **299**: 1355-1359 [PMID: 12610296 DOI: 10.1126/science.1079161]
  - 49 **Haigis MC**, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol* 2010; **5**: 253-295 [PMID: 20078221 DOI: 10.1146/annurev.pathol.4.110807.092250]
  - 50 **Singh SK**, Williams CA, Klarmann K, Burkett SS, Keller JR, Oberdoerffer P. Sirt1 ablation promotes stress-induced loss of epigenetic and genomic hematopoietic stem and progenitor cell maintenance. *J Exp Med* 2013; **210**: 987-1001 [PMID: 23630229 DOI: 10.1084/jem.20121608]
  - 51 **Kaidi A**, Weinert BT, Choudhary C, Jackson SP. Human SIRT6 promotes DNA end resection through CtIP deacetylation. *Science* 2010; **329**: 1348-1353 [PMID: 20829486 DOI: 10.1126/science.1192049]
  - 52 **Su X**, Paris M, Gi YJ, Tsai KY, Cho MS, Lin YL, Biernaskie JA, Sinha S, Prives C, Pevny LH, Miller FD, Flores ER. TAp63 prevents premature aging by promoting adult stem cell maintenance. *Cell Stem Cell* 2009; **5**: 64-75 [PMID: 19570515 DOI: 10.1016/j.stem.2009.04.003]
  - 53 **Lehmann AR**. DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. *Biochimie* 2003; **85**: 1101-1111 [PMID: 14726016 DOI: 10.1016/j.biochi.2003.09.010]
  - 54 **Schumacher B**, Garinis GA, Hoeijmakers JH. Age to survive: DNA damage and aging. *Trends Genet* 2008; **24**: 77-85 [PMID: 18192065 DOI: 10.1016/j.tig.2007.11.004]
  - 55 **Mohrin M**, Bourke E, Alexander D, Warr MR, Barry-Holson K, Le Beau MM, Morrison CG, Passegué E. Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. *Cell Stem Cell* 2010; **7**: 174-185 [PMID: 20619762 DOI: 10.1016/j.stem.2010.06.014]
  - 56 **Geißler S**, Textor M, Kühnisch J, Könning D, Klein O, Ode A, Pfützner T, Adjaye J, Kasper G, Duda GN. Functional comparison of chronological and in vitro aging: differential role of the cytoskeleton and mitochondria in mesenchymal stromal cells. *PLoS One* 2012; **7**: e52700 [PMID: 23285157 DOI: 10.1371/journal.pone.0052700]
  - 57 **Chen ML**, Logan TD, Hochberg ML, Shelat SG, Yu X, Wilding GE, Tan W, Kujoth GC, Prolla TA, Selak MA, Kundu M, Carroll M, Thompson JE. Erythroid dysplasia, megaloblastic anemia, and impaired lymphopoiesis arising from mitochondrial dysfunction. *Blood* 2009; **114**: 4045-4053 [PMID: 19734452 DOI: 10.1182/blood-2008-08-169474]
  - 58 **Liu J**, Cao L, Chen J, Song S, Lee IH, Quijano C, Liu H, Keyvanfar K, Chen H, Cao LY, Ahn BH, Kumar NG, Rovira II, Xu XL, van Lohuizen M, Motoyama N, Deng CX, Finkel T. Bmi1 regulates mitochondrial function and the DNA damage response pathway. *Nature* 2009; **459**: 387-392 [PMID: 19404261 DOI: 10.1038/nature08040]
  - 59 **Hong H**, Takahashi K, Ichisaka T, Aoi T, Kanagawa O, Nakagawa M, Okita K, Yamanaka S. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* 2009; **460**: 1132-1135 [PMID: 19668191]
  - 60 **Mohyeldin A**, Garzón-Muvdi T, Quiñones-Hinojosa A. Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell* 2010; **7**: 150-161 [PMID: 20682444 DOI: 10.1016/j.stem.2010.07.007]
  - 61 **Folmes CD**, Nelson TJ, Martinez-Fernandez A, Arrell DK, Lindor JZ, Dzeja PP, Ikeda Y, Perez-Terzic C, Terzic A. Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. *Cell Metab* 2011; **14**: 264-271 [PMID: 21803296 DOI: 10.1016/j.cmet.2011.06.011]
  - 62 **Kondoh H**, Lleonart ME, Gil J, Wang J, Degan P, Peters G, Martinez D, Carnero A, Beach D. Glycolytic enzymes can modulate cellular life span. *Cancer Res* 2005; **65**: 177-185 [PMID: 15665293]
  - 63 **Oberdoerffer P**, Sinclair DA. The role of nuclear architecture in genomic instability and ageing. *Nat Rev Mol Cell Biol* 2007; **8**: 692-702 [PMID: 17700626 DOI: 10.1038/nrm2238]
  - 64 **Campisi J**, Vijg J. Does damage to DNA and other macromolecules play a role in aging? If so, how? *J Gerontol A Biol Sci Med Sci* 2009; **64**: 175-178 [PMID: 19228786 DOI: 10.1093/gerona/gln065]
  - 65 **Rando TA**, Chang HY. Aging, rejuvenation, and epigenetic reprogramming: resetting the aging clock. *Cell* 2012; **148**: 46-57 [PMID: 22265401 DOI: 10.1016/j.cell.2012.01.003]
  - 66 **Liu B**, Yee KM, Tahk S, Mackie R, Hsu C, Shuai K. PIAS1 SUMO ligase regulates the self-renewal and differentiation of hematopoietic stem cells. *EMBO J* 2014; **33**: 101-113 [PMID: 24357619 DOI: 10.1002/embj.201283326]
  - 67 **Martynoga B**, Mateo JL, Zhou B, Andersen J, Achimastou A, Urbán N, van den Berg D, Georgopoulou D, Hadjir S, Wittbrodt J, Ettwiller L, Piper M, Gronostajski RM, Guillemot F. Epigenomic enhancer annotation reveals a key role for NFIX in neural stem cell quiescence. *Genes Dev* 2013; **27**: 1769-1786 [PMID: 23964093 DOI: 10.1101/gad.216804.113]
  - 68 **Venkatraman A**, He XC, Thorvaldsen JL, Sugimura R, Perry JM, Tao F, Zhao M, Christenson MK, Sanchez R, Yu JY, Peng L, Haug JS, Paulson A, Li H, Zhong XB, Clemens TL, Bartolomei MS, Li L. Maternal imprinting at the H19-Igf2 locus maintains adult haematopoietic stem cell quiescence. *Nature* 2013; **500**: 345-349 [PMID: 23863936 DOI: 10.1038/nature12303]
  - 69 **Wang T**, Nandakumar V, Jiang XX, Jones L, Yang AG, Huang XF, Chen SY. The control of hematopoietic stem cell maintenance, self-renewal, and differentiation by Mysl-mediated epigenetic regulation. *Blood* 2013; **122**: 2812-2822 [PMID: 24014243 DOI: 10.1182/blood-2013-03-489641]
  - 70 **Yilmaz ÖH**, Katajisto P, Lamming DW, Gültekin Y, Bauer-Rowe KE, Sengupta S, Birsoy K, Dursun A, Yilmaz VO, Selig M, Nielsen GP, Mino-Kenudson M, Zuberberg LR, Bhan AK, Deshpande V, Sabatini DM. mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. *Nature* 2012; **486**: 490-495 [PMID: 22722868 DOI: 10.1038/nature11163]
  - 71 **Pattappa G**, Thorpe SD, Jegard NC, Heywood HK, de Bruijn JD, Lee DA. Continuous and uninterrupted oxygen tension influences the colony formation and oxidative metabolism of human mesenchymal stem cells. *Tissue Eng Part C Methods* 2013; **19**: 68-79 [PMID: 22731854 DOI: 10.1089/ten.tec.2011.0734]
  - 72 **Takubo K**, Nagamatsu G, Kobayashi CI, Nakamura-Ishizu A, Kobayashi H, Ikeda E, Goda N, Rahimi Y, Johnson RS, Soga T, Hirao A, Suematsu M, Suda T. Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. *Cell Stem Cell* 2013; **12**: 49-61 [PMID: 23290136 DOI: 10.1016/j.stem.2012.10.011]
  - 73 **Vacanti NM**, Metallo CM. Exploring metabolic pathways that contribute to the stem cell phenotype. *Biochim Biophys Acta* 2013; **1830**: 2361-2369 [PMID: 22917650 DOI: 10.1016/j.bbagen.2012.08.007]
  - 74 **Chen C**, Liu Y, Liu Y, Zheng P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. *Sci Signal* 2009; **2**:



- ra75 [PMID: 19934433 DOI: 10.1126/scisignal.2000559]
- 75 **Jasper H**, Jones DL. Metabolic regulation of stem cell behavior and implications for aging. *Cell Metab* 2010; **12**: 561-565 [PMID: 21109189 DOI: 10.1016/j.cmet.2010.11.010]
- 76 **Kharas MG**, Okabe R, Ganis JJ, Gozo M, Khandan T, Paktinat M, Gilliland DG, Gritsman K. Constitutively active AKT depletes hematopoietic stem cells and induces leukemia in mice. *Blood* 2010; **115**: 1406-1415 [PMID: 20008787 DOI: 10.1182/blood-2009-06-229443]
- 77 **Kalaitzidis D**, Sykes SM, Wang Z, Punt N, Tang Y, Ragu C, Sinha AU, Lane SW, Souza AL, Clish CB, Anastasiou D, Gilliland DG, Scadden DT, Guertin DA, Armstrong SA. mTOR complex 1 plays critical roles in hematopoiesis and Pten-loss-evoked leukemogenesis. *Cell Stem Cell* 2012; **11**: 429-439 [PMID: 22958934 DOI: 10.1016/j.stem.2012.06.009]
- 78 **Magee JA**, Ikenoue T, Nakada D, Lee JY, Guan KL, Morrison SJ. Temporal changes in PTEN and mTORC2 regulation of hematopoietic stem cell self-renewal and leukemia suppression. *Cell Stem Cell* 2012; **11**: 415-428 [PMID: 22958933 DOI: 10.1016/j.stem.2012.05.026]
- 79 **Stanfel MN**, Shamieh LS, Kaeberlein M, Kennedy BK. The TOR pathway comes of age. *Biochim Biophys Acta* 2009; **1790**: 1067-1074 [PMID: 19539012 DOI: 10.1016/j.bbagen.2009.06.007]
- 80 **Selman C**, Tullet JM, Wieser D, Irvine E, Lingard SJ, Choudhury AI, Claret M, Al-Qassab H, Carmignac D, Ramadan F, Woods A, Robinson IC, Schuster E, Batterham RL, Kozma SC, Thomas G, Carling D, Okkenhaug K, Thornton JM, Partridge L, Gems D, Withers DJ. Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* 2009; **326**: 140-144 [PMID: 19797661 DOI: 10.1126/science.1177221]
- 81 **Gan B**, Hu J, Jiang S, Liu Y, Sahin E, Zhuang L, Fletcher-Sananikone E, Colla S, Wang YA, Chin L, Depinho RA. Lkb1 regulates quiescence and metabolic homeostasis of haematopoietic stem cells. *Nature* 2010; **468**: 701-704 [PMID: 21124456 DOI: 10.1038/nature09595]
- 82 **Shiraki N**, Shiraki Y, Tsuyama T, Obata F, Miura M, Nagae G, Aburatani H, Kume K, Endo F, Kume S. Methionine metabolism regulates maintenance and differentiation of human pluripotent stem cells. *Cell Metab* 2014; **19**: 780-794 [PMID: 24746804 DOI: 10.1016/j.cmet.2014.03.017]
- 83 **Yun DH**, Song HY, Lee MJ, Kim MR, Kim MY, Lee JS, Kim JH. Thromboxane A(2) modulates migration, proliferation, and differentiation of adipose tissue-derived mesenchymal stem cells. *Exp Mol Med* 2009; **41**: 17-24 [PMID: 19287196 DOI: 10.3858/emmm.2009.41.1.003]
- 84 **Garcia-Gonzalo FR**, Izpisua Belmonte JC. Albumin-associated lipids regulate human embryonic stem cell self-renewal. *PLoS One* 2008; **3**: e1384 [PMID: 18167543 DOI: 10.1371/journal.pone.0001384]
- 85 **Bracha AL**, Ramanathan A, Huang S, Ingber DE, Schreiber SL. Carbon metabolism-mediated myogenic differentiation. *Nat Chem Biol* 2010; **6**: 202-204 [PMID: 20081855 DOI: 10.1038/nchembio.301]
- 86 **Levy D**, Ruiz JL, Celestino AT, Silva SF, Ferreira AK, Isaac C, Bydlowski SP. Short-term effects of 7-ketocholesterol on human adipose tissue mesenchymal stem cells in vitro. *Biochem Biophys Res Commun* 2014; **446**: 720-725 [PMID: 24491549 DOI: 10.1016/j.bbrc.2014.01.132]
- 87 **Klotz B**, Mentrup B, Regensburger M, Zeck S, Schneidereit J, Schupp N, Linden C, Merz C, Ebert R, Jakob F. 1,25-dihydroxyvitamin D3 treatment delays cellular aging in human mesenchymal stem cells while maintaining their multipotent capacity. *PLoS One* 2012; **7**: e29959 [PMID: 22242193 DOI: 10.1371/journal.pone.0029959]
- 88 **Geng S**, Zhou S, Bi Z, Glowacki J. Vitamin D metabolism in human bone marrow stromal (mesenchymal stem) cells. *Metabolism* 2013; **62**: 768-777 [PMID: 23375059 DOI: 10.1016/j.metabol.2013.01.003]

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## Basic Study

## Mesenchymal stem cells and collagen patches for anterior cruciate ligament repair

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**Ethics approval:** The study protocol was approved by local Ethical authorities.

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**Data sharing:** Technical appendix, statistical code, and dataset are available from the corresponding author (Benjamin. Gantenbein@istb.unibe.ch).

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### Abstract

**AIM:** To investigate collagen patches seeded with mesenchymal stem cells (MSCs) and/or tenocytes (TCs) with regards to their suitability for anterior cruciate ligament (ACL) repair.

**METHODS:** Dynamic intraligamentary stabilization utilizes a dynamic screw system to keep ACL remnants in place and promote biological healing, supplemented by collagen patches. How these scaffolds interact with cells and what type of benefit they provide has not yet been investigated in detail. Primary ACL-derived TCs and human bone marrow derived MSCs were seeded onto two different types of 3D collagen scaffolds, Chondro-Gide® (CG) and Novocart® (NC). Cells were seeded onto the scaffolds and cultured for 7 d either as a pure populations or as "premix" containing a 1:1 ratio of TCs to MSCs. Additionally, as controls, cells were seeded in monolayers and in co-cultures on both sides of porous high-density membrane inserts (0.4 µm). We analyzed the patches by real time polymerase chain reaction, glycosaminoglycan (GAG), DNA and hydroxyproline (HYP) content. To determine cell spreading and adherence in the scaffolds microscopic imaging techniques, *i.e.*, confocal laser scanning microscopy (cLSM) and scanning electron microscopy (SEM), were applied.

**RESULTS:** CLSM and SEM imaging analysis confirmed cell adherence onto scaffolds. The metabolic cell activity revealed that patches promote adherence and proliferation of cells. The most dramatic increase in absolute metabolic cell activity was measured for CG samples seeded with tenocytes or a 1:1 cell premix. Analysis of DNA content and cLSM imaging also indicated MSCs were not proliferating as nicely as tenocytes on CG. The HYP to GAG ratio significantly changed for the premix group, resulting from a slightly lower GAG content, demonstrating that the cells are modifying the underlying matrix. Real-time quantitative



polymerase chain reaction data indicated that MSCs showed a trend of differentiation towards a more tenogenic-like phenotype after 7 d.

**CONCLUSION:** CG and NC are both cyto-compatible with primary MSCs and TCs; TCs seemed to perform better on these collagen patches than MSCs.

**Key words:** Anterior cruciate ligament rupture; Anterior cruciate ligament tenocyte; Dynamic intraligamentary stabilization system; Resazurin red assay; Mesenchymal stem cells; Real-time polymerase chain reaction; Histology; Scanning electron microscopy microscopy; Scanning electron microscopy

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**Core tip:** Commercially available porcine or bovine-derived collagen type I and type III patches are cyto-compatible for anterior cruciate ligament derived tenocytes (ACL-TCs) and for bone-marrow derived mesenchymal stem cells (MSCs). The combination of commercially available collagen patches with MSCs and ACL-TCs seems to be suitable for application with a dynamic intraligamentary stabilization system, which represents a novel technique to fix a ruptured ACL. However, co-culture of MSCs with ACL-TCs did show signs of differentiation of MSCs towards a more TC-like phenotype after a short-term culture of 7 d.

Gantenbein B, Gadhari N, Chan SCW, Kohl S, Ahmad SS. Mesenchymal stem cells and collagen patches for anterior cruciate ligament repair. *World J Stem Cells* 2015; 7(2): 521-534 Available from: URL: <http://www.wjgnet.com/1948-0210/full/v7/i2/521.htm> DOI: <http://dx.doi.org/10.4252/wjsc.v7.i2.521>

## INTRODUCTION

Anterior cruciate ligament (ACL) injuries represent one of the most common ligament injuries, estimated to impact 100000 to 200000 people annually in the United States<sup>[1-4]</sup>. In Germany, the incidence of ACL ruptures is estimated at 32 per 100000 in the general population; in the active sports community, this rate is more than twice as high, at 70 per 100000<sup>[1]</sup>. The ACL provides an essential mechanism for ensuring stability in the knee joint<sup>[5]</sup>. The instability resulting from ACL injury not only impairs the functionality of the joint, but can also result in secondary damage to other structures, leading to arthritic changes<sup>[1,6,7]</sup>. Current surgical treatment methods are thus focused on restoring stability of the knee, reducing pain and preventing osteoarthritis<sup>[1,8,9]</sup>. The cruciate ligaments do not heal spontaneously; this has been attributed to a lack of a fibrin-platelet clot formation in ACL defects, which may be due to the synovial fluid surrounding the

cruciate ligaments into which blood from the ruptured stumps disperses, as well as to high levels of plasmin concentrations known to be circulating in the synovial fluid<sup>[10]</sup>. Lack of spontaneous healing necessitates intervention to provide for alternative options to restore stability.

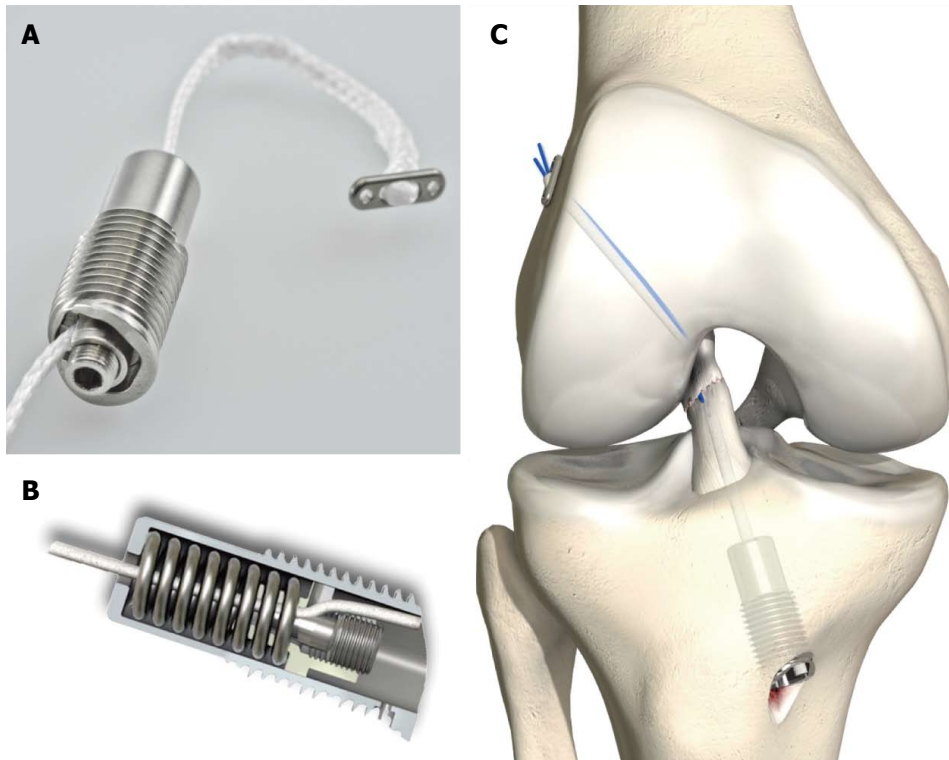
Given the clinical success of the dynamic intra-ligamentary stabilization approach in ACL repair<sup>[11-13]</sup>, the aim of this study was to provide insight as to whether the collagen patches-which are currently approved and marketed for cartilage and meniscus repair-are also suitable for ACL repair, and elucidate the extent to which they impact and interact with cells. For this purpose, we tested two commercially available collagen scaffolds with regards to cell viability, adherence and proliferation of seeded tenocytes (TCs), the primary cell type in ACL tissue<sup>[14]</sup>. TCs are considered to be a specific subset of the category of fibroblasts<sup>[14]</sup>. Ideally, the cells seeded on the scaffold would proliferate and modify the matrix, slowly reconstructing the original tissue.

Considering the difficulty of obtaining autologous ACL-derived TCs for common surgical use, we also used bone-marrow derived mesenchymal stem cells (MSCs), which can be easily harvested through bone marrow aspiration, cultured and stimulated to differentiate into a fibroblast phenotype<sup>[2,6,15]</sup>. Several studies propose MSCs proliferate faster than TCs, and also exhibit a higher survivability *in vivo*<sup>[15]</sup>. Additionally, current literature suggests that MSCs may have an anti-inflammatory effect, providing an additional benefit for their use in tissue engineering<sup>[16]</sup>. We were particularly interested whether the MSCs would be able to stimulate the TCs in this *in vitro* experiment set up.

Due to the composition of the scaffold materials, we hypothesized that the patches would provide a suitable environment for TCs and lead to cell adherence as well as proliferation and collagen deposition. Furthermore, we expected MSCs to differentiate into a TCs phenotype in co-culture and even more so if cultured on collagen scaffolds rather than cultured on culture inserts. Currently, no unique marker profile for ligaments exists; thus, tendon-like cells are commonly distinguished using a combination of markers, including scleraxis, tenomodulin, tenascin-C, collagen I and collagen III<sup>[6,14]</sup>.

Here, we present analyses on cell compatibility for primary ACL-derived TCs and bone-marrow derived MSCs seeded on collagen scaffolds. We investigated the phenotype of these cells using real time real time polymerase chain reaction (RT-PCR) and biochemical analysis in order to judge the suitability of cell-seeded collagen scaffolds for the Ligamys surgery application to "boost" the healing process and possibly to improve the surgery's outcome. To the best of our knowledge, no studies have been previously published with this aim.





**Figure 1** Dynamic intraligamentary stabilization screw called Ligamys® (Mathys, inc. Bettlach, Switzerland). A: Close-up of the outside of the screw made of titanium and illustrating with a mounted lace which mimics the polyethylene string that is mounted in the real surgery to stabilize the knee joint in case of an ACL rupture; B: Inside of the dynamic fixation screw with the spring that takes the dynamic load of the ACL and stabilizes the joint; C: Illustration of the exact position of the dynamic intraligamentary stabilization *in situ* in the knee joint. ACL: Anterior cruciate ligament.

## MATERIALS AND METHODS

### Scaffolds and sample preparation

Two types of commercially available and CE label approved bio-resorbable collagen scaffolds were tested in the experiments: Novocart® (NC) produced by Tetec AG (Reutlingen, Germany), a B. Braun AG Company, and Chondro-Gide® (CG), produced by Geistlich Pharma AG (Wolhusen, Switzerland). NC is a biphasic collagen-chondroitin sulfate matrix of bovine origin; CG represents a porcine collagen bilayer matrix. Both scaffolds were specifically developed for human chondrocytes and are used clinically in connection with autologous chondrocyte transplantation and autologous matrix-induced chondrogenesis for cartilage regeneration. Here, we investigated these scaffolds for application in ACL repair (Figures 1 and 2). The NC and CG scaffolds comprise a porous sponge and an adhering compact membrane to protect the cells and the lesion. Cells were seeded on the porous side as intended by its application. The sponge itself consists of fibers, building a three-dimensional structure with interconnecting pores.

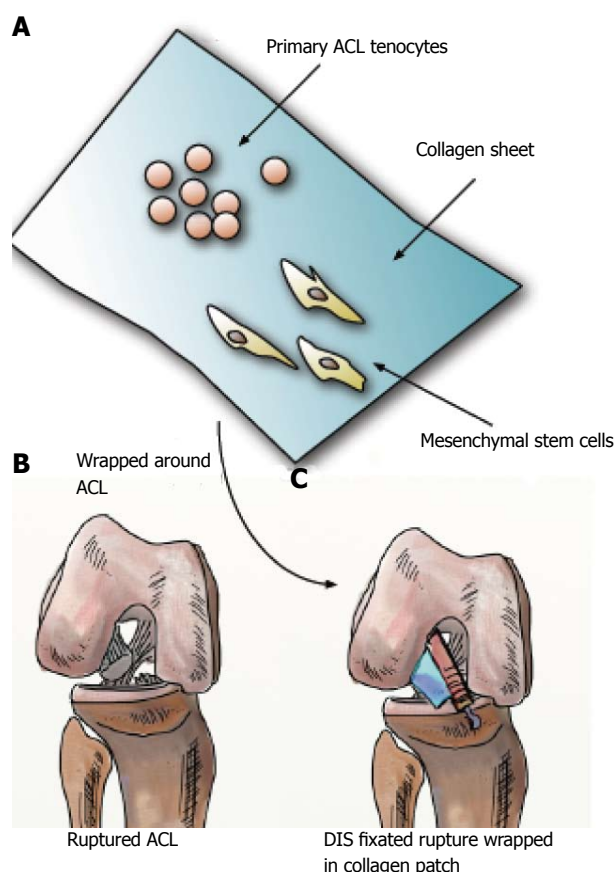
Prior to cell seeding, the scaffolds were cut in equally sized samples using an 8 mm sterile biopsy punch (Kai Medical, Polymed Inc., Glattbrugg, Switzerland) and then soaked in high glucose Dulbecco's Modified Eagle's Medium (HG-DMEM), (Gibco, Life Technologies, Zug, Switzerland) with 10% fetal calf serum (FCS) and 1 ×

penicillin-streptomycin (P/S) (all Sigma-Aldrich, Buchs, Switzerland). The patches were seeded with 40000 cells; in addition, material only controls were also cultured under the same culture conditions.

### Cell source and culture

Primary ACL derived TCs were isolated from ACL tissue obtained from full-knee prosthesis surgery (ethical approval was obtained from the local committee: KEK registration 22-12-13). Primary TCs were isolated by cutting the tissue in smaller pieces of approximately 4 mm<sup>3</sup> pieces, then washed in phosphate buffered saline (PBS) twice and digested overnight in collagenase 2 (Worthington, London, United Kingdom) at 37 °C, under constant shaking at 10 RPM. The released cells were filtered with a 100 µm cell strainer (BD Falcon, Switzerland) and seeded at a density of 1000 cells/cm<sup>2</sup> for monolayer expansion in HG-DMEM + 10% FCS. With written consent human bone marrow was obtained from patients ages 55-84 undergoing hip or spine surgery. The procedure was approved by the Ethics Office of the Canton of Bern (KEK # 187/10), all patients gave their informed consent prior to their inclusion in the study. Human MSCs were amplified from the mononuclear cell fraction after density gradient centrifugation (Histopaque-1077, Sigma) by selection for plastic adherence for 2-3 passages. The MSCs were expanded using α-Minimal Essential Medium (α-MEM) with 10% FCS, 100 µg/mL penicillin, 100 UI/mL





**Figure 2** Overview of current regenerative approaches to improve anterior cruciate ligament rupture treatment in combination of the dynamic intraligamentary stabilization approach. A: First mesenchymal stem cells and/or primary ACL-tenocytes are pre-seeded on collagen patches (shown in blue); B: Ruptured ACL; C: DIS approach takes over major mechanical load and stabilizes the knee, whereas collagen patch wrapped around the ACL provides an ideal solution to improve clinical outcome. ACL: Anterior cruciate ligament; DIS: Dynamic intraligamentary stabilization.

streptomycin, and 5 ng/mL bFGF-2<sup>[17]</sup>.

### Scaffold seeding

Scaffolds that were seeded contained either a single cell type-human MSCs or human TCs- or a combination of both cell types (Figure 3). For the samples containing co-cultures, the two cell types were mixed in a ratio of 1:1 prior to seeding; recent literature demonstrates that this provides the most effective ratio<sup>[16]</sup>. All patches were placed in 12 well plates, and the samples were incubated for approximately one hour prior to adding 2 mL medium to each well, in order to give the cells time to adhere to the patch.

### Monolayer cultures

In addition to the scaffold samples, we also grew monolayer cell cultures as well as co-cultures using translucent inserts (Falcon, 6 well cell culture inserts, 0.4  $\mu$ m PET high density pores, Falcon™, BD Biosciences, Allschwil) in 6 well plates (Falcon™). The single cell population monolayer controls served as reference samples for the real-time PCR results, while the

inserts allowed us to evaluate changes in MSCs gene expression in co-culture. Separating the MSCs and TCs cultures with inserts made it possible to attribute the relative gene expression results of cells in co-culture to the correct cell type. The pore size was chosen to facilitate cell-cell contact between the two cell types, without allowing for cell migration between the two cell populations. This setup allowed us to observe whether cell-cell contact permits differentiation of MSCs into TCs phenotype.

The co-culture for the gene expression analysis was set up as follows: culture inserts were seeded on the bottom with 100000 MSCs. Two hundred microlitre HG-DMEM medium with 10% FCS and 1  $\times$  P/S was added to the layer of MSCs. The inserts, bottom side facing up, were placed on a petri dish, covered with a beaker and placed in a 37 °C incubator for 1 h. Within this timeframe, the MSCs adhered to the insert, as has been shown in previous studies by our group<sup>[18]</sup>. Subsequently, the other side of the insert was also seeded with the same number of TCs.

### Resazurin sodium salt metabolic cell activity assay

Patches were incubated at 37 °C on a shaker for 5 h with resazurin red solution to measure mitochondrial activity as described in<sup>[19]</sup>. Resazurin is a nontoxic, cell-permeable compound that is blue in color and virtually non-fluorescent. Upon entry into cells, resazurin is reduced to resorufin, which emits very bright red fluorescence. The amount of fluorescence or absorbance is proportional to the number of living cells (the linearity of transformation was evaluated based on primary cells) and corresponds to the cell's metabolic activity. The amount of fluorescence was monitored with a Softmax® M5 Pro Multi wavelength fluorescence reader (Molecular Devices, distributed by Bucher Biotec, Switzerland) at excitation 547 nm and emission wavelength 582 nm.

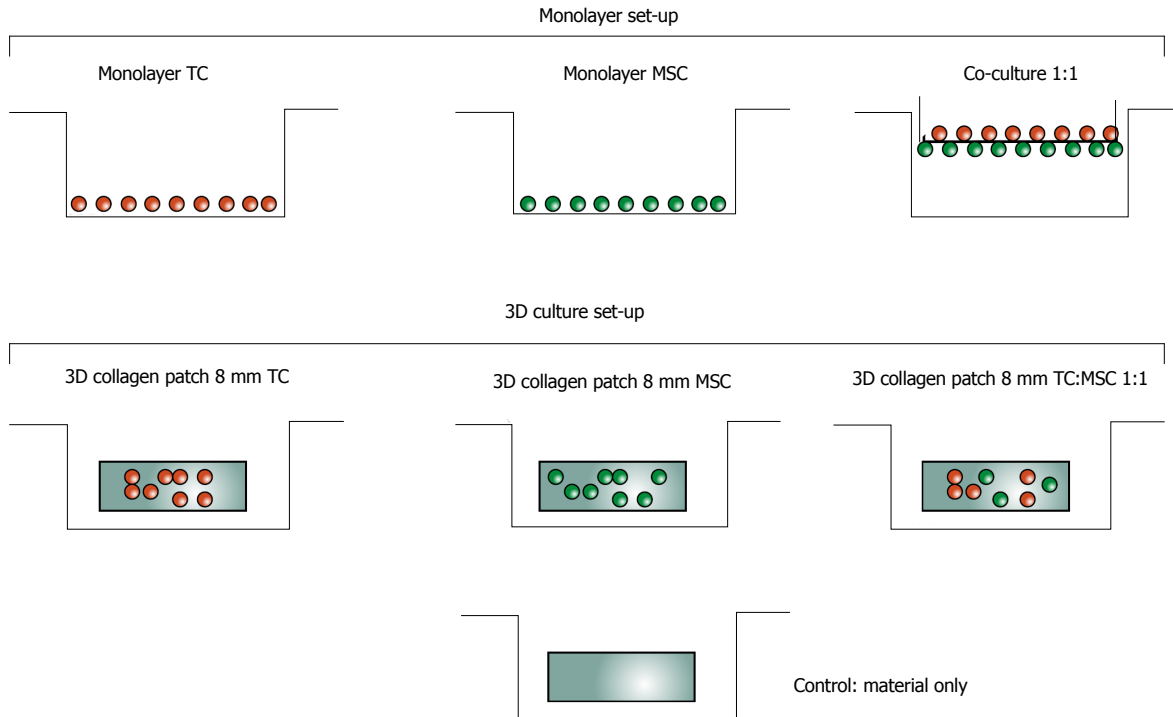
### Quantification of DNA, glycosaminoglycan and HYP content

For biochemical analysis, dry-weight of each sample was measured and then each sample was digested with papain. We used the dimethylmethyle blue (DMMB) dye method to analyze the digested samples for sulphated glycosaminoglycans (GAGs), using bovine chondroitin sulfate as standard. Total collagen content was determined based on the hydroxy-proline content after acid hydrolysis and reaction with p-dimethyl-amino-benz-aldehyde and chloramine-T<sup>[20,21]</sup>.

### RT-PCR

All samples were frozen down on day 0 and day 7 of each experiment, except for two samples collected during the pilot experiment, which were collected on day 8 (NC patch with TCs and NC patch with MSCs). RNA extraction was performed with TRI reagent [Molecular Research Center (MRC), Cincinnati,





**Figure 3 Experimental design of *in vitro* testing of cyto-compatibility.** All monolayer and collagen patch cultures were run for 7 d under standard culture conditions (37 °C at 100% humidity and 5% CO<sub>2</sub>) in high glucose Dulbecco's Modified Eagle Medium and 10% fetal calf serum. MSC: Mesenchymal stem cell; TC: Tenocyte.

MA, United States], polyacrylcarrier (MRC), 100% molecular grade ethanol, 1-bromo-3-chloropropane (BCP, Sigma Aldrich), GenElute miniprep kit, and the AMP-D1 DNase I Kit which contains both DNase I and 10 × DNase Digest Buffer (all Sigma Aldrich). The same process was followed for all samples, except that the scaffold samples were subject to an additional step, since those samples had been snap frozen in liquid nitrogen on day 7 of the experiments and placed in -80 °C without any RNA protective solution. In order to perform RNA extraction on those samples, the scaffold samples were first ground using a mortar pre-cooled with liquid nitrogen, then immediately placed in a solution containing 1 mL TRI and 5 µL polyacrylcarrier. The samples were then centrifuged at 16000 g to separate the scaffold debris from the solution. cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Cressier, Switzerland). For each sample, 20 µL reaction mix was set up containing 10 µL RNA template (about 0.5-1 µg total RNA), 4 µL 5 × iScript Reaction Mix, 1 µL iScript reverse transcriptase and 5 µL nuclease-free water. After the incubation, the cDNA samples were diluted with 40 µL 1 × TE Buffer.

We evaluated the relative gene expression for the genes we were interested in using human primers (all Microsynth, Balgach, Switzerland; see Table 1 for a detailed list of primers). Separate forward and reverse primers were used to set up the reaction mix, which also contained iQ SYBR Green Supermix2X (Bio-Rad) and RNase free water. We tested ligament fibroblast markers including scleraxis, tenomodulin, tenascin-C,

collagen I and III<sup>[6,22]</sup>. In addition, we tested for gene expression of two matrix metalloproteinases (MMPs, enzymes which regulate matrix turnover) MMP3 and 13, aggrecan and collagen type II. MMP3 (stromelysin 1) and MMP13 (collagenase 3) break down collagen and proteoglycans quickly and are known to play a role in ligament remodeling<sup>[23]</sup>. Collagen II was used to test that the cells did not differentiate into chondrocyte-like cells. There has been some evidence of increased amounts of proteoglycans in pathological tendons; therefore, gene expression of aggrecan was also evaluated<sup>[22]</sup>. To calculate relative gene expression, the Livak method ( $2^{-\Delta\Delta Ct}$ )<sup>[24]</sup> was used, which makes use of both a calibrator sample and a normalizing reference gene; we applied 18S (ribosomal RNA) as the reference gene for all samples<sup>[25]</sup>. Relative gene expression levels for samples were calibrated to day 0 reference samples. Day 7 TCs samples were compared relative to TCs at day 0; day 7 MSCs samples were compared relative to MSCs at day 0 (*i.e.*, expression = 1.0).

### Microscopy techniques

Confocal laser scanning microscopy (cLSM) was used to confirm visually that cells are able to adhere and proliferate on the scaffolds in the first two experiments. Samples for the cLSM were stained using Vybrant membrane tracker dyes DiO (DiO = green, for MSCs) and DiI (DiI = red, for TCs) (both Molecular Probes, Life Technologies, Basel, Switzerland); these dyes are lipophilic carbocyanines, which integrate into the membrane<sup>[26]</sup>. In addition, we also imaged the cells



**Table 1** Real-time polymerase chain reaction primers used for real-time polymerase chain reaction

Abbreviation	Gene name	Forward	Reverse
18S	18S Ribosomal RNA	CGA TGC GGC GGC GTT ATT C	TCT GTC AAT CCT GTC CGT GTC C
HsACAN	Core protein	CAT CAC TGC AGC TGT CAC	AGC AGC ACT ACC TCC TTC
Hs_col1	Collagen 1 A2	GTG GCA GTG ATG GAA GTG	CAC CAG TAA GGC CGT TTG
Hs_col2	Collagen 2 A1	AGCAAGAGCAAGGAGAAG	GGGAGCCAGATTGTCATC
Hs_col3	Collagen 3 A1	GTG AGC CTG GTA AGA ATG G	CCT GGA ACA CCT GGA ATA C
Hs_TNC	Tenascin C	CAC GCT GAG GTT GAT GTT C	GTT GAT GGT CGC TGG ATT G
Hs_SCX	Scleraxis A	CAC CAA CAG CGT GAA CAC	GCA GCG TCT CAA TCT TGG A
Hs_TNMD	Tenomodulin	ACA AGC AAG TGA GGA AGA A	GAC GGC AGT AAA TAC AAC AAT
Hs_MMP13	Collagenase 3	AGT GGT GGT GAT GAA GAT	CTA AGG TGT TAT CGT CAA GIT
Hs_MMP3	Matrix metalloproteinase 3	CAA GGC ATA GAG ACA ACA TAG A	GCA CAG CAA CAG TAG GAT

All primers were run at 61 °C Ta (annealing temperature) and a two-step protocol (95 °C for 10 s and 61 °C for 30 s) and 45 cycles.

on both sides of the culture inserts. This allowed us to confirm that the cells adhered to both sides of the culture insert and could therefore be evaluated with real-time PCR.

Further, additional samples were prepared during one experiment to be processed with scanning electron microscopy (SEM), as this method permits visualization of 3D structures on a very small scale. This technique also allowed us to see how the cells attach to the scaffold, as well as provided a more detailed visualization of the scaffolds themselves. On day 7, the samples were first washed with PBS to remove traces of serum, then fixed with 2.5% glutar-aldehyde. On the day of SEM sample preparation, the specimen were again washed with PBS prior to following a dehydration protocol using an alcohol series of increasing concentration, *i.e.*, 50%, 70%, 80%, 90% and 2 × 100%, with incubation periods of 10 min. Subsequently, the specimen were critical point-dried in a Bal-Tec CPD 030 (BalTec inc., Balzers, Liechtenstein) and sputtered with approximately 10 nm (30 mA for 30 s at 50 mm working distance), producing a 10 nm gold layer in a SCD 004 (BalTec inc.). Samples were then stored in an exsiccator until examination at 5 kV accelerating voltage in a Zeiss scanning electron microscope DSM 982 (Carl Zeiss, Jena, Germany).

### Statistical analysis

Data were analyzed using the Kruskal-Wallis test unless otherwise noted, followed by Dunn's multiple comparisons test using the PRISM software package (GraphPad, La Jolla, United States, version 6.0e). RT-PCR data was tested using a *t*-test statistic and mean gene expression was tested if significantly different from a hypothetical value of 1.0. A significance value of  $P < 0.05$  was specified to be significant. Where applicable groups were tested in 2-way ANOVA for treatment and culture time, multiple comparisons were then performed using Bonferroni's multiple testing. The statistics was checked by a biostatistics expert.

## RESULTS

The experiments were meant to provide initial insight

into whether NC and CG collagen patches, which are currently being used in cartilage repair, are suitable for ACL repair. Thus, adherence and proliferation of cells was tested, as well as the collagen deposition and differentiation potential of cells.

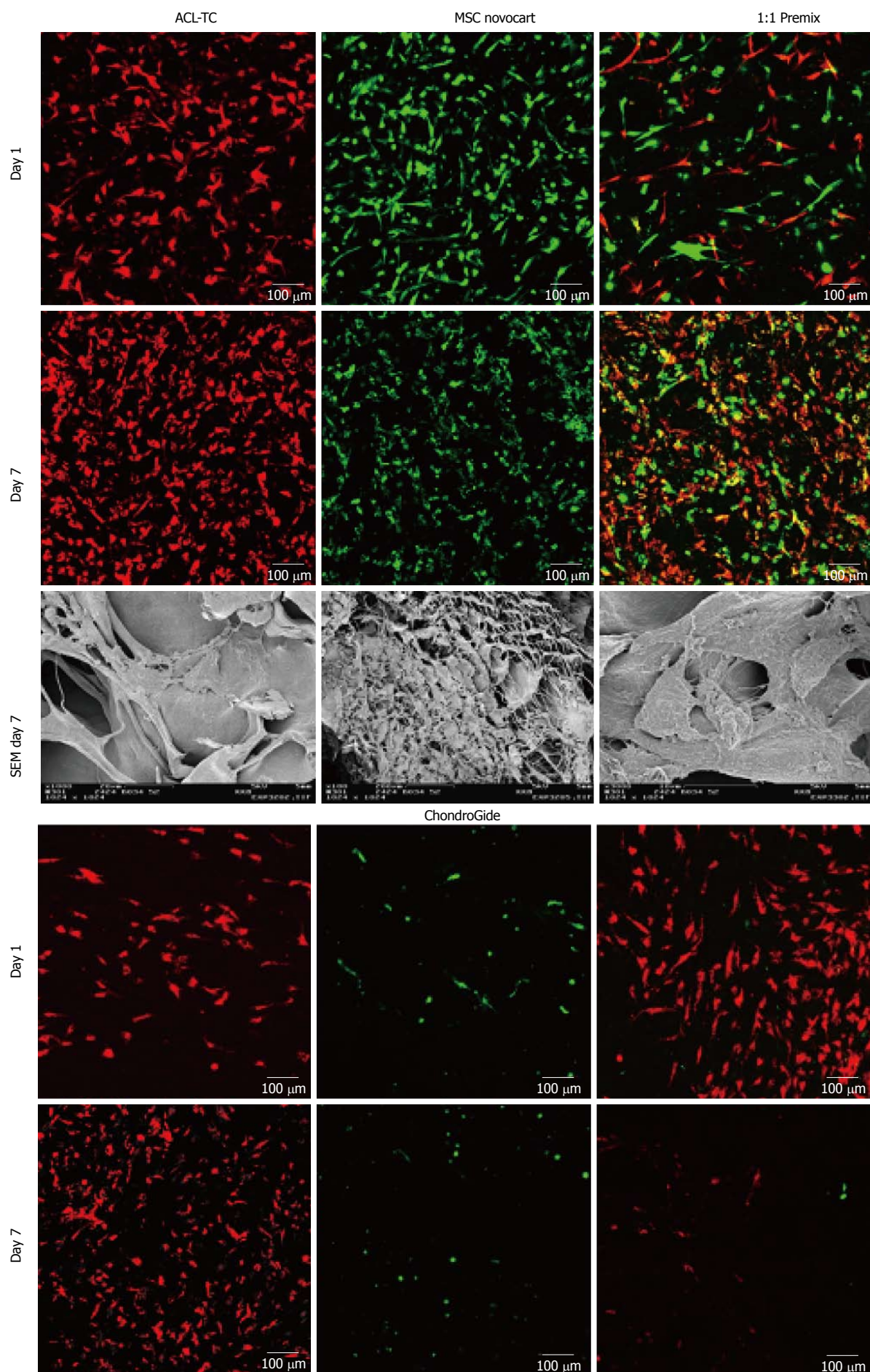
### Adherence, proliferation and matrix changes

CLSM and SEM imaging and the metabolic cell activity assay confirmed that both cell types, TCs and MSCs, adhere on the substrate and seem to proliferate on both patch types (Figure 4). The images seem to show, however, that MSCs decreased in cell number after 7 d.

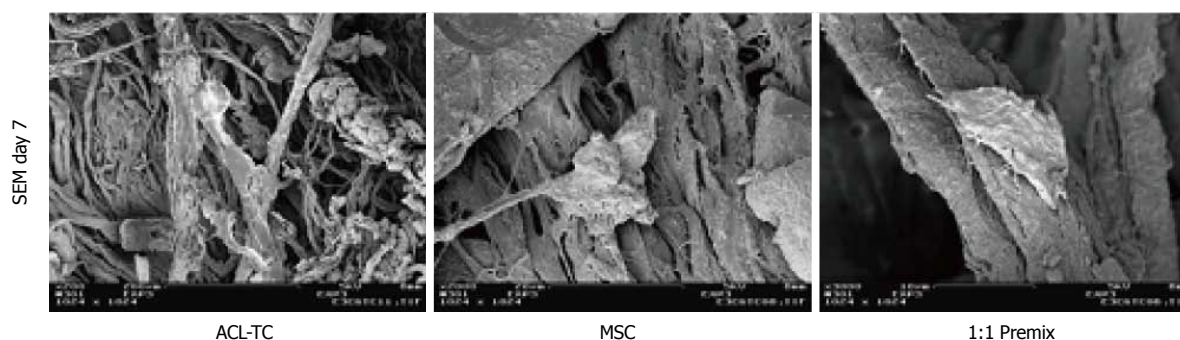
Noteworthy, DNA content (Hoechst) of collagen scaffolds contained a relatively high amount of porcine/bovine DNA (Figure 5), which was confirmed by DNase digestion (data not shown). There was no significant change in DNA content among the groups over the 7-d culture period. The metabolic cell activity assay (see Figure 6) revealed increased cell activity on day 7 vs day 1 for all cell treatment groups, and a slightly higher absolute level of cell activity for all CG samples versus NC patches on a given day. The relative increase in cell activity level from day 1 to day 7 in both patch types was similar (Figure 6).

The non-parametric Kruskal-Wallis tests showed that cell type did not have a significant impact on the relative change in metabolic activity for either of the two patch types (Figure 6). Furthermore, we found no significant differences between NC and CG samples in terms of the relative changes in metabolic cell activity between the two patch types. While both patch types also showed an increase in absolute cell activity, as measured in RFUs, from day 1 to day 7 for every cell type, this trend was not significant for NC patches, but significant at an overall level for CG patches ( $P = 0.0298$ ,  $n = 4$ ). Dunn's multiple comparisons test for CG however revealed no significant  $P$  values for individual treatment groups (TCs, MSCs, premix) with different treatment lengths (Figure 6). However, after testing for 2-way ANOVA for time and treatment we found a significant effect ( $P = 0.0001$ ) of culture time and found that activity increased over time for TCs and premixes ( $P = 0.0237$  and  $P = 0.0120$ , respectively)

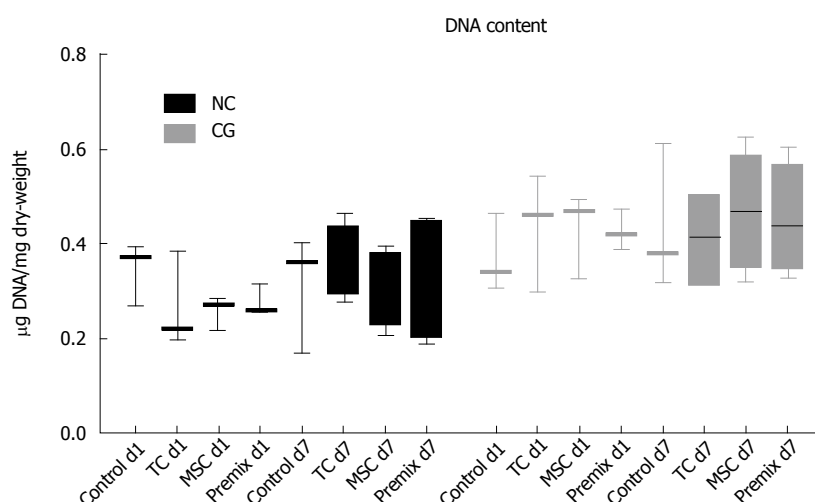




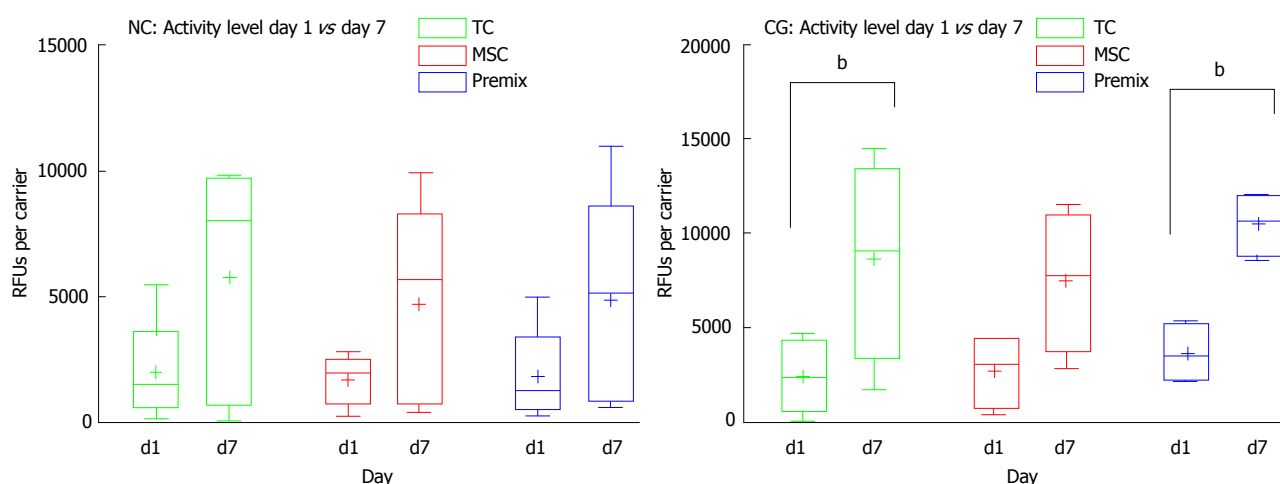




**Figure 4** Z-projections of confocal laser scanning microscope images of 200  $\mu\text{m}$  3D-stacks through the collagen patches of mesenchymal stem cells and anterior cruciate ligament-derived tenocytes on day 1 and after 7 d of culture on Novocart™ and Chondro-Gide™. Cells were stained with Vybrant™ DiO (DiO = green, MSCs) and DiI membrane tracker dyes (DiI = red, TCs) prior to seeding. Last row are scanning electron microscope pictures of the cell-seeded scaffolds after 7 d of culture showing adherence and shape of the MSCs and tenocytes on the scaffolds. Note that for Chondro-Gide™ patches these are only images from TCs seeded scaffolds. For the Novocart™ patches the right most image is from an experiment containing a premix of cells, and the left most and center SEM image are from experiments with MSCs seeded scaffolds. MSC: Mesenchymal stem cell; ACL-TC: Anterior cruciate ligament-derived tenocyte; SEM: Scanning electron microscope.



**Figure 5** DNA content of collagen patches. DNA content of collagen patches seeded with anterior cruciate ligament-derived tenocytes (TCs) and/or MSCs on Novocart™ and Chondro-Gide™ normalized per dry weight per carrier for the collagen patches for day 1 and day 7 of the experiment (values are means  $\pm$  SEM,  $n = 3$  experiments for day 1, 4 experiments for day 7). Note the large amount of DNA present in material controls. MSCs: Mesenchymal stem cells.



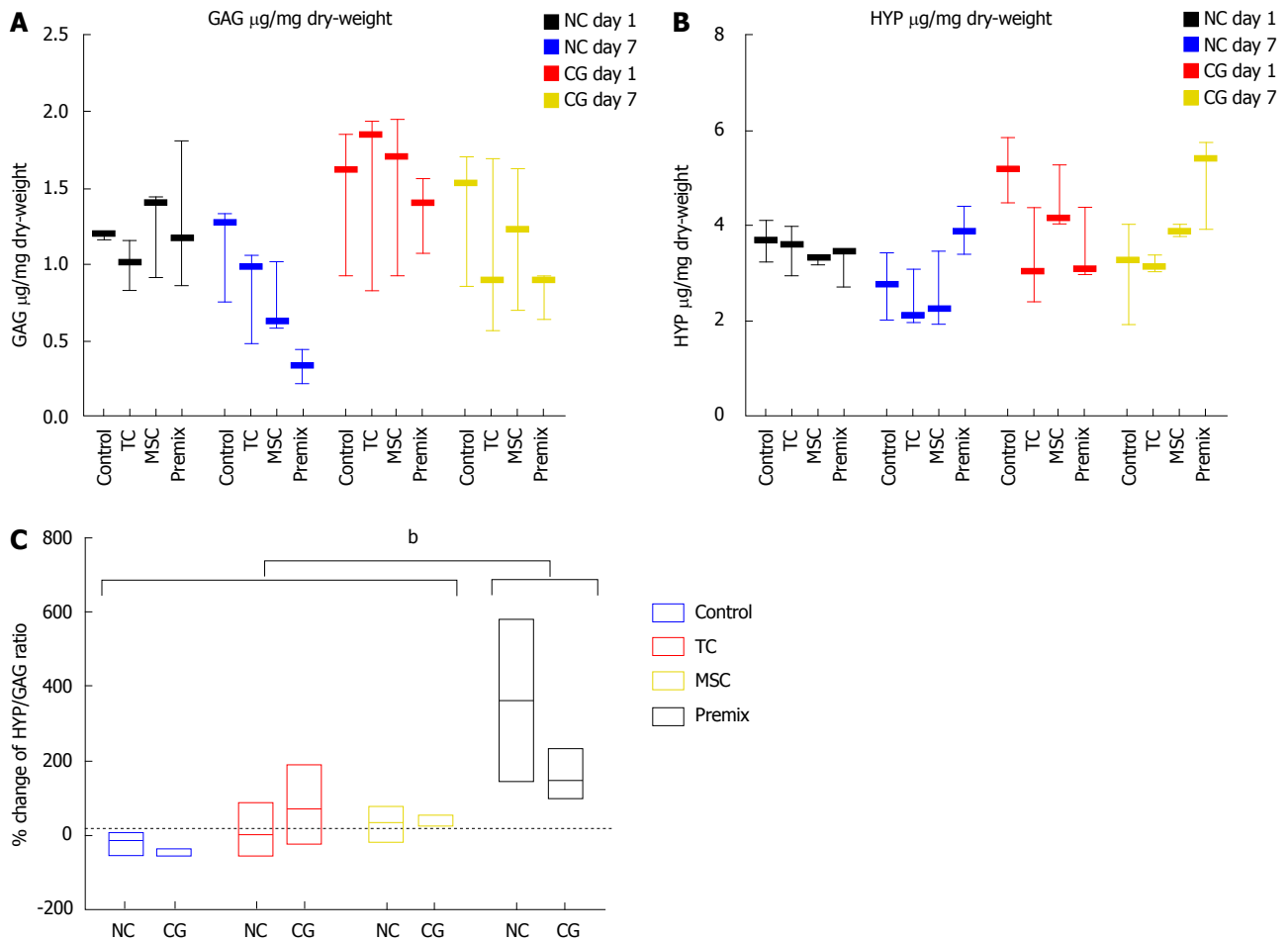
**Figure 6** Mitochondrial cell activity. Mitochondrial cell activity measured by resazurin red assay of collagen patches seeded with anterior cruciate ligament-derived tenocytes (TCs) and/or MSC on NC and CG normalized per dry weight carrier for the collagen patches for day 1 and day 7 of the experiment (values are means  $\pm$  SEM,  $n = 4$  experiments for CG and  $n = 5$  experiments for NC). b denotes  $P < 0.01$ . MSC: Mesenchymal stem cell; NC: Novocart™; CG: Chondro-Gide™; SEM: Scanning electron microscope.

(Figure 6).

Previous studies suggested that pathologic tendons exhibit higher GAG content<sup>[22]</sup>. We, therefore, measured

GAG content on day 1 and day 7 ( $n = 3$ ). In all samples, there was a decrease in the amount of GAG from day 1 to day 7 ( $\mu\text{g}$  per mg of dry-weight, corrected for controls





**Figure 7 Hydroxy-Proline and glycosaminoglycan content.** A: HYP content assay; B: GAG of collagen patches seeded with anterior cruciate ligament-TCs and/or MSCs on NC and CG normalized per dry weight carrier for the collagen patches for day 1 and day 7 of the experiment (values are means  $\pm$  SEM,  $n = 3$  experiments for day 1, 4 for day 7); C: Percentage change of HYP to GAG ratio relative to day 1 of NC and CG patches. b denotes a significant  $P < 0.01$ . GAG: Glycosaminoglycan; HYP: Hydroxy-proline; MSC: Mesenchymal stem cell; TC: Tenocytes; NC: Novocart™; CG: Chondro-Gide™; SEM: Scanning electron microscope.

using day 7 sample with no cells as a baseline for all samples). No significant difference was found between the two patch types or cell types in terms of the relative changes of GAG measured (Figure 7A).

Hydroxy-proline (HYP) levels were measured in order to assess collagen deposition of the different cell types on the scaffold (Figure 7B). Data revealed no significant change in HYP over time. In line with the other analyses, the different cell types were not found to have a significant effect on the percentage change of HYP measured, neither for NC patches nor for CG patches. Also, the Kruskal-Wallis test did not reveal a significant difference between the two patch types. However, when the ratio of HYP/GAG was considered, the 2-way ANOVA for scaffold and cell treatment showed a significant relative change for the Premix group ( $P = 0.005$ ) (Figure 7C).

Both cell types (TCs and MSCs) were analyzed individually with regards to the effect of different substrates (NC patch, CG patch, inserts or just well plates) on the relative gene expression for the genes of interest. A summary of the relative gene expression measurements is shown in Figure 8.

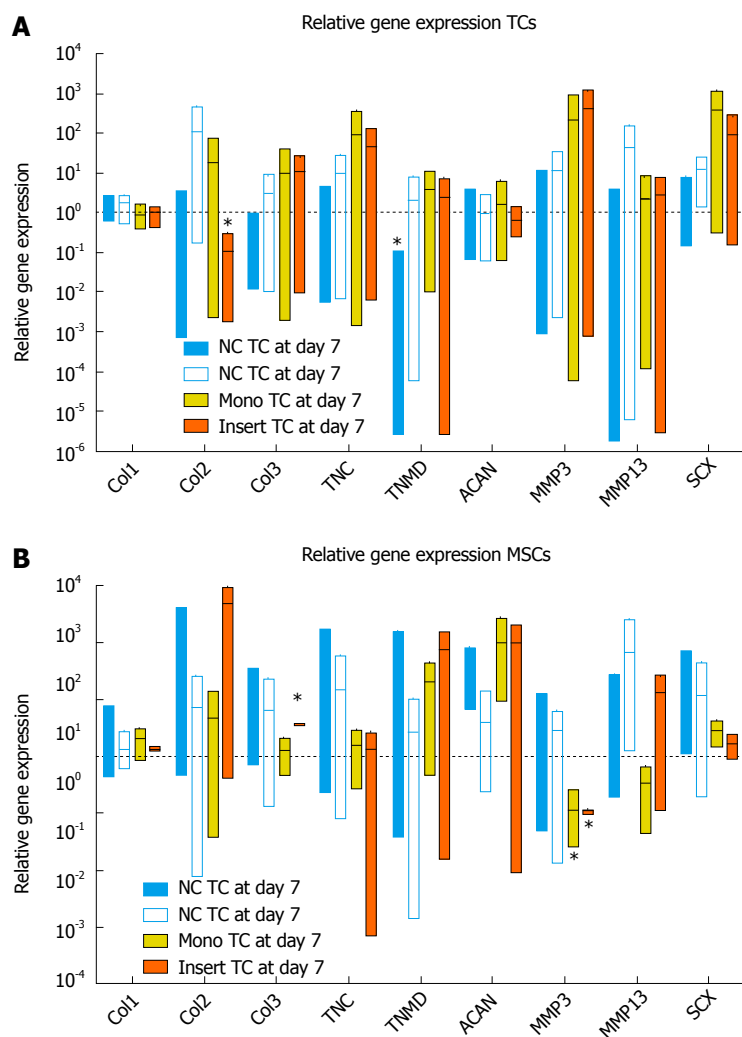
Relative gene expression revealed a significant down-regulation of TNMD for TCs grown on NC scaffolds ( $P < 0.0001$ ) and for col2 in TCs grown on culture inserts ( $P = 0.0121$ ) relative to day 0 controls (Figure 8A). Monolayer MSCs significantly down-regulated MMP3 ( $P = 0.0065$ ) after 7 d of culture, whereas MSCs grown on culture inserts significantly up-regulated col3 ( $P = 0.006$ ) and down-regulated MMP3 ( $P = 0.0065$ ).

## DISCUSSION

### TCs gene expression

In line with expectations, both patch types showed a slight up-regulation of collagen I gene expression for TCs in comparison to TCs grown in monolayers (Figure 8). Scleraxis expression trended up on patches vs d0 but was in line with monolayer results (Figure 8). At the same time, both patch types did not result in a significant up-regulation of collagen II or aggrecan, compared to monolayer (Figure 8). These factors suggest that the cells did not differentiate into cells of a chondrocyte phenotype. Based on the non-parametric Kruskal-Wallis test statistic, the differences in relative





**Figure 8** Relative gene expression of major extracellular matrix genes important for anterior cruciate ligament repair and mesenchymal stem cells differentiation grown on 3D collagen patches, i.e., Novocart™ and ChondroGide™. A: Gene expression of anterior cruciate ligament-derived tenocytes (TCs) relative to day 0 TCs; B: Gene expression of bone marrow-derived MSCs relative to day 0 MSCs (values are means  $\pm$  SEM,  $n = 4$  experiments for CG and  $n = 5$  for NC). MSC: Mesenchymal stem cell; NC: Novocart™; CG: ChondroGide™; SEM: Scanning electron microscope.

gene expression between TCs cultured on NC or CG patches or in monolayers are not significant, which is in line with our initial hypothesis. We did observe significant changes of relative gene expression when tested with one-sample *t*-test. While gene expression levels for TCs on NC patches were not vastly different from those cultured on CG or in monolayers, TCs grown on NC significantly expressed less tenomodulin (TNMD) and do seem to express slightly lower levels for the ligament markers collagen type 3 (col3), tenascin- C (TNC) and scleraxis (SCX) relative to those two substrates (see Figure 8A).

At first sight, especially when considering these data in light of the metabolic cell activity results, it may seem that the CG patches provide a better environment for TCs. The metabolic cell activity measurements showed that cells grown on CG patches vs NC patches exhibited a slightly higher (yet statistically insignificant) absolute cell activity on any given measurement day, as well as a somewhat higher relative increase in cell activity from day 1 to day 7 of the experiments. However, this aspect needs to be carefully examined further.

It is possible that the culture time was not long enough to clearly discern significant differences

between patch types. MSCs and TCs were cultured for up to 28 d and checked for cell adherence with the use of CLSM and the cell tracker dyes. Studies related to osteogenesis show that osteogenic differentiation of MSCs can take up to four weeks, with an initial early differentiation phase taking place between day 5 and day 14<sup>[27]</sup>. Conversely, it has also been shown that MSCs can show signs of differentiation into tenogenic phenotype after as little as seven days<sup>[28]</sup>. It is also possible that the cells on the NC scaffolds first partially de-differentiated. The down-regulation of tenomodulin (TNMD) has been observed for cells that were cultured two-dimensionally<sup>[29]</sup>. Since we observed a stronger down-regulation for cells cultured on NC than in monolayers, this may be interpreted as an indication of de-differentiation. This de-differentiation has been previously observed in monolayers as well as in organ cultures<sup>[15]</sup>.

Furthermore, scleraxis has been shown to modulate TNMD expression, such that the up-regulation of TNMD occurred several days after an up-regulation in scleraxis was observed<sup>[30]</sup>.

It is known that the structure of scaffolds can impact the cell morphology; ACL fibroblasts for instance orient parallel to collagen fibers. Cell shape



**Table 2** Summary of the donor tissue that was used to harvest and expand primary cells, *i.e.*, bone marrow derived mesenchymal stem cells and anterior cruciate ligament-derived tenocytes and stage of passage for 3D culture experiment

MSCs donor characteristics						TCs donor characteristics					
Experiment	ID	Passage	Donor gender	Donor age	Source	Experiment	ID	Passage	Donor gender	Donor age	Source
1	62	1	m	75	Lumbar	1	66	1	f	62	ACL
2	62	2	m	75	Lumbar	2	66	2	f	62	ACL
3	65	1	f	84	Lower limbs	3	67	1	f	38	ACL
4	47	2	m	64	Thoracal	4	67	2	f	38	ACL
5	48	2	m	55	Thoracal	5	70	1	f	74	ACL

MSC: Mesenchymal stem cell; TC: Tenocyte; ACL: Anterior cruciate ligament.

on the other hand can impact cell differentiation<sup>[30,31]</sup>. In fact, both patches were primarily constructed for use with chondrocytes. The manufacturer of NC patches clearly states that the patches are meant to induce a spherical morphology, which describes a chondrocyte phenotype. However, with SEM imaging we observed flat networks covering the NC patches. This observation was in line with the gene expression results, which demonstrate that the cells do not up-regulate genes, which are typical for chondrocyte phenotype. It would be interesting to re-evaluate all variables in experiments running for 14 or 21 d, to test whether this still holds true.

### MSCs phenotype

MSCs cultured on patches showed a trend towards up-regulation of ligament markers versus MSCs monolayers, particularly on NC scaffolds. For instance, collagen III tended to be up-regulated on NC, with a more inconsistent expression for MSCs on CG (Figure 8). Scleraxis tended to be higher expressed on both patches compared to MSCs cultured in monolayers or on inserts (Figure 8). These results seem to indicate that MSCs are developing into a TCs phenotype on the patches. MSCs cultured with TCs, however, consistently up-regulated col 3 expression, a sign for early differentiation of the MSCs towards a more TC-like phenotype. Again, additional time might be required in order to draw more conclusive insights about the potential for MSCs to differentiate on the various substrates. However, interpreting the results of the RT-PCR data demonstrating a significant increase of col 3 in presence of TCs on culture inserts together with the significantly increased HYP/GAG ratio of "premix" of TCs and MSCs on the 3D patches (Figure 7C) could be interpreted as an early differentiation of MSCs shutting down gene expression of GAG and increasing collagen expression. A study related to tenogenic differentiation of rat MSCs showed that a significant change in expression levels for some ligament markers could already be detected after 7 d (tenascin-C as well as scleraxis); however, other markers were not up-regulated until a few days later (collagen I and III)<sup>[28]</sup>.

There were some inherent limitations in the cell types used in our study. Cells had to be obtained from

patients whom may already exhibit certain morbidities. TCs were provided from patients undergoing knee surgeries– either total knee arthroplasties or ACL reconstruction. Patient number 67, for instance, underwent ACL reconstruction due to a traumatic rupture, where the stumps were intra-operatively removed prior to graft insertion. It is possible that the cells cultured from these stumps may naturally exhibit slightly different characteristics, a topic that should be explored further. Moreover, it is also possible that the age of the donor plays a role in terms of the differentiation potential of the MSCs.

In this respect, it is also worth mentioning the existence of native ACL-derived autochthonous stem cells (ACL-SC), which already may exist in the tissue<sup>[32]</sup>. Zhang *et al.*<sup>[32]</sup> described the isolation and characterization of such plastic-adherent cells, which possess stem cell characteristic surface markers from healthy tissue samples. However, it is unknown whether these cells might still be present in more degenerated human tissue as analyzed in this study. An additional aspect that should be considered is the donor characteristics of cells (Table 2). In four out of five trials we had male MSC donors and in all five experiments, we had female TC donors (Table 2). The gender and age of the donors may have a hidden effect on the outcomes, including proliferation rates or differentiation ability. However, current evidence of the impact of gender and age is inconclusive<sup>[33,34]</sup>.

### Imaging analysis

Confocal laser scanning microscopy (CLSM) images of CG patches showed less MSCs than TCs, both in the single cell culture of MSCs as well as when cultured together with TCs. The MSCs appear in a rounded shape in the CLSM images, as opposed to the TCs, which have a more elongated appearance. This is in line with the SEM results for the CG patches, on which we found rather roundish morphologies.

There seems a conflict in the data that the cell density on CG patches was decreasing for MSCs (Figure 4 lower panel) but DNA content (Figure 5) and cell activity (Figure 6) showed a steady-state and a trend of increased activity, respectively. We interpret this as a donor-specific phenomenon in the CLSM data.



**Extracellular matrix and collagen patch environment**

GAG and HYP values were also corrected for control values, as we registered significant amounts of initial GAG and HYP in our controls (Figure 7A and 7B), which was expected considering that collagen patches consist primarily of collagen 1 and 3. Correcting for the controls, we see a decrease in GAG per mg of dry-weight. This suggests that the MSCs are changing in phenotype and possibly produce less GAG over time if in direct 1:1 co-culture. Interestingly, comparing the relative change of the HYP/GAG ratio of day 7 relative to day 1 showed a significant effect of “premix” cells but there was no effect of the differences between the two scaffolds (Figure 7). The effect of cells was highly significant in the 2-way ANOVA ( $P = 0.005$ , Figure 7). Thus, premix of cells shifted the ratio between HYP/GAG towards lower production of GAG and slight increase of HYP.

In conclusion, the results of these 3D cell culture experiments demonstrate the potential of collagen patches for ACL repair<sup>[4]</sup>. Using biochemical and imaging methods, we were able to demonstrate that primary TCs and MSCs can both be grown on the NC and CG patches, which were originally designed to induce a chondrogenic phenotype. Both TCs and MSCs adhere to and proliferate on the patches. Moreover, MSCs seem to show a tenogenic differentiation potential on these scaffolds as indicated by the significant increase of collagen type 3. Future work might consider the importance of alternative stimuli to accelerate healing such as the application of platelet rich plasma (PRP) or platelet rich fibrin (PRF). The problem with ACL-derived TCs and MSCs, which have to be expanded first *ex vivo* on plastics, is their clinical approval. With PRP on the other hand are unsolved issues about the reproducibility and the ideal dose of platelet concentrations<sup>[35,36]</sup>.

Further studies should be performed to evaluate additional aspects. First of all, it would be beneficial to run the experiments with a longer culture time. This would permit a more in depth analysis of the induced cell morphologies on the different patches, which could alter the survivability of the cells as well as the differentiation potential. Additionally, it would allow us to observe whether this changes the gene expression profile of the cells. It is also likely that longer culturing duration would lead to more pronounced differences between cell and patch types and be able to further support the trends we have observed so far.

Furthermore, studies should also be performed using mechanical loading. It has been shown that mechanical strain induces MSCs to differentiate into a TCs phenotype<sup>[37,38]</sup>. It would be interesting to see whether the cells respond differently on the two patch types, subjected to equal parameters. Also, the mechanical properties of a scaffold are known to impact cell differentiation. In fact, several prior attempts at ligament or tendon repair have resulted in ectopic bone formation<sup>[30]</sup>. Thus, further experiments

testing the impact of the mechanical properties would be helpful in evaluating the full potential of these patches. However, in these studies, it would be crucial also to look at bone-specific markers such as osteocalcin<sup>[30]</sup>.

In conclusion, while certain aspects should be examined in more depth, the use of collagen scaffolds for ACL regeneration is promising<sup>[4]</sup>. The usage of a biological ACL repair model including collagen patches in a non-loading repair model seems a genius option to improve healing. What remains to be shown is whether mechanical loading improves the healing process or whether platelet rich fibrin PRF or PRP might be another option to be included at the site of rupture for the healing or whether it might be even hindering the healing process as has been recently proposed<sup>[35,36,39,40]</sup>.

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**COMMENTS****Background**

Mesenchymal stem cells (MSCs) and anterior cruciate ligament (ACL)-derived tenocyte (TCs) are both potential source cells to be applied for the cell seeding of the dynamic intraligamentary stabilization for the fixation of ruptured ACLs. Current treatment options for non-fusions of ruptured ACLs are very limited.

**Research frontiers**

ACL injury is a common disease and the repair of ACL remains a challenge due to its limited self-regenerative ability. Despite the progression of reconstructive techniques for ACL repair, an ideal biological scaffold for ACL reconstruction is needed to improve clinical outcome, especially in the case of non-unions.

**Innovations and breakthroughs**

In this study, the authors present novel data on the regenerative technique to use commercially Food and Drug Administration (FDA)-approved collagen 1 and 3 scaffolds along with ACL-derived TCs and MSCs. They provide microscopy imaging data, biochemical data, and gene expression data of human derived primary bone-marrow derived MSCs and ACL-derived tenocytes. There was not much known how cell therapy could accelerate and improve healing for ACL ruptures in combination with a recently developed dynamic anchoring technique called Ligamys®. The combination of regenerative approaches involving TCs and MSCs transplantation along with commercially available FDA-approved collagen patches seems a straightforward approach, as the membrane is highly biocompatible and does not carry the mechanical force.

**Applications**

Cyto-compatibility for the collagen patches is a tool to explain good clinical outcome.

**Peer-review**

This paper compared two different collagen patches seeding with either bone marrow mesenchymal stem cells or primary tenocytes for possible anterior cruciate ligament repair. Chongrogide is composed of porcine collagen I and III as a bilayer membrane. Novocart is a biphasic, three-dimensional collagen-based matrix from bovine. Both collagen sponges have been used for cartilage repair. ACL injury is a common disease and the repair of ACL remains a challenge due to its limited self regenerative ability. Despite the progression of reconstructive techniques for ACL repair, an ideal biological scaffold for ACL reconstruction is needed. Thus, this study is of importance and experiments strongly suggest that the patches of collagen may be useful for anterior cruciate ligament repair.



## REFERENCES

- 1 **Voigt C**, Schönaich M, Lill H. Anterior cruciate ligament reconstruction: state of the art. *Eur J Trauma* 2006; **32**: 332-339 [DOI: 10.1007/s00068-006-6118-x]
- 2 **Vunjak-Novakovic G**, Altman G, Horan R, Kaplan DL. Tissue engineering of ligaments. *Annu Rev Biomed Eng* 2004; **6**: 131-156 [PMID: 15255765 DOI: 10.1146/annurev.bioeng.6.040803.140037]
- 3 **Siegel L**, Vandenakker-Albanese C, Siegel D. Anterior cruciate ligament injuries: anatomy, physiology, biomechanics, and management. *Clin J Sport Med* 2012; **22**: 349-355 [PMID: 22695402 DOI: 10.1097/JSM.0b013e3182580cd0]
- 4 **Kiapour AM**, Murray MM. Basic science of anterior cruciate ligament injury and repair. *Bone Joint Res* 2014; **3**: 20-31 [PMID: 24497504 DOI: 10.1302/2046-3758.32.2000241]
- 5 **Markatos K**, Kaseta MK, Lallios SN, Korres DS, Efsthopoulos N. The anatomy of the ACL and its importance in ACL reconstruction. *Eur J Orthop Surg Traumatol* 2013; **23**: 747-752 [PMID: 23412211 DOI: 10.1007/s00590-012-1079-8]
- 6 **Petrigliano FA**, McAllister DR, Wu BM. Tissue engineering for anterior cruciate ligament reconstruction: a review of current strategies. *Arthroscopy* 2006; **22**: 441-451 [PMID: 16581458 DOI: 10.1016/j.arthro.2006.01.017]
- 7 **Saka T**. Principles of postoperative anterior cruciate ligament rehabilitation. *World J Orthop* 2014; **5**: 450-459 [PMID: 25232521 DOI: 10.5312/wjo.v5.i4.450]
- 8 **Luc B**, Gribble PA, Pietrosimone BG. Osteoarthritis Prevalence Following Anterior Cruciate Ligament Reconstruction: A Systematic Review and Numbers-Needed-to-Treat Analysis. *J Athl Train* 2014; **49**: 806-819 [PMID: 25232663 DOI: 10.4085/1062-6050-49.3.35]
- 9 **Murray MM**, Vavken P, Fleming BC. The ACL Handbook. NY: Springer, 2013
- 10 **Vavken P**, Murray MM. The potential for primary repair of the ACL. *Sports Med Arthrosc* 2011; **19**: 44-49 [PMID: 21293237 DOI: 10.1097/JSA.0b013e3182095e5d]
- 11 **Kohl S**, Evangelopoulos DS, Ahmad SS, Kohlhof H, Herrmann G, Bonel H, Egli S. A novel technique, dynamic intraligamentary stabilization creates optimal conditions for primary ACL healing: a preliminary biomechanical study. *Knee* 2014; **21**: 477-480 [PMID: 24405792 DOI: 10.1016/j.knee.2013.11.003]
- 12 **Egli S**, Kohlhof H, Zumstein M, Henle P, Hartel M, Evangelopoulos DS, Bonel H, Kohl S. Dynamic intraligamentary stabilization: novel technique for preserving the ruptured ACL. *Knee Surg Sports Traumatol Arthrosc* 2014 Mar 21; Epub ahead of print [PMID: 24651979 DOI: 10.1007/s00167-014-2949-x]
- 13 **Kohl S**, Evangelopoulos DS, Kohlhof H, Hartel M, Bonel H, Henle P, von Rechenberg B, Egli S. Anterior cruciate ligament rupture: self-healing through dynamic intraligamentary stabilization technique. *Knee Surg Sports Traumatol Arthrosc* 2013; **21**: 599-605 [PMID: 22437658 DOI: 10.1007/s00167-012-1958-x]
- 14 **Milz S**, Ockert B, Putz R. [Tenocytes and the extracellular matrix : a reciprocal relationship]. *Orthopade* 2009; **38**: 1071-1079 [PMID: 19885652]
- 15 **Lui PP**, Rui YF, Ni M, Chan KM. Tenogenic differentiation of stem cells for tendon repair-what is the current evidence? *J Tissue Eng Regen Med* 2011; **5**: e144-e163 [PMID: 21548133 DOI: 10.1002/term.424]
- 16 **Canseco JA**, Kojima K, Penvose AR, Ross JD, Obokata H, Gomoll AH, Vacanti CA. Effect on ligament marker expression by direct-contact co-culture of mesenchymal stem cells and anterior cruciate ligament cells. *Tissue Eng Part A* 2012; **18**: 2549-2558 [PMID: 22780864 DOI: 10.1089/ten.TEA.2012.0030]
- 17 **Solchaga LA**, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. *J Cell Physiol* 2005; **203**: 398-409 [PMID: 15521064 DOI: 10.1002/jcp.20238]
- 18 **Gantenbein-Ritter B**, Benneker LM, Alini M, Grad S. Differential response of human bone marrow stromal cells to either TGF- $\beta$ (1) or rhGDF-5. *Eur Spine J* 2011; **20**: 962-971 [PMID: 21086000 DOI: 10.1007/s00586-010-1619-z]
- 19 **Xiao J**, Zhang Y, Wang J, Yu W, Wang W, Ma X. Monitoring of cell viability and proliferation in hydrogel-encapsulated system by resazurin assay. *Appl Biochem Biotechnol* 2010; **162**: 1996-2007 [PMID: 20437208 DOI: 10.1007/s12010-010-8975-3]
- 20 **Farndale RW**, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* 1986; **883**: 173-177 [PMID: 3091074]
- 21 **Enobakhare BO**, Bader DL, Lee DA. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. *Anal Biochem* 1996; **243**: 189-191 [PMID: 8954546 DOI: 10.1006/abio.1996.0502]
- 22 **Samiric T**, Parkinson J, Ilic MZ, Cook J, Feller JA, Handley CJ. Changes in the composition of the extracellular matrix in patellar tendinopathy. *Matrix Biol* 2009; **28**: 230-236 [PMID: 19371780 DOI: 10.1016/j.matbio.2009.04.001]
- 23 **Attia E**, Bohnert K, Brown H, Bhargava M, Hannafin JA. Characterization of total and active matrix metalloproteinases-1, -3, and -13 synthesized and secreted by anterior cruciate ligament fibroblasts in three-dimensional collagen gels. *Tissue Eng Part A* 2014; **20**: 171-177 [PMID: 23879595 DOI: 10.1089/ten.TEA.2012.0669]
- 24 **Livak KJ**, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-408 [PMID: 11846609 DOI: 10.1006/meth.2001.1262]
- 25 **Goidin D**, Mamessier A, Staquet M-J, Schmitt D, Berthier-Vergnes O. Ribosomal 18S RNA Prevails over Glyceraldehyde-3-Phosphate Dehydrogenase and [beta]-Actin Genes as Internal Standard for Quantitative Comparison of mRNA Levels in Invasive and Noninvasive Human Melanoma Cell Subpopulations. *Anal Biochem* 2001; **295**: 17-21 [DOI: 10.1006/abio.2001.5171]
- 26 **Pawley J**. Handbook of biological confocal microscopy. NY: Springer, 2010
- 27 **Birmingham E**, Niebur GL, McHugh PE, Shaw G, Barry FP, McNamara LM. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. *Eur Cell Mater* 2012; **23**: 13-27 [PMID: 22241610]
- 28 **Luo Q**, Song G, Song Y, Xu B, Qin J, Shi Y. Indirect co-culture with tenocytes promotes proliferation and mRNA expression of tendon/ligament related genes in rat bone marrow mesenchymal stem cells. *Cytotechnology* 2009; **61**: 1-10 [PMID: 19842053 DOI: 10.1007/s10616-009-9233-9]
- 29 **Pauly S**, Klatte F, Strobel C, Schmidmaier G, Greiner S, Scheibel M, Wildemann B. Characterization of tendon cell cultures of the human rotator cuff. *Eur Cell Mater* 2010; **20**: 84-97 [PMID: 20661865]
- 30 **Kishore V**, Bullock W, Sun X, Van Dyke WS, Akkus O. Tenogenic differentiation of human MSCs induced by the topography of electrochemically aligned collagen threads. *Biomaterials* 2012; **33**: 2137-2144 [PMID: 22177622 DOI: 10.1016/j.biomaterials.2011.11.066]
- 31 **Seiler C**, Gazdhar A, Reyes M, Benneker LM, Geiser T, Siebenrock KA, Gantenbein-Ritter B. Time-lapse microscopy and classification of 2D human mesenchymal stem cells based on cell shape picks up myogenic from osteogenic and adipogenic differentiation. *J Tissue Eng Regen Med* 2014; **8**: 737-746 [PMID: 22815264 DOI: 10.1002/term.1575]
- 32 **Zhang J**, Pan T, Im HJ, Fu FH, Wang JH. Differential properties of human ACL and MCL stem cells may be responsible for their differential healing capacity. *BMC Med* 2011; **9**: 68 [PMID: 21635735 DOI: 10.1186/1741-7015-9-68]
- 33 **Fossett E**, Khan WS, Longo UG, Smitham PJ. Effect of age and gender on cell proliferation and cell surface characterization of synovial fat pad derived mesenchymal stem cells. *J Orthop Res* 2012; **30**: 1013-1018 [PMID: 22228598 DOI: 10.1002/jor.22057]
- 34 **Rust PA**, Kalsi P, Briggs TW, Cannon SR, Blunn GW. Will mesenchymal stem cells differentiate into osteoblasts on allograft? *Clin Orthop Relat Res* 2007; **457**: 220-226 [PMID: 17146367 DOI: 10.1097/BLO.0b013e31802e7e8f]
- 35 **Yoshida R**, Cheng M, Murray MM. Increasing platelet concentration in platelet-rich plasma inhibits anterior cruciate ligament cell function



- in three-dimensional culture. *J Orthop Res* 2014; **32**: 291-295 [PMID: 24122902 DOI: 10.1002/jor.22493]
- 36 **Fleming BC**, Proffen BL, Vavken P, Shalvoy MR, Machan JT, Murray MM. Increased platelet concentration does not improve functional graft healing in bio-enhanced ACL reconstruction. *Knee Surg Sports Traumatol Arthrosc* 2014 Mar 18; Epub ahead of print [PMID: 24633008 DOI: 10.1007/s00167-014-2932-6]
  - 37 **Pietschmann MF**, Frankewycz B, Schmitz P, Docheva D, Sievers B, Jansson V, Schieker M, Müller PE. Comparison of tenocytes and mesenchymal stem cells seeded on biodegradable scaffolds in a full-size tendon defect model. *J Mater Sci Mater Med* 2013; **24**: 211-220 [PMID: 23090834 DOI: 10.1007/s10856-012-4791-3]
  - 38 **Scott A**, Danielson P, Abraham T, Fong G, Sampaio AV, Underhill TM. Mechanical force modulates scleraxis expression in bioartificial tendons. *J Musculoskelet Neuronal Interact* 2011; **11**: 124-132 [PMID: 21625049]
  - 39 **Hutchinson ID**, Rodeo SA, Perrone GS, Murray MM. Can Platelet-Rich Plasma Enhance Anterior Cruciate Ligament and Meniscal Repair? *J Knee Surg* 2015; **28**: 19-28 [PMID: 25101873 DOI: 10.1055/s-0034-1387166]
  - 40 **Murray MM**, Fleming BC. Use of a bioactive scaffold to stimulate anterior cruciate ligament healing also minimizes posttraumatic osteoarthritis after surgery. *Am J Sports Med* 2013; **41**: 1762-1770 [PMID: 23857883 DOI: 10.1177/0363546513483446]

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