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EDITORIAL

Moving forward on the pathway of cell-based therapies in ischemic heart disease and heart failure – time for new recommendations?

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Abstract

Although substantial advances have been made in treating ischemic heart disease and subsequent heart failure, the overall morbidity and mortality from these conditions remain high. Stem cell-based therapy has emerged as a promising approach for prompting cardiac rejuvenation. Various cell types have been tested in the clinical arena, proving consistent safety results. As for efficiency outcomes, contradictory findings have been reported, partly due to inconsistency in study protocols but also due to poor survival, engraftment and differentiation of transplanted cells in the hostile milieu of the ischemic host tissue. Studies have varied in terms of route of delivery, type and dose of implanted stem cells, patient selection and randomization, and assessment of therapeutic effect. Founded on the main achievements and challenges within almost 20 years of research, a number of official documents have been published by leading experts in the field. Core recommendations have focused on developing and optimizing effective strategies to enrich cell retention and their regenerative potential. Issued consensus and position papers have stemmed from an unmet need to provide a harmonized framework for future research, resulting in improved therapeutic application of cell-based therapies for cardiac regeneration and repair.

Key words: Stem cell therapy; Ischemic heart disease; Heart failure; Cardiac regeneration; Recommendations

Core tip: Ischemic heart disease and resulting heart failure remain a major public health problem worldwide in spite of therapeutic progresses. Almost two decades ago, stem cell-based therapy appeared as a promising method to stimulate cardiac regeneration. Based on the main findings and challenges faced during clinical trials within this timeframe, a number of consensus and position papers have been issued by key opinion leaders, with the specific aim to empower cell-based cardiac repair and regeneration in patients with the aforesaid maladies.

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INTRODUCTION

In spite of standard-of-care therapies, ischemic heart disease (IHD) remains one of the leading causes of early death and disease burden worldwide, leading to almost 9 million deaths and 170 million disability-adjusted life years globally in 2017[1]. The poor prognosis is related to the reduced endogenous regenerative ability of the adult human heart. Evidence-based disease management greatly improves patient outcomes, but it does not completely prevent myocyte injury and consequent adverse cardiac remodeling. Ongoing efforts are being made to develop alternative strategies to prompt the restoration of both cardiac structure and function. Advances in understanding stem cell (SC) biology have led to the development of stem cell-based therapy (SCT), which holds high therapeutic promise. The rationale behind SCT is that the supplied cells will facilitate the generation of functional cardiomyocytes and new blood vessels, either by exogenous regenerative responses or by activating endogenous renewal mechanisms[2].

THE PAST

Since the first in-man SCT for IHD[3], a substantial number of clinical trials (CTs) have been finalized and comprehensive reviews and meta-analyzes have been published, yielding inconsistent results[4,5]. But when it comes to papers expressing opinion and recommendations from expert authorities, their number is not so impressive. Since current guidelines on SCT in IHD and heart failure (HF) are lacking, experts in the field provided harmonized statements in order to move forward the clinical application of cell-based therapies for cardiac regeneration and repair; three position papers and two consensus documents have been put out in the last 13 years (Figure 1).

The first document of its kind was presented in 2006, when the Task Force of the European Society of Cardiology (ESC) published a consensus document on the use of autologous cell therapy for repair of the heart[6]. Although the 2006 paper is rather obsolete given the existence of an updated version[7], it has the merit of establishing a framework for upcoming research.

It took a decade until new papers were issued, a timespan in which important data have been offered by completed CTs. Provided recommendations have been formulated to address the main limitations raised within prior hallmark studies, such as reduced survival and engraftment of delivered cells in ischemic myocardium, lack of effective differentiation of adult SCs into mature and functional cardiomyocytes, insufficient activation of resident cardiac SCs, inadequate electrophysiological integration of the implanted cells with native myocardium, and the use of inappropriate end-points for assessing the outcomes of SCT. Hence, continual development of carrier materials and priming strategies (such as genetic and pharmacological modification) to improve SC retention, survival and differentiation has been recommended. A particularly important aspect is related to the type of SC to be transplanted, which should be carefully chosen. Due to adverse events, the skeletal myoblast is no longer of interest. First-generation cells, such as bone marrow - derived mononuclear cells (commonly referred to as BM-MNCs) or mesenchymal stem cells (MSCs), are considered to prompt endogenous repair mechanisms, while second-generation cells, such as pluripotent SCs and cardiac stem cells/cardiac progenitor cells (CSCs/CPCs), are believed to hold exogenous regenerative potential and actually replace the injured myocardium. Therefore, diverse cell types or a mixture of cell types have been suggested to be tested in randomized CTs. Nevertheless, possible confounders such as gender, age, comorbidities, and daily medications, should permanently be taken into account. Last but not least, the necessity of employing "hard clinically meaningful endpoints" to determine the actual impact on disease burden has been emphasized[2,7,8].

Notably, disease-specific recommendations have been also envisaged[7]. On the

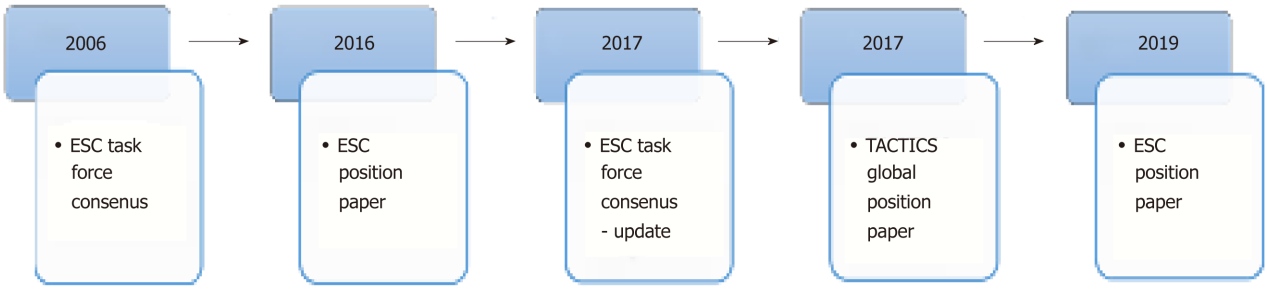

Figure 1 Timeline of expert opinions in cell-based therapies for cardiac regeneration and repair. ESC: European Society of Cardiology; TACTICS: Transnational AllianCe for regenerative Therapies In Cardiovascular Syndromes.

subject of launching additional autologous bone marrow cell CTs in acute myocardial infarction settings, the consensus was to await results from the BAMI trial[9]. BAMI was designed as the largest phase III randomized CT with the precise goal to provide a conclusive answer whether BM-MNCs plus standard of care therapy can lead to a 25% reduction in mortality when compared to best medical care alone[10]. It is to emphasize that the study protocol was substantially revised, as the accrual rate was significantly impaired (375 randomized patients instead of the initial target of 3000 patients). Still, the results are eagerly awaited, and the study is being reconsidered as an estimation trial with the aim to assess the treatment effect and event rates in the SCT group. Until now, successful standardization of the bone marrow procurement and cell manufacturing technique has been reported. The full findings will be released after October 2019 (the estimated study completion date). In regard to SCT in chronic HF, the recommendation to use cardiopoietic cells - either primary or engineered - is reiterated. In view of the recognized safety of SCT, repeated administration should be planned in order to achieve improved long-term clinical outcome[7].

Of note, some of the aforementioned recommendations have already been translated into practice. For example, Bartunek and colleagues used a combination of cardiogenic growth factors to direct patient-derived MSCs toward a cardiopoietic phenotype[11,12]. Lineage specified MSCs proved to exert beneficial effects on cardiac remodeling, exercise capacity and quality of life[13,14].

Furthermore, a four-arm randomized CT has been designed with the aim to compare the restorative capacity of autologous bone marrow-derived MSCs and c-kit$^+$ CPCs, either alone or in combination, in patients with ischemic HF. The estimated study completion date is May 2020[15].

While the aforesaid studies employed adult SCs, there was a single CT that used human embryonic SC-derived CD15+ Isl-1+ progenitors to treat patients with severe ischemic HF (the ESCORT study)[16]. Regardless of study limitations (*i.e.* small sample size, lack of blinded assessment, confounding effect of the concomitant coronary artery bypass grafting), the trial provided proof of concept for further robust studies.

THE PRESENT

In light of today's knowledge, the very recent document published on behalf of ESC has focused on strategies to boost cell delivery and retention within native area by combined administration of cells, biologically active molecules and bio-materials (*e.g.*, hydrogels, cell sheets, prefabricated matrices, microspheres, and injectable matrices)[17]. Cutting-edge tissue engineering (TE) approaches have been shown to increase the long-term cell retention of more than 80%, and for that reason they have emerged as valuable tools to advance cell therapies for IHD and HF. The use of materials that do not trigger inflammatory or foreign body responses (such as naturally derived polymers with an anti-inflammatory activity, extracellular matrix components, and materials with controlled release of anti-inflammatory/immunosuppressive molecules) is favored.

Also, the therapeutic potential held by human induced pluripotent stem cells (hiPSCs) is emphasized. Preclinical research revealed that transplanted hiPSC-derived cardiomyocytes (hiPSC-CMs) were able to persist, mature and proliferate within the host myocardium, causing improved cardiac function in recipient animals[18-20]. For an enhanced regenerative outcome, combinations of cells and bio-materials have been employed. For example, in a porcine ischemic cardiomyopathy model, transplantation of hiPSC-CMs cell sheets together with an omentum flap as a source of blood supply

yielded better results compared with hiPSC-CM administration alone[21]. Similarly, co-transplantation of multiple hiPSC-derived cardiovascular cell types (*i.e.* cardiomyocytes, endothelial cells and smooth muscle cells) with a 3D fibrin patch impregnated with a pro-survival factor resulted in reduced cardiomyocyte apoptosis, diminished infarct size, and improved cardiac function[22]. However, the use of iPSC-CMs is not without risks (*i.e.* graft-related arrhythmias). For a safe and effective iPSC-based therapy, targeted cardiomyocyte subtype specification and functional maturation are of the essence. Accordingly, sustained efforts have been made to attain specialized, mature hiPSC-CM phenotypes, which could be further used for human engineered heart muscle constructs[23-25].

Another topic evoked by the authors of the ESC position paper refers to prompting cardiac regeneration by cell-free *in situ* strategies, such as injection of materials containing instructive signals for cardiac cell reprogramming or SC-derived secretome survival factors. In particular, direct cellular reprogramming of cardiac fibroblasts seems most appealing, given their abundance in infarcted myocardium. Indeed, prior studies have demonstrated that fibroblasts can be driven directly into cardiomyocytes by distinct combinations of lineage-significant transcription factors or microRNAs[26-30]. Of note, induced *in situ* fibroblast reprogramming improved cardiac function in animal myocardial infarction models, with 30%-40% increase in left ventricle ejection fraction and reduction of fibrotic scar by up to 50%[31].

At present, with very few exceptions (Table 1[16,32-34]), the use of tissue-engineered constructs for myocardial regeneration is still in the preclinical phase. To expedite TE and cell-based therapies for cardiac repair, the experts from the ESC Working Group on Cellular Biology of the Heart have issued several key statements. Hence, more effective TE strategies to increase cell retention should be further developed and optimized (including 3D printing to augment the biological ability of TE products). Of note, the whole fabrication of products should be conducted in agreement with regulatory demands, comprising proof of concept in rodent and large animal models[17].

THE FUTURE

With BAMI's results being expected to be released and pluripotent SC-cardiac derivatives entering the clinical arena, it seems like these are exciting times for mending broken hearts. Hence, one can only ask oneself: What next?

Table 1 Ongoing / completed human clinical trials for cardiac tissue engineering

Clinical trial name	Trial identifier	Target sample size	Status
Epicardial Infarct Repair Using CorMatrix®-ECM: Clinical Feasibility Study (EIR)	ClinicalTrials.gov Identifier: NCT02887768[32]	8	Completed
Transplantation of Human Embryonic Stem Cell-derived Progenitors in Severe Heart Failure (ESCORT)	ClinicalTrials.gov Identifier: NCT02057900[16]	10	Completed
Myocardial Assistance by Grafting a New Bioartificial Upgraded Myocardium (MAGNUM Trial)	-	20	Completed
A Study of VentriGel in Post-MI Patients	ClinicalTrials.gov Identifier: NCT02305602[33]	15	Active, not recruiting
Clinical trial of human (allogeneic) induced pluripotent stem cell-derived cardiomyocyte sheet for severe cardiomyopathy	UMIN-CTR Clinical Trial ID: UMIN000032989[34]	3	Not yet recruiting

REFERENCES

1 **GBD 2017 Disease and Injury Incidence and Prevalence Collaborators**. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* 2018; **392**: 1789-1858 [PMID: 30496104 DOI: 10.1016/S0140-6736(18)32279-7]

2 **Fernández-Avilés F**, Sanz-Ruiz R, Climent AM, Badimon L, Bolli R, Charron D, Fuster V, Janssens S, Kastrup J, Kim HS, Lüscher TF, Martin JF, Menasché P, Simari RD, Stone GW, Terzic A, Willerson JT, Wu JC; TACTICS (Transnational Alliance for Regenerative Therapies in Cardiovascular Syndromes) Writing Group; Authors/Task Force Members. Chairpersons; Basic Research Subcommittee; Translational Research Subcommittee; Challenges of Cardiovascular Regenerative Medicine Subcommittee; Tissue Engineering Subcommittee; Delivery, Navigation, Tracking and Assessment Subcommittee; Clinical Trials Subcommittee; Regulatory and funding strategies subcommittee; Delivery, Navigation, Tracking and Assessment Subcommittee. Global position paper on cardiovascular regenerative medicine. *Eur Heart J* 2017; **38**: 2532-2546 [PMID: 28575280 DOI: 10.1093/eurheartj/ehx248]

3 **Strauer BE**, Brehm M, Zeus T, Gattermann N, Hernandez A, Sorg RV, Kögler G, Wernet P. Intracoronary, human autologous stem cell transplantation for myocardial regeneration following myocardial infarction. *Dtsch Med Wochenschr* 2001; **126**: 932-938 [PMID: 11523014 DOI: 10.1055/s-2001-16579-2]

4 **Dorobantu M**, Popa-Fotea NM, Popa M, Rusu I, Micheu MM. Pursuing meaningful end-points for stem cell therapy assessment in ischemic cardiac disease. *World J Stem Cells* 2017; **9**: 203-218 [PMID: 29321822 DOI: 10.4252/wjsc.v9.i12.203]

5 **Micheu MM**, Dorobantu M. Fifteen years of bone marrow mononuclear cell therapy in acute myocardial infarction. *World J Stem Cells* 2017; **9**: 68-76 [PMID: 28491241 DOI: 10.4252/wjsc.v9.i4.68]

6 **Bartunek J**, Dimmeler S, Drexler H, Fernández-Avilés F, Galinanes M, Janssens S, Martin J, Mathur A, Menasche P, Priori S, Strauer B, Tendera M, Wijns W, Zeiher A; task force of the European Society of Cardiology. The consensus of the task force of the European Society of Cardiology concerning the clinical investigation of the use of autologous adult stem cells for repair of the heart. *Eur Heart J* 2006; **27**: 1338-1340 [PMID: 16543252 DOI: 10.1093/eurheartj/ehi793]

7 **Mathur A**, Fernández-Avilés F, Dimmeler S, Hauskeller C, Janssens S, Menasche P, Wojakowski W, Martin JF, Zeiher A; BAMI Investigators. The consensus of the Task Force of the European Society of Cardiology concerning the clinical investigation of the use of autologous adult stem cells for the treatment of acute myocardial infarction and heart failure: update 2016. *Eur Heart J* 2017; **38**: 2930-2935 [PMID: 28204458 DOI: 10.1093/eurheartj/ehw640]

8 **Madonna R**, Van Laake LW, Davidson SM, Engel FB, Hausenloy DJ, Lecour S, Leor J, Perrino C, Schulz R, Ytrehus K, Landmesser U, Mummery CL, Janssens S, Willerson J, Eschenhagen T, Ferdinandy P, Sluijter JP. Position Paper of the European Society of Cardiology Working Group Cellular Biology of the Heart: cell-based therapies for myocardial repair and regeneration in ischemic heart disease and heart failure. *Eur Heart J* 2016; **37**: 1789-1798 [PMID: 27055812 DOI: 10.1093/eurheartj/ehw113]

9 **Mathur A**. The Effect of Intracoronary Reinfusion of Bone Marrow-derived Mononuclear Cells(BM-MNC) on All Cause Mortality in Acute Myocardial Infarction. [accessed. 2019; ClinicalTrials.gov [Internet]. United States National Library of Medicine Available from: http://clinicaltrials.gov/show/ NCT01569178 ClinicalTrials.gov Identifier: NCT01569178

10 **Mathur A**, Arnold R, Assmus B, Bartunek J, Belmans A, Bönig H, Crea F, Dimmeler S, Dowlut S, Fernández-Avilés F, Galiñanes M, Garcia-Dorado D, Hartikainen J, Hill J, Hogardt-Noll A, Homsy C, Janssens S, Kala P, Kastrup J, Martin J, Menasche P, Miklik R, Mozid A, San Román JA, Sanz-Ruiz R, Tendera M, Wojakowski W, Ylä-Herttuala S, Zeiher A. The effect of intracoronary infusion of bone marrow-derived mononuclear cells on all-cause mortality in acute myocardial infarction: rationale and design of the BAMI trial. *Eur J Heart Fail* 2017; **19**: 1545-1550 [PMID: 28948706 DOI: 10.1002/ejhf.829]

11 **Bartunek J**, Terzic A. C-Cure Clinical Trial. [accessed. 2019; ClinicalTrials.gov [Internet]. United States National Library of Medicine Available from: http://clinicaltrials.gov/show/ NCT00810238 ClinicalTrials.gov Identifier: NCT00810238

12 **Terzic A**, Bartunek J. Safety and Efficacy of Autologous Cardiopoietic Cells for Treatment of Ischemic Heart Failure. [accessed. 2019; ClinicalTrials.gov [Internet]. United States National Library of Medicine Available from: http://clinicaltrials.gov/show/ NCT01768702 ClinicalTrials.gov Identifier: NCT01768702

13 **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, Homsy 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]

14 **Bartunek J**, Terzic A, Davison BA, Filippatos GS, Radovanovic S, Beleslin B, Merkely B, Musialek P, Wojakowski W, Andreka P, Horvath IG, Katz A, Dolatabadi D, El Nakadi B, Arandjelovic A, Edes I, Seferovic PM, Obradovic S, Vanderheyden M, Jagic N, Petrov I, Atar S, Halabi M, Gelev VL, Shochat MK, Kasprzak JD, Sanz-Ruiz R, Heyndrickx GR, Nyolczas N, Legrand V, Guédès A, Heyse A, Moccetti T, Fernandez-Aviles F, Jimenez-Quevedo P, Bayes-Genis A, Hernandez-Garcia JM, Ribichini F, Gruchala M, Waldman SA, Teerlink JR, Gersh BJ, Povsic TJ, Henry TD, Metra M, Hajjar RJ, Tendera M, Behfar A, Alexandre B, Seron A, Stough WG, Sherman W, Cotter G, Wijns W; CHART Program. Cardiopoietic cell therapy for advanced ischaemic heart failure: results at 39 weeks of the prospective, randomized, double blind, sham-controlled CHART-1 clinical trial. *Eur Heart J* 2017; **38**: 648-660 [PMID: 28025189 DOI: 10.1093/eurheartj/ehw543]

15 **Bolli R**, Hare JM, March KL, Pepine CJ, Willerson JT, Perin EC, Yang PC, Henry TD, Traverse JH, Mitrani RD, Khan A, Hernandez-Schulman I, Taylor DA, DiFede DL, Lima JAC, Chugh A, Loughran J, Vojvodic RW, Sayre SL, Bettencourt J, Cohen M, Moyé L, Ebert RF, Simari RD; Cardiovascular Cell Therapy Research Network (CCTRN). Rationale and Design of the CONCERT-HF Trial (Combination of Mesenchymal and c-kit+ Cardiac Stem Cells As Regenerative Therapy for Heart Failure). *Circ Res* 2018; **122**: 1703-1715 [PMID: 29703749 DOI: 10.1161/CIRCRESAHA.118.312978]

16 **Menasché P**. *Transplantation of Human Embryonic Stem Cell-derived Progenitors in Severe Heart Failure. [accessed* 2019; ClinicalTrials.gov [Internet]. United States National Library of Medicine Available from: http://clinicaltrials.gov/show/ NCT02057900 ClinicalTrials.gov Identifier: NCT02057900

17 **Madonna R**, Van Laake LW, Botker HE, Davidson SM, De Caterina R, Engel FB, Eschenhagen T, Fernandez-Aviles F, Hausenloy DJ, Hulot JS, Lecour S, Leor J, Menasché P, Pesce M, Perrino C, Prunier F, Van Linthout S, Ytrehus K, Zimmermann WH, Ferdinandy P, Sluijter JPG. ESC Working Group on Cellular Biology of the Heart: position paper for Cardiovascular Research: tissue engineering strategies combined with cell therapies for cardiac repair in ischaemic heart disease and heart failure. *Cardiovasc Res* 2019; **115**: 488-500 [PMID: 30657875 DOI: 10.1093/cvr/cvz010]

18 **Funakoshi S**, Miki K, Takaki T, Okubo C, Hatani T, Chonabayashi K, Nishikawa M, Takei I, Oishi A, Narita M, Hoshijima M, Kimura T, Yamanaka S, Yoshida Y. Enhanced engraftment, proliferation, and therapeutic potential in heart using optimized human iPSC-derived cardiomyocytes. *Sci Rep* 2016; **6**: 19111 [PMID: 26743035 DOI: 10.1038/srep19111]

19 **Shiba Y**, Gomibuchi T, Seto T, Wada Y, Ichimura H, Tanaka Y, Ogasawara T, Okada K, Shiba N, Sakamoto K, Ido D, Shiina T, Ohkura M, Nakai J, Uno N, Kazuki Y, Oshimura M, Minami I, Ikeda U. Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. *Nature* 2016; **538**: 388-391 [PMID: 27723741 DOI: 10.1038/nature19815]

20 **Rojas SV**, Kensah G, Rotaermel A, Baraki H, Kutschka I, Zweigerdt R, Martin U, Haverich A, Gruh I, Martens A. Transplantation of purified iPSC-derived cardiomyocytes in myocardial infarction. *PLoS One* 2017; **12**: e0173222 [PMID: 28493867 DOI: 10.1371/journal.pone.0173222]

21 **Kawamura M**, Miyagawa S, Fukushima S, Saito A, Miki K, Funakoshi S, Yoshida Y, Yamanaka S, Shimizu T, Okano T, Daimon T, Toda K, Sawa Y. Enhanced Therapeutic Effects of Human iPS Cell Derived-Cardiomyocyte by Combined Cell-Sheets with Omental Flap Technique in Porcine Ischemic Cardiomyopathy Model. *Sci Rep* 2017; **7**: 8824 [PMID: 28821761 DOI: 10.1038/s41598-017-08869-z]

22 **Ye L**, Chang YH, Xiong Q, Zhang P, Zhang L, Somasundaram P, Lepley M, Swingen C, Su L, Wendel JS, Guo J, Jang A, Rosenbush D, Greder L, Dutton JR, Zhang J, Kamp TJ, Kaufman DS, Ge Y, Zhang J. Cardiac repair in a porcine model of acute myocardial infarction with human induced pluripotent stem cell-derived cardiovascular cells. *Cell Stem Cell* 2014; **15**: 750-761 [PMID: 25479750 DOI: 10.1016/j.stem.2014.11.009]

23 **Lee JH**, Protze SI, Laksman Z, Backx PH, Keller GM. Human Pluripotent Stem Cell-Derived Atrial and Ventricular Cardiomyocytes Develop from Distinct Mesoderm Populations. *Cell Stem Cell* 2017; **21**: 179-194.e4 [PMID: 28777944 DOI: 10.1016/j.stem.2017.07.003]

24 **Lemme M**, Ulmer BM, Lemoine MD, Zech ATL, Flenner F, Ravens U, Reichenspurner H, Rol-Garcia M, Smith G, Hansen A, Christ T, Eschenhagen T. Atrial-like Engineered Heart Tissue: An In Vitro Model of the Human Atrium. *Stem Cell Reports* 2018; **11**: 1378-1390 [PMID: 30416051 DOI: 10.1016/j.stemcr.2018.10.008]

25 **Zhao Y**, Rafatian N, Feric NT, Cox BJ, Aschar-Sobbi R, Wang EY, Aggarwal P, Zhang B, Conant G, Ronaldson-Bouchard K, Pahnke A, Protze S, Lee JH, Davenport Huyer L, Jekic D, Wickeler A, Naguib HE, Keller GM, Vunjak-Novakovic G, Broeckel U, Backx PH, Radisic M. A Platform for Generation of Chamber-Specific Cardiac Tissues and Disease Modeling. *Cell* 2019; **176**: 913-927.e18 [PMID: 30686581 DOI: 10.1016/j.cell.2018.11.042]

26 **Qian L**, Huang Y, Spencer CI, Foley A, Vedantham V, Liu L, Conway SJ, Fu JD, Srivastava D. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature* 2012; **485**: 593-598 [PMID: 22522929 DOI: 10.1038/nature11044]

27 **Song K**, Nam YJ, Luo X, Qi X, Tan W, Huang GN, Acharya A, Smith CL, Tallquist MD, Neilson EG, Hill JA, Bassel-Duby R, Olson EN. Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature* 2012; **485**: 599-604 [PMID: 22660318 DOI: 10.1038/nature11139]

28 **Addis RC**, Epstein JA. Induced regeneration--the progress and promise of direct reprogramming for heart repair. *Nat Med* 2013; **19**: 829-836 [PMID: 23836233 DOI: 10.1038/nm.3225]

29 **Jayawardena TM**, Egemnazarov B, Finch EA, Zhang L, Payne JA, Pandya K, Zhang Z, Rosenberg P, Mirotsou M, Dzau VJ. MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. *Circ Res* 2012; **110**: 1465-1473 [PMID: 22539765 DOI: 10.1161/CIRCRESAHA.112.269035]

30 **Jayawardena TM**, Finch EA, Zhang L, Zhang H, Hodgkinson CP, Pratt RE, Rosenberg PB, Mirotsou M, Dzau VJ. MicroRNA induced cardiac reprogramming in vivo: evidence for mature cardiac myocytes and improved cardiac function. *Circ Res* 2015; **116**: 418-424 [PMID: 25351576 DOI: 10.1161/CIRCRESAHA.116.304510]

31 **Rosengart TK**, Patel V, Sellke FW. Cardiac stem cell trials and the new world of cellular reprogramming: Time to move on. *J Thorac Cardiovasc Surg* 2018; **155**: 1642-1646 [PMID: 29397153 DOI: 10.1016/j.jtcvs.2017.11.104]

32 **Fedak PW**. Epicardial Infarct Repair Using CorMatrixÂ®-ECM: Clinical Feasibility Study. [accessed 2019 May 30]. In: ClinicalTrials.gov [Internet]. United States National Library of Medicine. Available from: URL: http://clinicaltrials.gov/show/ NCT02887768 ClinicalTrials.gov Identifier: NCT02887768

33 **Ventrix Inc**. A Study of VentriGel in Post-MI Patients. [accessed 2019 May 30]. In: ClinicalTrials.gov [Internet]. United States National Library of Medicine. Available from: URL: http://clinicaltrials.gov/show/ NCT02305602 ClinicalTrials.gov Identifier: NCT02305602

34 **Sawa Y**. Clinical trial of human (allogeneic) induced pluripotent stem cell-derived cardiomyocyte sheet for severe cardiomyopathy. [accessed 2019 May 30]. In: upload.umin.ac.jp [Internet]. Available from: URL: https://upload.umin.ac.jp/ R000037108 UMIN-CTR Clinical Trial ID: UMIN000032989

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OPINION REVIEWS

Neural regeneration by regionally induced stem cells within post-stroke brains: Novel therapy perspectives for stroke patients

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Abstract

Ischemic stroke is a critical disease which causes serious neurological functional loss such as paresis. Hope for novel therapies is based on the increasing evidence of the presence of stem cell populations in the central nervous system (CNS) and the development of stem-cell-based therapies for stroke patients. Although mesenchymal stem cells (MSCs) represented initially a promising cell source, only a few transplanted MSCs were present near the injured areas of the CNS. Thus, regional stem cells that are present and/or induced in the CNS may be ideal when considering a treatment following ischemic stroke. In this context, we have recently showed that injury/ischemia-induced neural stem/progenitor cells (iNSPCs) and injury/ischemia-induced multipotent stem cells (iSCs) are present within post-stroke human brains and post-stroke mouse brains. This indicates that iNSPCs/iSCs could be developed for clinical applications treating patients with stroke. The present study introduces the traits of mouse and human iNSPCs, with a focus on the future perspective for CNS regenerative therapies using novel iNSPCs/iSCs.

Key words: Ischemic stroke; Stroke patients; Central nervous system; Neural stem/progenitor cells; Multipotent stem cells; Stem-cell-based therapies

Core tip: Ischemic stroke is a critical disease that is accompanied by serious symptoms, such as paresis. Until recently, it was believed that areas affected by stroke mainly

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consist of necrotic and inflammatory cells. However, we have recently demonstrated that novel ischemia-induced stem cells can be isolated from not only mouse brains after stroke but also human brains after stroke. These stem cells exhibited the multipotency and differentiated into electrophysiologically functional neurons. In this article, we introduce the future perspectives for patients suffering from ischemic stroke using these regionally derived stem cells.

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INTRODUCTION

Cerebrovascular diseases, including stroke, are a leading cause of death worldwide. Owing to recent therapeutic advances such as reperfusion therapies by intravenous administration of recombinant tissue plasminogen activator (IV t-PA) and neuroendovascular treatment, including mechanical thrombectomy[1-3], some patients can recover from stroke without sequelae. With the increased implementation of these therapies, it is speculated that more stroke patients can benefit from them. In addition, the therapeutic time window of IV t-PA was extended to 4.5 h[4]. Moreover, there is a possibility, when guided by imaging, for the IV t-PA indication to be expanded in patients with acute ischemic stroke of unknown onset[5]. As for mechanical thrombectomy, the therapeutic time window was expanded up to 16 h from onset or to 24 h if the acute stroke patients had a mismatch between the ischemic core and hypoperfusion area[6,7]. However, many patients with stroke are not eligible for these therapies because of excluding factors (*e.g.*, time after onset and portion of vascular obstruction). Currently, approximately 13%-20% of acute ischemic stroke patients are potentially eligible for mechanical thrombectomy[7,8]. In patients who had mechanical thrombectomy, the rate of good clinical outcome was below 50%[3]. Alternatively, patients receive rehabilitation, but many continue to suffer from various sequelae such as paresis.

Thus, more attention is paid to reparative medicines, particularly to those based on stem cell therapies. Various types of stem cells, including neural stem/progenitor cells (NSPCs)[9-12], mesenchymal stem cells (MSCs)[13,14] (*e.g.*, bone marrow-derived MSCs, adipose-derived MSCs [15,16]), embryonic stem (ES) cell-derived NSPCs[17], and induced pluripotent stem (iPS) cell-derived NSPCs[18], are considered as candidates for cell transplantation following ischemic stroke.

Although the central nervous system (CNS), brain and spinal cord, was long considered not to have regeneration potential after injury, accumulating evidence indicate that the adult CNS contains NSPCs[19,20]. Therefore, CNS repair might be achieved through endogenous stem cells. However, no concrete evidence showing that stem-cell-based therapies by NSPCs are clinically useful for patients with various CNS diseases, including stroke, was reported. Although the reason remains unclear, increasing evidence shows that the traits of not only stem cells themselves but also a stem-cell niche surrounding stem cells (*e.g.*, endothelial cells) alter after ischemia/hypoxia and differ among the developing ages of mice in the CNS[21-24]. Thus, the lack of data may be due to the NSPCs being derived not from pathological but from normal conditions (*e.g.*, developmental fetal NSPCs)[9,10] and investigation having focused on the reparative mechanism not emerging from the pathological CNS.

INSPCS/ISCS DERIVED FROM MICE ISCHEMIC BRAINS

In our laboratory, we aimed to develop a method to isolate and utilize endogenous NSPCs specifically induced by brain injury such as ischemic stroke (injury/ischemia-induced NSPC; iNSPC). We used a mouse model of cerebral infarction whose post-ischemic areas were highly reproducible[25,26]. As a result, we demonstrated for the first time that, although mature neural cells such as neurons, astrocytes, and oligodendrocytes underwent cell death within ischemic regions, iNSPCs that had the

potential to differentiate into these cells developed within the same areas[27]. In addition, we have shown that activation of iNSPCs promoted neural repair and functional recovery following ischemic stroke[22,28].

BRAIN PERICYTES FOLLOWING ISCHEMIA: DO THEY FUNCTION AS NSPCS?

Many types of cells, including astrocytes in the subventricular zone (SVZ)[29,30], reactive astrocytes[31], resident glia[32], oligodendrocyte precursor cells (OPCs)[33,34], and ependymal cells[35,36], have been reported as NSPC candidates. Although the origin of iNSPCs remains unclear, previous studies showed that several types of NSPCs such as SVZ astrocytes[37,38] and OPCs[39,40] reside near blood vessels, in close association with endothelial cells. We have previously shown that nestin$^+$ iNSPCs within ischemic areas express various pericyte markers such as platelet-derived growth factor receptor beta (PDGFRβ), neuronal/glial 2 (NG2), and alpha smooth muscle actin (αSMA)[21,24,41]. Importantly, nestin$^+$ cells were absent from non-ischemic areas in the cortex of adult mice, indicating that normal pericytes in the adult brain do not express nestin. Thus, we proposed that brain pericytes, localized near blood vessels, are potentially giving rise to iNSPCs after injuries such as ischemic stroke[24,42].

Pericytes are localized near blood vessels and form a neurovascular unit (NVU) together with endothelial cells and neural lineage cells (neurons and astrocytes). Pericytes are heterogeneous cells: although PDGFRβ, NG2, nestin,αSMA, CD146, Glast, Tbx18, and regulator of G protein signaling 5[24,43-51] are expressed on pericytes, none of those are specific markers. Birbrair *et al*[44] divided skeletal-muscle-derived pericytes into two subtypes (nestin$^-$/NG2$^+$ type-1 pericytes and nestin$^+$/NG2$^+$ type-2 pericytes). Using their proposed categorization, iNSPCs would be classified as type-2 pericytes as they express both nestin and NG2. In addition, Birbrair *et al*[52] reported that nestin$^+$/NG2$^+$ type-2 pericytes have NG2$^+$ glia-like traits. However, NG2$^+$ glia is identical to OPCs[53], and both pericytes and OPCs express common markers, including NG2 and PDGFRα[54]. Thus, the precise connection between iNSPCs and resident glia should be determined in further studies (Figure 1).

BRAIN PERICYTES FOLLOWING ISCHEMIA: DO THEY FUNCTION AS MULTIPOTENT STEM CELLS?

Brain pericytes are a key component of the NVU and play an important role in maintaining this unit[55]. Even after severe stress such as ischemic stroke, cells forming the NVU, including pericytes[42] and endothelial cells[23], survive, suggesting that these cells play an essential role under pathological conditions as well as under normal conditions.

Besides endothelial cells[56-59], pericytes possess plasticity[54,60] and function as multipotent stem cells as well[43,44,47,61-67]. Therefore, we investigated whether iNSPCs maintain their multipotency under pathological conditions. We found out that iNSPCs can differentiate into not only neural but also mesenchymal lineages, including osteoblasts, adipocytes, and chondrocytes[21,41]. Thus, under ischemic conditions following stroke, brain pericytes might convert into injury/ischemia-induced multipotent stem cells (iSCs) by acquiring the stemness, thereby producing iNSPCs (Figure 1). Consistent with our previous reports[21,41], using a mouse model of cerebral infarction, other groups have also shown that brain pericytes following ischemia display the potential to differentiate into multilineage cells[68]. We also showed that iSCs share angioblast features and give rise to hematopoietic cell lineages such as microglia[21,41]. Consistent with these reports, a recent study showed that brain pericytes and endothelial cells share certain traits[69]. Interestingly, a subtype of pericytes was reported to be derived from hematopoietic lineages, including microglia[70-72]. Thus, the relationship among iSCs, pericytes, and hematopoietic lineages remains to be elucidated in future studies.

It remains unclear whether brain pericytes behave as multipotent stem cells *in vivo*. Ideally, this should be clarified in mice using pericyte markers. A recent study using genetic mapping by the Cre-loxP system failed to demonstrate that Tbx18$^+$ brain pericytes function as multipotent stem cells *in vivo* following mild injury, although they behave as multipotent stem cells *in vitro*[50]. However, phenotypes of cells expressing certain genes (*e.g.*, nestin) in transgenic mice differ depending on the intron regions in which a tag (*e.g.*, green fluorescent protein) is inserted[73-75]. Accumulating evidence also shows that genetic mapping techniques by the Cre-loxP

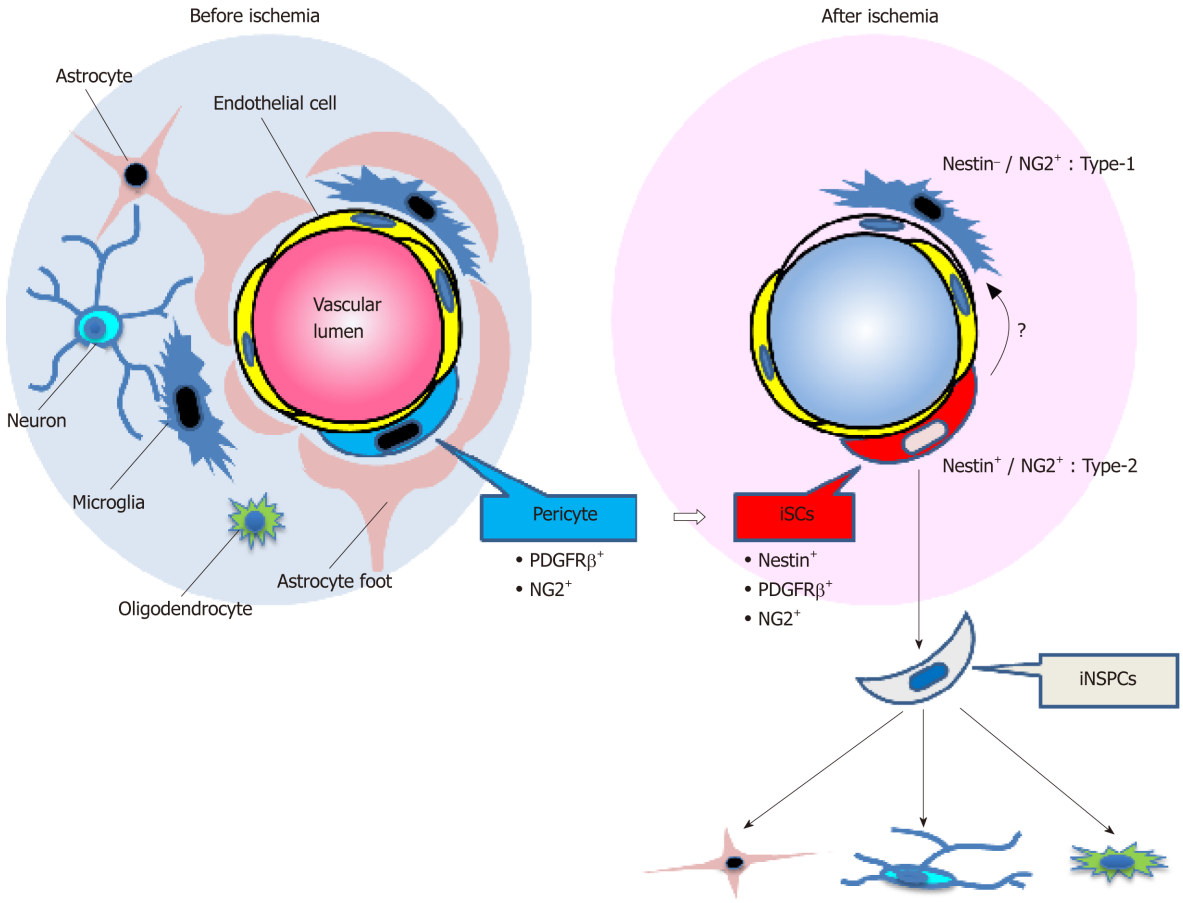

Figure 1 Schematic representation of the fate of injury/ischemia-induced multipotent stem cells and injury/ischemia-induced neural stem/progenitor cells following ischemic stroke. Under ischemic conditions following stroke, brain pericytes, which constitute the neurovascular unit together with endothelial cells and neural lineage cells, may convert into induced multipotent stem cells (iSCs) by acquiring stemness. iSCs may generate induced neural stem/progenitor cells, which have the potential to differentiate into various neural lineage cells, including neurons, astrocytes, and oligodendrocytes. NG2: Neuronal/glial 2; iSCs: Injury/ischemia-induced multipotent stem cells; PDGFRβ: Platelet-derived growth factor receptor beta; iNSPCs: Injury/ischemia-induced neural stem/progenitor cells.

system present several pitfalls[76-78]. For example, gene expression patterns and localizations of certain genes (*e.g.*, nestin) are different depending on the reporter mice used for crossbreeding[78]. Additionally, recombination efficiency following tamoxifen treatment differs among the developing stages of mice[77]. Furthermore, we have previously demonstrated that induction of iNSPCs/iSCs varies with the degree of ischemic stimuli and that a severe injury is essential for inducing iNSPCs/iSCs[42]. Therefore, whether brain pericytes function as multipotent stem cells following injury *in vivo* should be carefully investigated in further studies.

Moreover, to confirm that iSCs are multipotent, it is necessary to show that iSCs derived from a single-cell type can differentiate into multiple cell types. We previously proposed that iSCs might be composed of subpopulations each specifically differentiating into neural or mesenchymal lineages[79]. If so, these subpopulations once isolated could be useful for clinical applications. For example, the subpopulation that can predominantly differentiate into neuronal lineages would be used for neural repair following CNS injuries. However, the precise relations between iNSPCs and iSCs should be clarified in further studies (Figure 1).

BRAIN PERICYTES FOLLOWING ISCHEMIA: HOW DO THEY ACQUIRE THE STEMNESS?

Although the mechanism by which brain pericytes acquire multipotency under ischemic conditions remains unclear, we have previously demonstrated that brain pericytes display up-regulated expression of various stem cell and undifferentiated cell markers when they are incubated under oxygen–glucose deprivation (OGD) that mimics ischemia/hypoxia[21,41]. In general, pericytes have the characteristics of mesenchymal lineages, and NSPCs have traits of epithelial lineages. Following OGD stimuli, we showed that the mesenchymal-epithelial transition (MET) was facilitated

in brain pericytes as demonstrated by the up-regulated expression of the *Sox2* gene[21,41].

These findings suggest that iNSPCs/iSCs are derived from brain PCs having developed stemness through cellular reprogramming and MET. In support of this viewpoint, accumulating evidence shows that brain PCs reprogrammed by gene transduction (*e.g.*, *Sox2* gene) acquire neural lineage traits, including NSPC and neuron phenotypes[48,80].

In addition to the NSPC marker nestin, iNSPCs/iSCs express various stem cell and undifferentiated cell markers, including Sox2, Nanog, c-myc, and Klf4. However, iNSPCs/iSCs lack *Oct 3/4* gene expression, which is essential in producing iPS cells[21,24,81], even though iNSPCs/iSCs can differentiate into neural and mesenchymal lineages. Therefore, iNSPCs/iSCs differ from pluripotent stem cells such as iPS cells and ES cells. We also found out that it is not easy for somatic adult pericytes to be reprogrammed into a pluripotent state even when subjected to severe stress such as ischemia[21]. However, a recent study showed that an injury stimulus did convert skeletal muscle cells into a pluripotent state[82]. Thus, whether injury stimuli can induce somatic cells to become pluripotent cells should be carefully investigated in future studies.

BRAIN PERICYTES FOLLOWING ISCHEMIA: ARE THEY IDENTICAL TO OTHER TYPES OF MULTIPOTENT STEM CELLS THAT RESIDE NEAR BLOOD VESSELS?

Akin to pericytes, previous studies showed that multipotent stem cells such as MSCs[83-87] and neural crest stem cells (NCSCs)[88] reside in the perivascular regions of multiple organs. These cells also differentiate into various lineages, including neural and mesenchymal lineages, consistent with the traits of iNSPCs/iSCs.

Comparing iNSPCs/iSCs with other types of multipotent stem cells such as bone-marrow-derived MSCs, iNSPCs/iSCs differentiate into mesenchymal lineages, including osteoblasts and adipocytes as well as MSCs. Using multi-electrode arrays[89], we recently reported that iNSPCs/iSCs, but not MSCs, have the potential to differentiate into electrophysiologic-functional neurons[90]. On the basis of their developmental origin in multiple organs, the majority of non-CNS pericytes originate from the mesoderm. However, brain pericytes are likely neural crest derivatives[91,92].

The cells of the neural crest originate from the neural tube through the epithelial-mesenchymal transition. The cells of the neural crest are multipotent stem cells (NSCs) that share both neural and mesenchymal traits[79,93,94].

Considering their origin, iNSPCs/iSCs have a stronger neural phenotype than MSCs. Thus, it is likely that iNSPCs/iSCs are stem cells which differ from previously reported ones. However, recent studies show that the traits of MSCs vary among organs[87]. Thus, brain MSCs might have features differing from those of MSCs derived from other organs (*e.g.*, bone-marrow-derived MSCs)[95], and further investigations are necessary regarding the relations among iNSPCs/iSCs, brain pericytes, and brain MSCs.

INSPCS/ISCS DERIVED FROM HUMAN ISCHEMIC BRAINS

To translate the non-clinical findings obtained in mouse iNSPCs/iSCs into clinical applications, it is essential to understand the traits of human iNSPCs/iSCs obtained from patients with stroke.

Using brain samples obtained from stroke patients who needed both decompressive craniectomy and partial lobectomy as a life-saving therapy for diffuse cerebral infarction, we attempted to isolate human iNSPCs/iSCs. We detected iNSPCs/iSCs within post-stroke areas of the human brains, consistent with those of mouse brains[21,24,41,90].

Isolation and characterization of human iNSPCs/iSCs from stroke patients

Recently, we have reported the traits of iNSPCs/iSCs obtained from two patients with cerebral infarction[96]. The samples obtained from two elderly patients displayed gross necrosis and histological cell death. Immunohistochemical analysis showed that, although mature neural cells disappear within post-stroke areas, nestin$^+$ cells were present within these areas. The nestin$^+$ cells localized near blood cells and expressed pericyte markers such as NG2 and αSMA. After the cells isolated from post-ischemic human tissues were incubated in medium with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), many proliferative cells emerged, and they

expressed the dividing cell marker Ki67. The cells isolated from post-ischemic human tissues expressed not only nestin but also the pericyte markers NG2, PDGFRβ, and αSMA. However, these nestin$^+$ cells did not express endothelial cells and astrocytes markers. These findings indicate that brain pericytes convert into nestin$^+$ iNSPCs/iSCs within post-stroke human brains, consistent with mouse brains[21].

Next, we examined the multipotency of human iNSPCs/iSCs. Even after several passages, nestin$^+$ iNSPCs/iSCs retained the expression of various stem cell and undifferentiated cell markers, including Sox2, c-myc, and Klf4. When they were incubated under conditions to promote the differentiation into mesoderm lineages such as osteoblasts, adipocytes, and chondrocytes, they differentiated into these cells, respectively. They also formed neurosphere-like cells under floating cultures and differentiated into Tuj-1$^+$ and MAP2$^+$ neuronal cells. These findings demonstrate that iNSPCs/iSCs are present within post-stroke human brains as well as in post-stroke mouse brains.

However, more precise traits of human iNSPCs/iSCs remain unclear, including their multipotency potential to differentiate into functional neurons. To address this question, we are now investigating the features of human iNSPCs/iSCs obtained from additional post-ischemic cerebral samples. Our preliminary study shows that human iNSPCs/iSCs expanded from a single-cell lineage mainly differentiated into Tuj1$^+$ neurons under neuronal differentiation conditions, and they differentiated into fatty acid binding protein 4 (FABP4)$^+$ adipocytes under adipogenic differentiation conditions. Our recent study also reveals that human iNSPCs/iSCs have the potential to differentiate into functional neurons[97]. These results indicate that iNSPCs/iSCs (at least a sub-population) function as multipotent stem cells that differentiate into neuronal cells. Therefore, these cells should be renamed iSCs rather than iNSPCs because they can differentiate into various cell lineages other than neural.

Other questions remain. For example, the traits of iNSPCs/iSCs may differ from the time of injury onset to surgery. Also, iNSPC/iSC features may vary among CNS regions (*e.g.*, cerebrum, cerebellum, brainstem, spinal cord). Regarding the latter question, our recent study demonstrated that iNSPCs/iSCs could be isolated from the cerebellum[97] as well as the cerebrum[96]. Comparative gene expression profiles showed that although the cerebellar iNSPCs/iSCs resembled cerebral iNSPCs/iSCs, they expressed certain cerebellum-specific genes[97]. Thus, further studies are needed using additional samples to identify comprehensively the traits of iNSPCs/iSCs.

THE PROSPECTS OF REGENERATIVE THERAPIES USING INSPCS/ISCS

Evidence showing that iNSPCs/iSCs are present within post-stroke human brains suggests that stem-cell-based therapies using iNSPCs/iSCs could contribute to neural repair in patients with stroke in the future. Two strategies for clinical applications using iNSPCs/iSCs could be implemented as follows.

A strategy targeting exogenously transplanted NSPCs/iSCs

The first strategy implies to transplant exogenous iNSPCs/iSCs within or near post-ischemic areas (Figure 2A). iNSPCs/iSCs isolated from ischemic areas exhibit high proliferative activities in a medium containing bFGF and EGF[96]. Thus, after a satisfactory expansion of iNSPCs/iSCs, the autologous transplantation of iNSPCs/iSCs could be performed during subacute and chronic periods. This therapy presents the advantage to repeatedly transplant iNSPCs/iSCs that satisfy certain cell profiles. Another advantage is that the cell number (*e.g.*, low dose of cells and high dose of cells) and the transplant location (*e.g.*, within ischemic areas, around ischemic areas, and non-ischemic areas) can be chosen.

On the other hand, there are several disadvantages. For example, several weeks are required to prepare enough iNSPCs/iSCs *in vitro*, not allowing iNSPC/iSC transplantation in stroke patients during acute phases. Furthermore, iNSPCs/iSCs cannot be obtained from any stroke patients. Currently, iNSPCs/iSCs can only be obtained from patients who needed both decompressive craniectomy and partial lobectomy as a life-saving therapy for diffuse cerebral infarction. It is ethically impossible to get iNSPCs/iSCs from patients with small infarcted areas (*e.g.*, lacunar infarction). Therefore, only a small portion of stroke patients would be eligible for this treatment in the future.

Currently, we are investigating the safety (*e.g.*, tumorigenesis onset and formation) and efficiency (*e.g.*, cell survival, neuronal differentiation, and functional improvement) upon transplantation of human iNSPCs/iSCs in mice post-stroke. Theoretically, the above-mentioned problems would be solved if iNSPCs/iSCs are

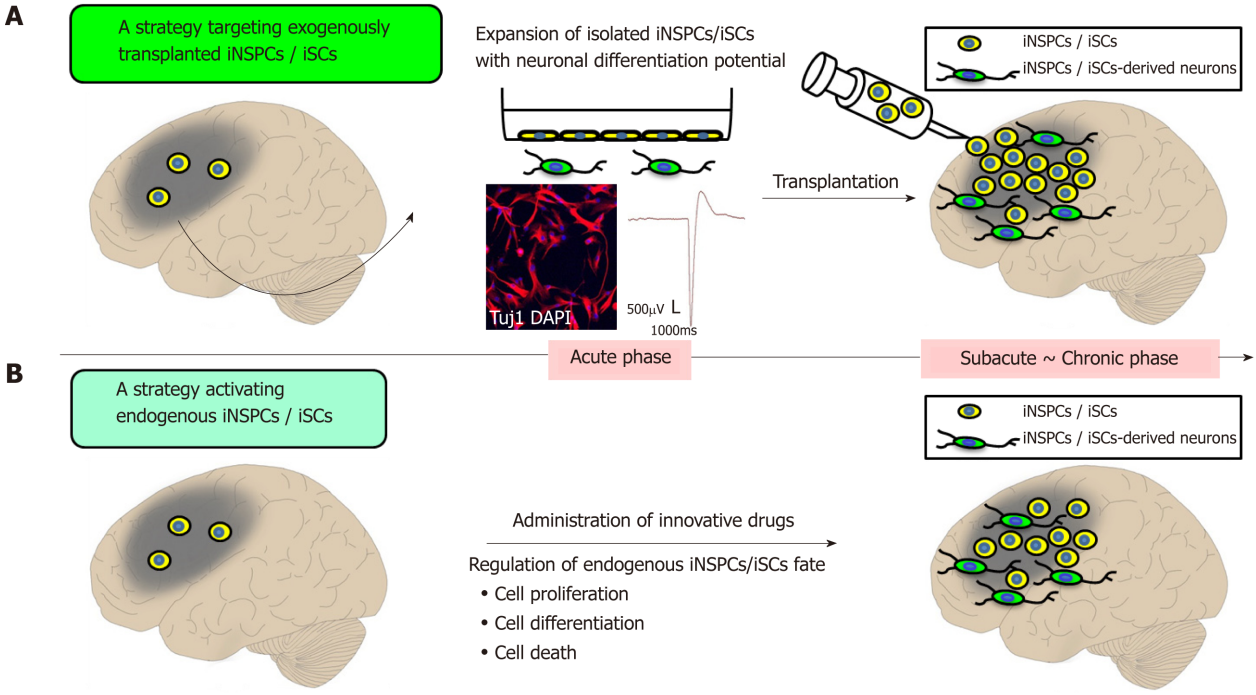

Figure 2 Prospects of regenerative therapy using injury/ischemia-induced neural stem/progenitor cells and injury/ischemia-induced multipotent stem cells. A: Strategic targeting of exogenously transplanted iNSPCs/iSCs. iNSPCs/iSCs exhibit high proliferative activity and differentiate into electrophysiologically-functional neurons in vitro. Thus, it is expected that transplanted iNSPCs/iSCs can differentiate into neuronal cells in vivo, thereby promoting central nervous system repair; B: A strategy for activating endogenous iNSPCs/iSCs. Administration of bioactive molecules has the potential to promote neural repair by regulating cell proliferation, cell differentiation, and cell death of endogenous iNSPCs/iSCs. iSCs: Injury/ischemia-induced multipotent stem cells; iNSPCs: Injury/ischemia-induced neural stem/progenitor cells.

expandable in allograft and autograft transplantations. However, we have to carefully evaluate whether iNSPCs/iSCs can be utilized as an allograft because iNSPCs/iSCs are stem cells that originated from brains that differ from stem cells derived from non-CNS (*e.g.*, bone marrow-derived MCS).

These problems may be solved using iNSPCs/iSCs derived from iPS cells. For example, using iPS-cell-derived iNSPCs/iSCs obtained from skin fibroblasts of stroke patients, patients may receive an autologous transplantation therapy using iNSPCs/iSCs. However, when making iPS cells, new problems could emerge, such as tumor formation.

A strategy activating endogenous iNSPCs/iSCs

The second strategy involves identifying the factors regulating the fate of iNSPCs/iSCs (*e.g.*, factors promoting cell proliferation and differentiation, and factors inhibiting cell death) and to develop those as innovative drugs (Figure 2B).

Using a mouse model of cerebral infarction, we previously showed that iNSPCs/iSCs isolated from ischemic areas differentiated into electrophysiologic-functional neurons and did express mature neuronal markers[27]. *In vivo*, the number of nestin$^+$ iNSPCs/iSCs peaked around post-stroke day 3 and then gradually decreased. In addition, immature newly born neurons were identified within and near ischemic areas at post-stroke day 3, and their numbers decreased thereafter as well[24,42,49].

This suggests that, although iNSPCs/iSCs are present within ischemic areas, several factors regulate their survival, proliferation, and differentiation. In support of this viewpoint, we have previously demonstrated that the endothelial cells residing around iNSPCs/iSCs promote their survival, proliferation, and neuronal differentiation[22,28]. This suggests that endothelial-derived trophic factors exhibit a positive effect on iNSPCs/iSCs. Alternatively, endothelial cells and/or the extracellular matrix produced by endothelial cells[98] may function as a niche for iNSPCs/iSCs, as it is the case with NSPCs[99].

Further investigations are needed to understand the factors involved in the regulation of iNSPCs/iSCs. However, our previous studies indicated that a subset of lymphocytes that infiltrated into ischemic areas during acute phases inhibited the survival of iNSPCs/iSCs[100,101]. In addition, our preliminary study showed that inflammatory cells such as microglia/macrophages rapidly increase at the time when

nestin$^+$ iNSPCs/iSCs disappear. These findings indicate that iNSPC/iSC regulation also relies on environmental factors surrounding them (*e.g.*, inflammatory cells), and both intrinsic and extrinsic factors play an essential role in neural regeneration.

CONCLUSION

Our studies showed that iNSPCs/iSCs are present within post-stroke areas of mouse and human brains. Further studies are needed to identify the traits, fate, proliferation, and differentiation factors of iNSPCs/iSCs for their clinical applications. However, iNSPCs/iSCs represent a cornerstone in contributing to CNS repair because they are stem cells that develop within ischemic areas following CNS injuries. Evidence of the presence of iNSPCs/iSCs within post-ischemic human brains is encouraging for the development of new stem-cell-based therapies for stroke patients.

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REFERENCES

1 **Mokin M**, Kass-Hout T, Kass-Hout O, Dumont TM, Kan P, Snyder KV, Hopkins LN, Siddiqui AH, Levy EI. Intravenous thrombolysis and endovascular therapy for acute ischemic stroke with internal carotid artery occlusion: a systematic review of clinical outcomes. *Stroke* 2012; **43**: 2362-2368 [PMID: 22811456 DOI: 10.1161/STROKEAHA.112.655621]

2 **Zaidat OO**, Suarez JI, Sunshine JL, Tarr RW, Alexander MJ, Smith TP, Enterline DS, Selman WR, Landis DM. Thrombolytic therapy of acute ischemic stroke: correlation of angiographic recanalization with clinical outcome. *AJNR Am J Neuroradiol* 2005; **26**: 880-884 [PMID: 15814938]

3 **Goyal M**, Menon BK, van Zwam WH, Dippel DW, Mitchell PJ, Demchuk AM, Dávalos A, Majoie CB, van der Lugt A, de Miquel MA, Donnan GA, Roos YB, Bonafe A, Jahan R, Diener HC, van den Berg LA, Levy EI, Berkhemer OA, Pereira VM, Rempel J, Millán M, Davis SM, Roy D, Thornton J, Román LS, Ribó M, Beumer D, Stouch B, Brown S, Campbell BC, van Oostenbrugge RJ, Saver JL, Hill MD, Jovin TG; HERMES collaborators. Endovascular thrombectomy after large-vessel ischaemic stroke: a meta-analysis of individual patient data from five randomised trials. *Lancet* 2016; **387**: 1723-1731 [PMID: 26898852 DOI: 10.1016/S0140-6736(16)00163-X]

4 **Hacke W**, Kaste M, Bluhmki E, Brozman M, Dávalos A, Guidetti D, Larrue V, Lees KR, Medeghri Z, Machnig T, Schneider D, von Kummer R, Wahlgren N, Toni D; ECASS Investigators. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N Engl J Med* 2008; **359**: 1317-1329 [PMID: 18815396 DOI: 10.1056/NEJMoa0804656]

5 **Thomalla G**, Simonsen CZ, Boutitie F, Andersen G, Berthezene Y, Cheng B, Cheripelli B, Cho TH, Fazekas F, Fiehler J, Ford I, Galinovic I, Gellissen S, Golsari A, Gregori J, Günther M, Guibernau J, Häusler KG, Hennerici M, Kemmling A, Marstrand J, Modrau B, Neeb L, Perez de la Ossa N, Puig J, Ringleb P, Roy P, Scheel E, Schonewille W, Serena J, Sunaert S, Villringer K, Wouters A, Thijs V, Ebinger M, Endres M, Fiebach JB, Lemmens R, Muir KW, Nighoghossian N, Pedraza S, Gerloff C; WAKE-UP Investigators. MRI-Guided Thrombolysis for Stroke with Unknown Time of Onset. *N Engl J Med* 2018; **379**: 611-622 [PMID: 29766770 DOI: 10.1056/NEJMoa1804355]

6 **Nogueira RG**, Jadhav AP, Haussen DC, Bonafe A, Budzik RF, Bhuva P, Yavagal DR, Ribo M, Cognard C, Hanel RA, Sila CA, Hassan AE, Millan M, Levy EI, Mitchell P, Chen M, English JD, Shah QA, Silver FL, Pereira VM, Mehta BP, Baxter BW, Abraham MG, Cardona P, Veznedaroglu E, Hellinger FR, Feng L, Kirmani JF, Lopes DK, Jankowitz BT, Frankel MR, Costalat V, Vora NA, Yoo AJ, Malik AM, Furlan AJ, Rubiera M, Aghaebrahim A, Olivot JM, Tekle WG, Shields R, Graves T, Lewis RJ, Smith WS, Liebeskind DS, Saver JL, Jovin TG; DAWN Trial Investigators. Thrombectomy 6 to 24 Hours after Stroke with a Mismatch between Deficit and Infarct. *N Engl J Med* 2018; **378**: 11-21 [PMID: 29129157 DOI: 10.1056/NEJMoa1706442]

7 **Albers GW**, Marks MP, Kemp S, Christensen S, Tsai JP, Ortega-Gutierrez S, McTaggart RA, Torbey MT, Kim-Tenser M, Leslie-Mazwi T, Sarraj A, Kasner SE, Ansari SA, Yeatts SD, Hamilton S, Mlynash M, Heit JJ, Zaharchuk G, Kim S, Carrozzella J, Palesch YY, Demchuk AM, Bammer R, Lavori PW, Broderick JP, Lansberg MG; DEFUSE 3 Investigators. Thrombectomy for Stroke at 6 to 16 Hours with Selection by Perfusion Imaging. *N Engl J Med* 2018; **378**: 708-718 [PMID: 29364767 DOI: 10.1056/NEJMoa1713973]

8 **Chia NH**, Leyden JM, Newbury J, Jannes J, Kleinig TJ. Determining the Number of Ischemic Strokes Potentially Eligible for Endovascular Thrombectomy: A Population-Based Study. *Stroke* 2016; **47**: 1377-1380 [PMID: 26987869 DOI: 10.1161/STROKEAHA.116.013165]

9 **Darsalia V**, Kallur T, Kokaia Z. Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged rat striatum. *Eur J Neurosci* 2007; **26**: 605-614 [PMID: 17686040 DOI: 10.1111/j.1460-9568.2007.05702.x]

10 **Kelly S**, Bliss TM, Shah AK, Sun GH, Ma M, Foo WC, Masel J, Yenari MA, Weissman IL, Uchida N, Palmer T, Steinberg GK. Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. *Proc Natl Acad Sci U S A* 2004; **101**: 11839-11844 [PMID: 15280535 DOI: 10.1073/pnas.0404474101]

11 **Hicks AU**, Hewlett K, Windle V, Chernenko G, Ploughman M, Jolkkonen J, Weiss S, Corbett D. Enriched environment enhances transplanted subventricular zone stem cell migration and functional recovery after

stroke. *Neuroscience* 2007; **146**: 31-40 [PMID: 17320299 DOI: 10.1016/j.neuroscience.2007.01.020]
12 **Kameda M**, Shingo T, Takahashi K, Muraoka K, Kurozumi K, Yasuhara T, Maruo T, Tsuboi T, Uozumi T, Matsui T, Miyoshi Y, Hamada H, Date I. Adult neural stem and progenitor cells modified to secrete GDNF can protect, migrate and integrate after intracerebral transplantation in rats with transient forebrain ischemia. *Eur J Neurosci* 2007; **26**: 1462-1478 [PMID: 17880388 DOI: 10.1111/j.1460-9568.2007.05776.x]
13 **Honma T**, Honmou O, Iihoshi S, Harada K, Houkin K, Hamada H, Kocsis JD. Intravenous infusion of immortalized human mesenchymal stem cells protects against injury in a cerebral ischemia model in adult rat. *Exp Neurol* 2006; **199**: 56-66 [PMID: 15967439 DOI: 10.1016/j.expneurol.2005.05.004]
14 **Bang OY**, Lee JS, Lee PH, Lee G. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann Neurol* 2005; **57**: 874-882 [PMID: 15929052 DOI: 10.1002/ana.20501]
15 **Huang H**, Lin F, Jiang J, Chen Y, Mei A, Zhu P. Effects of intra-arterial transplantation of adipose-derived stem cells on the expression of netrin-1 and its receptor DCC in the peri-infarct cortex after experimental stroke. *Stem Cell Res Ther* 2017; **8**: 223 [PMID: 29017609 DOI: 10.1186/s13287-017-0671-6]
16 **Zhao K**, Li R, Gu C, Liu L, Jia Y, Guo X, Zhang W, Pei C, Tian L, Li B, Jia J, Cheng H, Xu H, Li L. Intravenous Administration of Adipose-Derived Stem Cell Protein Extracts Improves Neurological Deficits in a Rat Model of Stroke. *Stem Cells Int* 2017; **2017**: 2153629 [PMID: 28265288 DOI: 10.1155/2017/2153629]
17 **Kimura H**, Yoshikawa M, Matsuda R, Toriumi H, Nishimura F, Hirabayashi H, Nakase H, Kawaguchi S, Ishizaka S, Sakaki T. Transplantation of embryonic stem cell-derived neural stem cells for spinal cord injury in adult mice. *Neurol Res* 2005; **27**: 812-819 [PMID: 16354541 DOI: 10.1179/016164105X63629]
18 **Salewski RP**, Mitchell RA, Li L, Shen C, Milekovskaia M, Nagy A, Fehlings MG. Transplantation of Induced Pluripotent Stem Cell-Derived Neural Stem Cells Mediate Functional Recovery Following Thoracic Spinal Cord Injury Through Remyelination of Axons. *Stem Cells Transl Med* 2015; **4**: 743-754 [PMID: 25979861 DOI: 10.5966/sctm.2014-0236]
19 **Alvarez-Buylla A**, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. *J Neurosci* 2002; **22**: 629-634 [PMID: 11826091 DOI: 10.1523/JNEUROSCI.22-03-00629.2002]
20 **Kuhn HG**, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci* 1996; **16**: 2027-2033 [PMID: 8604047 DOI: 10.1523/JNEUROSCI.16-06-02027.1996]
21 **Nakagomi T**, Kubo S, Nakano-Doi A, Sakuma R, Lu S, Narita A, Kawahara M, Taguchi A, Matsuyama T. Brain vascular pericytes following ischemia have multipotential stem cell activity to differentiate into neural and vascular lineage cells. *Stem Cells* 2015; **33**: 1962-1974 [PMID: 25694098 DOI: 10.1002/stem.1977]
22 **Nakano-Doi A**, Nakagomi T, Fujikawa M, Nakagomi N, Kubo S, Lu S, Yoshikawa H, Soma T, Taguchi A, Matsuyama T. Bone marrow mononuclear cells promote proliferation of endogenous neural stem cells through vascular niches after cerebral infarction. *Stem Cells* 2010; **28**: 1292-1302 [PMID: 20517983 DOI: 10.1002/stem.454]
23 **Nakano-Doi A**, Sakuma R, Matsuyama T, Nakagomi T. Ischemic stroke activates the VE-cadherin promoter and increases VE-cadherin expression in adult mice. *Histol Histopathol* 2018; **33**: 507-521 [PMID: 29205257 DOI: 10.14670/HH-11-952]
24 **Nakagomi T**, Molnár Z, Nakano-Doi A, Taguchi A, Saino O, Kubo S, Clausen M, Yoshikawa H, Nakagomi N, Matsuyama T. Ischemia-induced neural stem/progenitor cells in the pia mater following cortical infarction. *Stem Cells Dev* 2011; **20**: 2037-2051 [PMID: 21838536 DOI: 10.1089/scd.2011.0279]
25 **Kasahara Y**, Ihara M, Nakagomi T, Momota Y, Stern DM, Matsuyama T, Taguchi A. A highly reproducible model of cerebral ischemia/reperfusion with extended survival in CB-17 mice. *Neurosci Res* 2013; **76**: 163-168 [PMID: 23603509 DOI: 10.1016/j.neures.2013.04.001]
26 **Taguchi A**, Kasahara Y, Nakagomi T, Stern DM, Fukunaga M, Ishikawa M, Matsuyama T. A Reproducible and Simple Model of Permanent Cerebral Ischemia in CB-17 and SCID Mice. *J Exp Stroke Transl Med* 2010; **3**: 28-33 [PMID: 20865060 DOI: 10.6030/1939-067X-3.1.28]
27 **Nakagomi T**, Taguchi A, Fujimori Y, Saino O, Nakano-Doi A, Kubo S, Gotoh A, Soma T, Yoshikawa H, Nishizaki T, Nakagomi N, Stern DM, Matsuyama T. Isolation and characterization of neural stem/progenitor cells from post-stroke cerebral cortex in mice. *Eur J Neurosci* 2009; **29**: 1842-1852 [PMID: 19473237 DOI: 10.1111/j.1460-9568.2009.06732.x]
28 **Nakagomi N**, Nakagomi T, Kubo S, Nakano-Doi A, Saino O, Takata M, Yoshikawa H, Stern DM, Matsuyama T, Taguchi A. Endothelial cells support survival, proliferation, and neuronal differentiation of transplanted adult ischemia-induced neural stem/progenitor cells after cerebral infarction. *Stem Cells* 2009; **27**: 2185-2195 [PMID: 19557831 DOI: 10.1002/stem.161]
29 **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]
30 **Jackson EL**, Garcia-Verdugo JM, Gil-Perotin S, Roy M, Quinones-Hinojosa A, VandenBerg S, Alvarez-Buylla A. PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. *Neuron* 2006; **51**: 187-199 [PMID: 16846854 DOI: 10.1016/j.neuron.2006.06.012]
31 **Shimada IS**, Peterson BM, Spees JL. Isolation of locally derived stem/progenitor cells from the peri-infarct area that do not migrate from the lateral ventricle after cortical stroke. *Stroke* 2010; **41**: e552-e560 [PMID: 20671247 DOI: 10.1161/STROKEAHA.110.589010]
32 **Yokoyama A**, Sakamoto A, Kameda K, Imai Y, Tanaka J. NG2 proteoglycan-expressing microglia as multipotent neural progenitors in normal and pathologic brains. *Glia* 2006; **53**: 754-768 [PMID: 16534776 DOI: 10.1002/glia.20332]
33 **Gaughwin PM**, Caldwell MA, Anderson JM, Schwiening CJ, Fawcett JW, Compston DA, Chandran S. Astrocytes promote neurogenesis from oligodendrocyte precursor cells. *Eur J Neurosci* 2006; **23**: 945-956 [PMID: 16519659 DOI: 10.1111/j.1460-9568.2006.04625.x]
34 **Kondo T**, Raff M. Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. *Science* 2000; **289**: 1754-1757 [PMID: 10976069 DOI: 10.1126/science.289.5485.1754]
35 **Carlén M**, Meletis K, Göritz C, Darsalia V, Evergren E, Tanigaki K, Amendola M, Barnabé-Heider F, Yeung MS, Naldini L, Honjo T, Kokaia Z, Shupliakov O, Cassidy RM, Lindvall O, Frisén J. Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke. *Nat Neurosci* 2009; **12**: 259-267 [PMID: 19234458 DOI: 10.1038/nn.2268]

36 **Moreno-Manzano V**, Rodríguez-Jiménez FJ, García-Roselló M, Laínez S, Erceg S, Calvo MT, Ronaghi M, Lloret M, Planells-Cases R, Sánchez-Puelles JM, Stojkovic M. Activated spinal cord ependymal stem cells rescue neurological function. *Stem Cells* 2009; **27**: 733-743 [PMID: 19259940 DOI: 10.1002/stem.24]

37 **Kojima T**, Hirota Y, Ema M, Takahashi S, Miyoshi I, Okano H, Sawamoto K. Subventricular zone-derived neural progenitor cells migrate along a blood vessel scaffold toward the post-stroke striatum. *Stem Cells* 2010; **28**: 545-554 [PMID: 20073084 DOI: 10.1002/stem.306]

38 **Tavazoie M**, Van der Veken L, Silva-Vargas V, Louissaint M, Colonna L, Zaidi B, Garcia-Verdugo JM, Doetsch F. A specialized vascular niche for adult neural stem cells. *Cell Stem Cell* 2008; **3**: 279-288 [PMID: 18786415 DOI: 10.1016/j.stem.2008.07.025]

39 **Maki T**, Maeda M, Uemura M, Lo EK, Terasaki Y, Liang AC, Shindo A, Choi YK, Taguchi A, Matsuyama T, Takahashi R, Ihara M, Arai K. Potential interactions between pericytes and oligodendrocyte precursor cells in perivascular regions of cerebral white matter. *Neurosci Lett* 2015; **597**: 164-169 [PMID: 25936593 DOI: 10.1016/j.neulet.2015.04.047]

40 **Seo JH**, Maki T, Maeda M, Miyamoto N, Liang AC, Hayakawa K, Pham LD, Suwa F, Taguchi A, Matsuyama T, Ihara M, Kim KW, Lo EH, Arai K. Oligodendrocyte precursor cells support blood-brain barrier integrity via TGF-β signaling. *PLoS One* 2014; **9**: e103174 [PMID: 25078775 DOI: 10.1371/journal.pone.0103174]

41 **Sakuma R**, Kawahara M, Nakano-Doi A, Takahashi A, Tanaka Y, Narita A, Kuwahara-Otani S, Hayakawa T, Yagi H, Matsuyama T, Nakagomi T. Brain pericytes serve as microglia-generating multipotent vascular stem cells following ischemic stroke. *J Neuroinflammation* 2016; **13**: 57 [PMID: 26952098 DOI: 10.1186/s12974-016-0523-9]

42 **Nakata M**, Nakagomi T, Maeda M, Nakano-Doi A, Momota Y, Matsuyama T. Induction of Perivascular Neural Stem Cells and Possible Contribution to Neurogenesis Following Transient Brain Ischemia/Reperfusion Injury. *Transl Stroke Res* 2017; **8**: 131-143 [PMID: 27352866 DOI: 10.1007/s12975-016-0479-1]

43 **Dore-Duffy P**, Katychev A, Wang X, Van Buren E. CNS microvascular pericytes exhibit multipotential stem cell activity. *J Cereb Blood Flow Metab* 2006; **26**: 613-624 [PMID: 16421511 DOI: 10.1038/sj.jcbfm.9600272]

44 **Birbrair A**, Zhang T, Wang ZM, Messi ML, Enikolopov GN, Mintz A, Delbono O. Skeletal muscle pericyte subtypes differ in their differentiation potential. *Stem Cell Res* 2013; **10**: 67-84 [PMID: 23128780 DOI: 10.1016/j.scr.2012.09.003]

45 **Morikawa S**, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am J Pathol* 2002; **160**: 985-1000 [PMID: 11891196 DOI: 10.1016/S0002-9440(10)64920-6]

46 **Mitchell TS**, Bradley J, Robinson GS, Shima DT, Ng YS. RGS5 expression is a quantitative measure of pericyte coverage of blood vessels. *Angiogenesis* 2008; **11**: 141-151 [PMID: 18038251 DOI: 10.1007/s10456-007-9085-x]

47 **Birbrair A**, Zhang T, Wang ZM, Messi ML, Olson JD, Mintz A, Delbono O. Type-2 pericytes participate in normal and tumoral angiogenesis. *Am J Physiol Cell Physiol* 2014; **307**: C25-C38 [PMID: 24788248 DOI: 10.1152/ajpcell.00084.2014]

48 **Karow M**, Sánchez R, Schichor C, Masserdotti G, Ortega F, Heinrich C, Gascón S, Khan MA, Lie DC, Dellavalle A, Cossu G, Goldbrunner R, Götz M, Berninger B. Reprogramming of pericyte-derived cells of the adult human brain into induced neuronal cells. *Cell Stem Cell* 2012; **11**: 471-476 [PMID: 23040476 DOI: 10.1016/j.stem.2012.07.007]

49 **Nakagomi T**, Molnár Z, Taguchi A, Nakano-Doi A, Lu S, Kasahara Y, Nakagomi N, Matsuyama T. Leptomeningeal-derived doublecortin-expressing cells in poststroke brain. *Stem Cells Dev* 2012; **21**: 2350-2354 [PMID: 22339778 DOI: 10.1089/scd.2011.0657]

50 **Guimarães-Camboa N**, Cattaneo P, Sun Y, Moore-Morris T, Gu Y, Dalton ND, Rockenstein E, Masliah E, Peterson KL, Stallcup WB, Chen J, Evans SM. Pericytes of Multiple Organs Do Not Behave as Mesenchymal Stem Cells In Vivo. *Cell Stem Cell* 2017; **20**: 345-359.e5 [PMID: 28111199 DOI: 10.1016/j.stem.2016.12.006]

51 **Göritz C**, Dias DO, Tomilin N, Barbacid M, Shupliakov O, Frisén J. A pericyte origin of spinal cord scar tissue. *Science* 2011; **333**: 238-242 [PMID: 21737741 DOI: 10.1126/science.1203165]

52 **Birbrair A**, Zhang T, Wang ZM, Messi ML, Enikolopov GN, Mintz A, Delbono O. Skeletal muscle neural progenitor cells exhibit properties of NG2-glia. *Exp Cell Res* 2013; **319**: 45-63 [PMID: 22999866 DOI: 10.1016/j.yexcr.2012.09.008]

53 **Moyon S**, Liang J, Casaccia P. Epigenetics in NG2 glia cells. *Brain Res* 2016; **1638**: 183-198 [PMID: 26092401 DOI: 10.1016/j.brainres.2015.06.009]

54 **Santos GSP**, Magno LAV, Romano-Silva MA, Mintz A, Birbrair A. Pericyte Plasticity in the Brain. *Neurosci Bull* 2019; **35**: 551-560 [PMID: 30367336 DOI: 10.1007/s12264-018-0296-5]

55 **Armulik A**, Genové G, Mäe M, Nisancioglu MH, Wallgard E, Niaudet C, He L, Norlin J, Lindblom P, Strittmatter K, Johansson BR, Betsholtz C. Pericytes regulate the blood-brain barrier. *Nature* 2010; **468**: 557-561 [PMID: 20944627 DOI: 10.1038/nature09522]

56 **Zeisberg EM**, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, Chandraker A, Yuan X, Pu WT, Roberts AB, Neilson EG, Sayegh MH, Izumo S, Kalluri R. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med* 2007; **13**: 952-961 [PMID: 17660828 DOI: 10.1038/nm1613]

57 **Yu W**, Liu Z, An S, Zhao J, Xiao L, Gou Y, Lin Y, Wang J. The endothelial-mesenchymal transition (EndMT) and tissue regeneration. *Curr Stem Cell Res Ther* 2014; **9**: 196-204 [PMID: 24524794 DOI: 10.2174/1574888X09666140213154144]

58 **Susienka MJ**, Medici D. Vascular endothelium as a novel source of stem cells for bioengineering. *Biomatter* 2013; 3 [PMID: 23603799 DOI: 10.4161/biom.24647]

59 **Kovacic JC**, Mercader N, Torres M, Boehm M, Fuster V. Epithelial-to-mesenchymal and endothelial-to-mesenchymal transition: from cardiovascular development to disease. *Circulation* 2012; **125**: 1795-1808 [PMID: 22492947 DOI: 10.1161/CIRCULATIONAHA.111.040352]

60 **Berthiaume AA**, Grant RI, McDowell KP, Underly RG, Hartmann DA, Levy M, Bhat NR, Shih AY. Dynamic Remodeling of Pericytes In Vivo Maintains Capillary Coverage in the Adult Mouse Brain. *Cell Rep* 2018; **22**: 8-16 [PMID: 29298435 DOI: 10.1016/j.celrep.2017.12.016]

61 **Crisan M**, Chen CW, Corselli M, Andriolo G, Lazzari L, Péault B. Perivascular multipotent progenitor cells in human organs. *Ann N Y Acad Sci* 2009; **1176**: 118-123 [PMID: 19796239 DOI:

10.1111/j.1749-6632.2009.04967.x]
62 **Kabara M**, Kawabe J, Matsuki M, Hira Y, Minoshima A, Shimamura K, Yamauchi A, Aonuma T, Nishimura M, Saito Y, Takehara N, Hasebe N. Immortalized multipotent pericytes derived from the vasa vasorum in the injured vasculature. A cellular tool for studies of vascular remodeling and regeneration. *Lab Invest* 2014; **94**: 1340-1354 [PMID: 25329003 DOI: 10.1038/labinvest.2014.121]
63 **Birbrair A**, Zhang T, Wang ZM, Messi ML, Enikolopov GN, Mintz A, Delbono O. Role of pericytes in skeletal muscle regeneration and fat accumulation. *Stem Cells Dev* 2013; **22**: 2298-2314 [PMID: 23517218 DOI: 10.1089/scd.2012.0647]
64 **Birbrair A**, Zhang T, Wang ZM, Messi ML, Mintz A, Delbono O. Pericytes: multitasking cells in the regeneration of injured, diseased, and aged skeletal muscle. *Front Aging Neurosci* 2014; **6**: 245 [PMID: 25278877 DOI: 10.3389/fnagi.2014.00245]
65 **Farrington-Rock C**, Crofts NJ, Doherty MJ, Ashton BA, Griffin-Jones C, Canfield AE. Chondrogenic and adipogenic potential of microvascular pericytes. *Circulation* 2004; **110**: 2226-2232 [PMID: 15466630 DOI: 10.1161/01.CIR.0000144457.55518.E5]
66 **Dar A**, Domev H, Ben-Yosef O, Tzukerman M, Zeevi-Levin N, Novak A, Germanguz I, Amit M, Itskovitz-Eldor J. Multipotent vasculogenic pericytes from human pluripotent stem cells promote recovery of murine ischemic limb. *Circulation* 2012; **125**: 87-99 [PMID: 22095829 DOI: 10.1161/CIRCULATIONAHA.111.048264]
67 **Doherty MJ**, Ashton BA, Walsh S, Beresford JN, Grant ME, Canfield AE. Vascular pericytes express osteogenic potential in vitro and in vivo. *J Bone Miner Res* 1998; **13**: 828-838 [PMID: 9610747 DOI: 10.1359/jbmr.1998.13.5.828]
68 **Gouveia A**, Seegobin M, Kannangara TS, He L, Wondisford F, Comin CH, Costa LDF, Béïque JC, Lagace DC, Lacoste B, Wang J. The aPKC-CBP Pathway Regulates Post-stroke Neurovascular Remodeling and Functional Recovery. *Stem Cell Reports* 2017; **9**: 1735-1744 [PMID: 29173896 DOI: 10.1016/j.stemcr.2017.10.021]
69 **Smyth LCD**, Rustenhoven J, Park TI, Schweder P, Jansson D, Heppner PA, O'Carroll SJ, Mee EW, Faull RLM, Curtis M, Dragunow M. Unique and shared inflammatory profiles of human brain endothelia and pericytes. *J Neuroinflammation* 2018; **15**: 138 [PMID: 29751771 DOI: 10.1186/s12974-018-1167-8]
70 **Yamamoto S**, Muramatsu M, Azuma E, Ikutani M, Nagai Y, Sagara H, Koo BN, Kita S, O'Donnell E, Osawa T, Takahashi H, Takano KI, Dohmoto M, Sugimori M, Usui I, Watanabe Y, Hatakeyama N, Iwamoto T, Komuro I, Takatsu K, Tobe K, Niida S, Matsuda N, Shibuya M, Sasahara M. A subset of cerebrovascular pericytes originates from mature macrophages in the very early phase of vascular development in CNS. *Sci Rep* 2017; **7**: 3855 [PMID: 28634350 DOI: 10.1038/s41598-017-03994-1]
71 **Yamazaki T**, Nalbandian A, Uchida Y, Li W, Arnold TD, Kubota Y, Yamamoto S, Ema M, Mukouyama YS. Tissue Myeloid Progenitors Differentiate into Pericytes through TGF-β Signaling in Developing Skin Vasculature. *Cell Rep* 2017; **18**: 2991-3004 [PMID: 28329690 DOI: 10.1016/j.celrep.2017.02.069]
72 **Fujita Y**, Ihara M, Ushiki T, Hirai H, Kizaka-Kondoh S, Hiraoka M, Ito H, Takahashi R. Early protective effect of bone marrow mononuclear cells against ischemic white matter damage through augmentation of cerebral blood flow. *Stroke* 2010; **41**: 2938-2943 [PMID: 20947840 DOI: 10.1161/STROKEAHA.110.596379]
73 **Suzuki S**, Namiki J, Shibata S, Mastuzaki Y, Okano H. The neural stem/progenitor cell marker nestin is expressed in proliferative endothelial cells, but not in mature vasculature. *J Histochem Cytochem* 2010; **58**: 721-730 [PMID: 20421592 DOI: 10.1369/jhc.2010.955609]
74 **Namiki J**, Suzuki S, Masuda T, Ishihama Y, Okano H. Nestin protein is phosphorylated in adult neural stem/progenitor cells and not endothelial progenitor cells. *Stem Cells Int* 2012; **2012**: 430138 [PMID: 23028390 DOI: 10.1155/2012/430138]
75 **Bernal A**, Arranz L. Nestin-expressing progenitor cells: function, identity and therapeutic implications. *Cell Mol Life Sci* 2018; **75**: 2177-2195 [PMID: 29541793 DOI: 10.1007/s00018-018-2794-z]
76 **Birbrair A**, Borges IDT, Gilson Sena IF, Almeida GG, da Silva Meirelles L, Gonçalves R, Mintz A, Delbono O. How Plastic Are Pericytes? *Stem Cells Dev* 2017; **26**: 1013-1019 [PMID: 28490256 DOI: 10.1089/scd.2017.0044]
77 **Liang H**, Hippenmeyer S, Ghashghaei HT. A Nestin-cre transgenic mouse is insufficient for recombination in early embryonic neural progenitors. *Biol Open* 2012; **1**: 1200-1203 [PMID: 23259054 DOI: 10.1242/bio.20122287]
78 **Sun MY**, Yetman MJ, Lee TC, Chen Y, Jankowsky JL. Specificity and efficiency of reporter expression in adult neural progenitors vary substantially among nestin-CreER(T2) lines. *J Comp Neurol* 2014; **522**: 1191-1208 [PMID: 24519019 DOI: 10.1002/cne.23497]
79 **Takagi T**, Yoshimura S, Sakuma R, Nakano-Doi A, Matsuyama T, Nakagomi T. Novel Regenerative Therapies Based on Regionally Induced Multipotent Stem Cells in Post-Stroke Brains: Their Origin, Characterization, and Perspective. *Transl Stroke Res* 2017; **8**: 515-528 [PMID: 28744717 DOI: 10.1007/s12975-017-0556-0]
80 **Karow M**, Camp JG, Falk S, Gerber T, Pataskar A, Gac-Santel M, Kageyama J, Brazovskaja A, Garding A, Fan W, Riedemann T, Casamassa A, Smiyakin A, Schichor C, Götz M, Tiwari VK, Treutlein B, Berninger B. Direct pericyte-to-neuron reprogramming via unfolding of a neural stem cell-like program. *Nat Neurosci* 2018; **21**: 932-940 [PMID: 29915193 DOI: 10.1038/s41593-018-0168-3]
81 **Nakagomi T**, Nakano-Doi A, Narita A, Matsuyama T. Concise Review: Are Stimulated Somatic Cells Truly Reprogrammed into an ES/iPS-Like Pluripotent State? Better Understanding by Ischemia-Induced Multipotent Stem Cells in a Mouse Model of Cerebral Infarction. *Stem Cells Int* 2015; **2015**: 630693 [PMID: 25945100 DOI: 10.1155/2015/630693]
82 **Vojnits K**, Pan H, Mu X, Li Y. Characterization of an Injury Induced Population of Muscle-Derived Stem Cell-Like Cells. *Sci Rep* 2015; **5**: 17355 [PMID: 26611864 DOI: 10.1038/srep17355]
83 **Paul G**, Özen I, Christophersen NS, Reinbothe T, Bengzon J, Visse E, Jansson K, Dannaeus K, Henriques-Oliveira C, Roybon L, Anisimov SV, Renström E, Svensson M, Haegerstrand A, Brundin P. The adult human brain harbors multipotent perivascular mesenchymal stem cells. *PLoS One* 2012; **7**: e35577 [PMID: 22523602 DOI: 10.1371/journal.pone.0035577]
84 **Crisan M**, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Péault B. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008; **3**: 301-313 [PMID: 18786417 DOI: 10.1016/j.stem.2008.07.003]
85 **Esteves CL**, Sheldrake TA, Dawson L, Menghini T, Rink BE, Amilon K, Khan N, Péault B, Donadeu FX. Equine Mesenchymal Stromal Cells Retain a Pericyte-Like Phenotype. *Stem Cells Dev* 2017; **26**: 964-972

[PMID: 28376684 DOI: 10.1089/scd.2017.0017]
86 **Ozen I**, Boix J, Paul G. Perivascular mesenchymal stem cells in the adult human brain: a future target for neuroregeneration? *Clin Transl Med* 2012; **1**: 30 [PMID: 23369339 DOI: 10.1186/2001-1326-1-30]
87 **Vezzani B**, Pierantozzi E, Sorrentino V. Not All Pericytes Are Born Equal: Pericytes from Human Adult Tissues Present Different Differentiation Properties. *Stem Cells Dev* 2016; **25**: 1549-1558 [PMID: 27549576 DOI: 10.1089/scd.2016.0177]
88 **Kubota Y**, Takubo K, Hirashima M, Nagoshi N, Kishi K, Okuno Y, Nakamura-Ishizu A, Sano K, Murakami M, Ema M, Omatsu Y, Takahashi S, Nagasawa T, Shibuya M, Okano H, Suda T. Isolation and function of mouse tissue resident vascular precursors marked by myelin protein zero. *J Exp Med* 2011; **208**: 949-960 [PMID: 21536740 DOI: 10.1084/jem.20102187]
89 **Dranias MR**, Ju H, Rajaram E, VanDongen AM. Short-term memory in networks of dissociated cortical neurons. *J Neurosci* 2013; **33**: 1940-1953 [PMID: 23365233 DOI: 10.1523/JNEUROSCI.2718-12.2013]
90 **Sakuma R**, Takahashi A, Nakano-Doi A, Sawada R, Kamachi S, Beppu M, Takagi T, Yoshimura S, Matsuyama T, Nakagomi T. Comparative Characterization of Ischemia-Induced Brain Multipotent Stem Cells with Mesenchymal Stem Cells: Similarities and Differences. *Stem Cells Dev* 2018; **27**: 1322-1338 [PMID: 29999479 DOI: 10.1089/scd.2018.0075]
91 **Morse DE**, Cova JL. Pigmented cells in the leptomeninges of the cat. *Anat Rec* 1984; **210**: 125-132 [PMID: 6486479 DOI: 10.1002/ar.1092100115]
92 **Etchevers HC**, Vincent C, Le Douarin NM, Couly GF. The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. *Development* 2001; **128**: 1059-1068 [PMID: 11245571]
93 **Nagoshi N**, Shibata S, Nakamura M, Matsuzaki Y, Toyama Y, Okano H. Neural crest-derived stem cells display a wide variety of characteristics. *J Cell Biochem* 2009; **107**: 1046-1052 [PMID: 19479900 DOI: 10.1002/jcb.22213]
94 **Nakagomi T**, Nakano-Doi A, Kawamura M, Matsuyama T. Do Vascular Pericytes Contribute to Neurovasculogenesis in the Central Nervous System as Multipotent Vascular Stem Cells? *Stem Cells Dev* 2015; **24**: 1730-1739 [PMID: 25900222 DOI: 10.1089/scd.2015.0039]
95 **Appaix F**, Nissou MF, van der Sanden B, Dreyfus M, Berger F, Issartel JP, Wion D. Brain mesenchymal stem cells: The other stem cells of the brain? *World J Stem Cells* 2014; **6**: 134-143 [PMID: 24772240 DOI: 10.4252/wjsc.v6.i2.134]
96 **Tatebayashi K**, Tanaka Y, Nakano-Doi A, Sakuma R, Kamachi S, Shirakawa M, Uchida K, Kageyama H, Takagi T, Yoshimura S, Matsuyama T, Nakagomi T. Identification of Multipotent Stem Cells in Human Brain Tissue Following Stroke. *Stem Cells Dev* 2017; **26**: 787-797 [PMID: 28323540 DOI: 10.1089/scd.2016.0334]
97 **Beppu M**, Nakagomi T, Takagi T, Nakano-Doi A, Sakuma R, Kuramoto Y, Tatebayashi K, Matsuyama T, Yoshimura S. Isolation and Characterization of Cerebellum-Derived Stem Cells in Poststroke Human Brain. *Stem Cells Dev* 2019; **28**: 528-542 [PMID: 30767605 DOI: 10.1089/scd.2018.0232]
98 **Baeten KM**, Akassoglou K. Extracellular matrix and matrix receptors in blood-brain barrier formation and stroke. *Dev Neurobiol* 2011; **71**: 1018-1039 [PMID: 21780303 DOI: 10.1002/dneu.20954]
99 **Kazanis I**, ffrench-Constant C. Extracellular matrix and the neural stem cell niche. *Dev Neurobiol* 2011; **71**: 1006-1017 [PMID: 21898854 DOI: 10.1002/dneu.20970]
100 **Saino O**, Taguchi A, Nakagomi T, Nakano-Doi A, Kashiwamura S, Doe N, Nakagomi N, Soma T, Yoshikawa H, Stern DM, Okamura H, Matsuyama T. Immunodeficiency reduces neural stem/progenitor cell apoptosis and enhances neurogenesis in the cerebral cortex after stroke. *J Neurosci Res* 2010; **88**: 2385-2397 [PMID: 20623538 DOI: 10.1002/jnr.22410]
101 **Takata M**, Nakagomi T, Kashiwamura S, Nakano-Doi A, Saino O, Nakagomi N, Okamura H, Mimura O, Taguchi A, Matsuyama T. Glucocorticoid-induced TNF receptor-triggered T cells are key modulators for survival/death of neural stem/progenitor cells induced by ischemic stroke. *Cell Death Differ* 2012; **19**: 756-767 [PMID: 22052192 DOI: 10.1038/cdd.2011.145]

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REVIEW

Orchestrating stem cell fate: Novel tools for regenerative medicine

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Abstract

Mesenchymal stem cells are undifferentiated cells able to acquire different phenotypes under specific stimuli. *In vitro* manipulation of these cells is focused on understanding stem cell behavior, proliferation and pluripotency. Latest advances in the field of stem cells concern epigenetics and its role in maintaining self-renewal and differentiation capabilities. Chemical and physical stimuli can modulate cell commitment, acting on gene expression of Oct-4, Sox-2 and Nanog, the main stemness markers, and tissue-lineage specific genes. This activation or repression is related to the activity of chromatin-remodeling factors and epigenetic regulators, new targets of many cell therapies. The aim of this review is to afford a view of the current state of *in vitro* and *in vivo* stem cell applications, highlighting the strategies used to influence stem cell commitment for current and future cell therapies. Identifying the molecular mechanisms controlling stem cell fate could open up novel strategies for tissue repairing processes and other clinical applications.

Key words: Stem cells; Epigenetics; Self-renewal; *In vitro* differentiation; Physical stimuli; Stem cell fate; Clinical practice; Cell transplantation

Core tip: The latest advances in the field of stem cells concern epigenetics and its role in

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self-renewal and differentiation capability. Activation or silencing of genes controlling stemness and tissue-lineage specification are related to chromatin-remodeling factors and epigenetic regulators. In this review, we focused on the principal epigenetic markers that regulate stem cell pluripotency, *in vitro* manipulation and the current state-of-the-art *in vivo* applications of human mesenchymal stem cells.

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INTRODUCTION

Stem cells are known for their self-renewal and their capability to differentiate into various lineages, participating in tissue regeneration after damage[1]. Since human embryonic stem cells (ESCs) are isolated from the inner cell mass of the blastocyst[2] their application *in vitro* and *in vivo* is burdened by ethical issues, causing researchers to turn their interests toward other sources[3,4]. Mesenchymal stem cells, defined by other authors as mesenchymal stromal cells[5], have shown a high proliferative potential *in vitro*, being identified as the elements that maintain the bone marrow microenvironment, improve hematopoiesis and give rise to various cell lineages[6,7]. The most common source for human mesenchymal stem cells (hMSCs) is the bone marrow, usually obtained from the iliac crest of adult patients. Bone marrow-derived stem cells (BM-MSCs) can be separated from the tissue by centrifugation in a density gradient media and, once placed in culture, they can be easily induced to differentiate towards different phenotypes[8]. MSCs are found in many other adult tissues, including the dental pulp[9], adipose tissue (ASCs)[10], umbilical cord blood[11] and Wharton's jelly of umbilical cord[12]. Despite some differences in terms of growth kinetics and pluripotency, donor age- and -gender-related features[13,14], MSCs can differentiate under a variety of external cues, acting to replace damaged cells and maintain tissue homeostasis[15]. In order to reduce manipulation of the stromal fraction, minimize enzymatic digestion and ensure maximum yield in culture, the interest of researchers has turned to the optimization of MSC isolation protocols[16,17]. In particular, new devices have been developed for adipose tissue, based only upon mechanical forces, thus allowing a micro-fragmented tissue fraction in one-step that is enriched in hMSCs and pericytes[18,19]. Stem cells represent an important model to study the molecular pathways involved in disease onset and progression and to develop drug delivery system and differentiation processes, in view of a successful application in tissue engineering and clinical practice[20,21]. In this review, we summarize the influence of specific chemical and physical agents able to affect stem cell behavior and fate, pointing out the current development of hMSCs applications *in vivo*.

EPIGENETIC REGULATION OF SELF-RENEWAL AND PLURIPOTENCY

Stem cell differentiation is an essential complex process involved in the maintenance of tissues homeostasis, being in turn orchestrated by a wide range of signaling pathways[22]. *In vitro* differentiation involves different molecular mechanisms influencing the expression of the main markers of stemness: Octamer-binding transcription factor 4 (Oct-4), sex determining region Y-box 2 (Sox-2) and Homeobox protein Nanog[23,24]. These transcription factors are essential for maintaining stem cell pluripotency and are also involved in adult somatic cell reprogramming[25,26].

Epigenetics refers to the range of heritable changes in the structure of chromatin able to affect gene expression and represents the molecular reaction to all the environmental changes[27]. These chromatin modifications are orchestrated by different kind of enzymes, such as DNA methyltransferases (DNMTs), or enzymes controlling post-translational histone modification, as Histone deacetylase (HDACs) and histone acetyltransferases[28]. Epigenetic mechanisms are involved in the progression from the undifferentiated to differentiated state, through silencing of self-

renewal genes and activation of differentiation markers. The onset of these specific gene expression patterns is stimulated by developmental and environmental stimuli, causing changes in the chromatin structure, thus allowing a specific transcriptional program, with a mechanism not fully clarified yet[29-31]. Therefore, epigenetics has a central role not only during embryogenesis but also in maintaining tissue homeostasis and controlling the regenerative potential through adulthood[32]. Wang *et al*[33] demonstrated that HDAC6 takes part in dental MSC differentiation and osteoblast maturation by maintaining dental and periodontal tissue homeostasis. Interactions between the HDAC Sirtuin 6 (Sirt6) and Ten-eleven translocation (Tet) enzyme are directly involved in the regulation of Oct-4, Sox-2 and Nanog genes, finely tuning pluripotency and differentiation balance in ESCs[34]. Santaniello *et al*[35] (2018) demonstrated that a combination of melatonin and vitamin D activates HDAC1 and the (NAD)-dependent deacetylases Sirtuins 1 and 2 in ASCs. The final effect was an inhibition of adipogenic differentiation, even when cells were cultured in a medium able to prime adipogenic differentiation[35].

Exposure of human amniotic fluid stem cells to DNMT inhibitors induces cardiomyogenic differentiation via chromatin remodeling, upregulation of cardiac-related genes and repression of HDAC1 expression[36]. In addition, a combination of DNMT and HDAC inhibitors counteracts cancer stem cell growth, reducing the tumor mass in mouse mammary tumor models, thus increasing mice survival, and unfolding novel epigenetic-based therapies for drug-resistant breast cancer[37]. DNA methylation plays a key role in maintaining the undifferentiated state in stem cells by silencing the differentiation genes, and it is also implicated in somatic cell reprogramming[38,39]. All of these classes of enzymes promote changes in chromatin structure, exerting a crucial role in regulating the balance between pluripotency and differentiation[40]. On the whole, continuous efforts to unravel epigenetic regulation holds promise for continuous innovation in strategies aimed at controlling stem cell pluripotency and tissue homeostasis. MicroRNAs (miRNAs), small non-coding RNAs, have been discovered as regulators of different signaling pathways, stem cell pluripotency and somatic cell reprogramming[41]. The modulation of cell differentiation by miRNAs could be used to treat various kind of diseases, including myocardial infarction, neurodegenerative and muscle diseases[42]. Moreover, epigenetic mechanisms could unravel many deregulated cellular dynamics, as those involved in cancer, aging and age-related diseases[43] (Figure 1).

IN VITRO MODULATION OF STEM CELL BEHAVIOR

In the last years, several molecules capable of orchestrating the multilineage repertoire of stem cells have been largely used to generate specific conditioned media[44,45]. Within this context, some authors found that medium conditioned by factors such as activin A, bone morphogenetic protein 4 (BMP4), vascular endothelial growth factor (VEGF) or Dickkopf-related protein 1, can optimize cardiac development in mouse and human stem cell lines[46,47]. BMP4 itself, in combination with inhibitors of the Activin/Nodal signaling pathways, induces differentiation of ESCs into trophoblastic cells, which show similar trophectoderm profile and are able to secrete placental hormones[48]. Concerning the use of chemistry to push stem cells to specific phenotypes, molecules that can affect the epigenetic code to activate a molecular differentiation program have largely been used. Ventura *et al*[49,50] described for the first time how a hyaluronan mixed ester of butyric and retinoic acids (HBR) increases the transcription of cardiogenic genes, acting through the epigenetic regulation of a cardiogenesis program *in vitro*. HBR was also able to promote cardiac regeneration in infarcted rat hearts, decreasing the number of apoptotic cardiomyocytes without the need for stem cell transplantation[49-52]. More recently, a mixture of HBR and melatonin was successfully employed to induce an osteogenic phenotype in dental pulp stem cells, suggesting the use of this cocktail for future *in vivo* orthopedic and dental applications[53].

MODULATION OF STEM CELL COMMITMENT BY PHYSICAL STIMULI

Electromagnetic fields can interact with cells, tissues and biological systems in general[54,55] and are able to influence phenotypic features, gene expression patterns and differentiation in MSCs, acting in a dose and time-dependent manner[56,57]. It has been shown that 7 d of MSC growth on an electroconductive polymeric substrate was sufficient to promote Nestin and β-3 Tubulin upregulation and the appearance of

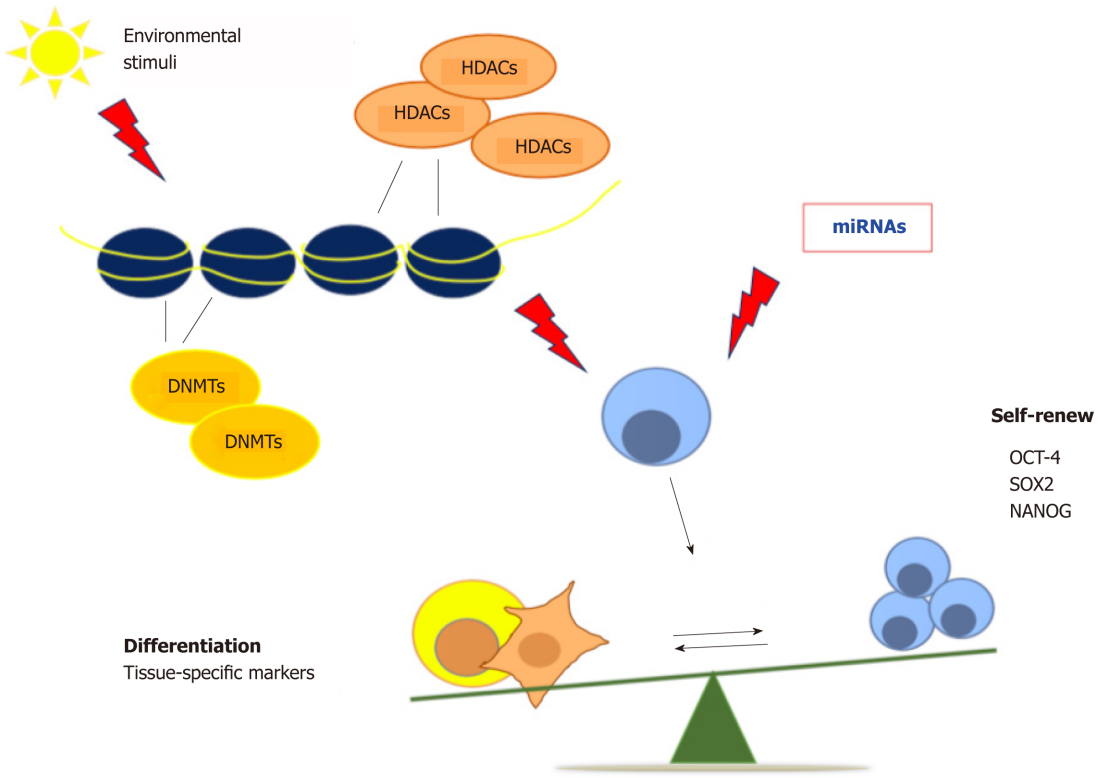

Figure 1 Epigenetic regulation of stem cell fate. Chromatin remodeling affects cell behavior and regulates the balance between pluripotency and differentiation. HDACs: Histone deacetylases; Oct-4: Octamer-binding transcription factor 4; Sox-2: Sex determining region Y-box 2; NANOG: Nanog homeobox.

neural-like morphological extensions[58]. MSCs can be employed to improve cartilage regeneration[59]. Synthetic scaffolds and biopolymers are incorporated in stem cell cultures to induce their growth, mimicking the stem cell niche[60]. Biomaterials provide a physical environment that can control cell function. The interaction between stem cells and these surfaces modulates multiple processes such as cell migration, proliferation and differentiation, as well as extracellular matrix deposition, providing dynamic signaling able to regulate cell behavior[61,62]. Non-invasive electrical stimulation therapy exerts an important role in controlling calcium channels, thus regulating the intracellular calcium concentration during chondrogenic and osteogenic stem cell differentiation, and opening novel approaches to improve tissue repair *in vivo*[63,64]. Extracorporeal shock wave therapy (ESWT) is largely used to treat orthopedic diseases, including tendinopathies or bone disorders, as well as wound healing stimulation in radiation-damaged skin[65,66]. ESWT stimulates angiogenesis, neovascularization, and recruitment of MSCs, inducing their proliferation and differentiation. These processes have been shown to involve ATP release and increased extracellular signal–regulated kinases Erk1/2 and p38 MAPK activation, which is responsible for the proliferative and reparative effects[67]. Human and rat ASCs exposed to repetitive ESWT retained all cell surface markers and exhibited increased multipotency into osteogenic and adipogenic lineages[68].

Radio electric fields asymmetrically conveyed by a medical device, referred to radioelectric asymmetric conveyer (REAC), are able to induce the transcription of GATA-4, Nkx-2.5, VEGF, hepatocyte growth factor (HGF), Von Willebrand factor (vWF), neurogenin-1, and myoD, genes orchestrating different tissue lineages, both in mouse embryonic and human adult stem cells[69,70]. Moreover, REAC exposure counteracted MSC senescence by downregulating the expression of p16INK4, ARF, p53, and p21, involved in cell cycle regulation, reducing the number of senescence associated-beta-galactosidase positive cells, while also preserving TERT expression and telomere length[71-74]. Radio electric conveyed fields allowed for the direct reprogramming of human skin fibroblasts toward cardiac and neurogenic lineages and synergistically enhanced the cardiogenic commitment in induced pluripotent stem cells (iPSCs) cultured in cardiogenic medium[47,75]. In addition, radio electric conveyed fields were sufficient to induce the neurogenic phenotype in PC12 cells, a model for dopaminergic neuron studies[76]. Finally, concerning cell reprogramming, several authors have shown that mechanical stimuli such as equiaxial stretching have an important role in reprogramming somatic cells into iPSCs, with the formation of a

great number of iPSC colonies without using common viral mediated gene transduction[77]. These findings showed the prominence of physical stimuli in opening up new strategies for cell manipulation and regenerative medicine[78,79].

BIOACTIVE MOLECULES IN ORCHESTRATING CELL DIFFERENTIATION

The use of nutraceuticals has recently been largely employed in regenerative medicine.

A wide range of natural molecules and compounds has been described as capable to orchestrate stem cell commitment. Known as nutraceuticals or functional foods, these molecules are largely used for their therapeutic or preventive effects[80,81]. Melatonin, the hormone secreted by the pineal gland, regulates many physiological functions such as circadian rhythm, hemostasis and the immune system. An alteration in its secretion is related to the onset of pathological manifestations[82,83]. *In vitro* studies with MSCs demonstrated that melatonin exerts anti-oxidant and anti-apoptotic effects, regulating the expression of pro- and anti-apoptotic proteins, ameliorating the outcome of stem cell transplantation[84,85]. Mendivil-Perez *et al*[86] demonstrated that melatonin in transplanted mice was able to induce proliferation and differentiation of neural stem cells into oligodendrocytes and astrocytes, reducing oxidative stress produced by mitochondrial activity. Oxidative stress has a crucial role in osteogenesis inhibition and in aging-related osteoporosis[87]. MSCs exposed to melatonin exhibit increased calcium stores and osteogenic differentiation. These events include the recruitment of AMP-activated protein kinase (AMPK), Runt-related transcription factor 2 and Forkhead box O3, with the latter usually being downregulated under stress conditions[88]. AMPK activation is also involved in the regulation of adipogenesis. It regulates the expression of peroxisome proliferator-activated receptor γ (PPARγ), the main adipogenic orchestrator gene and a molecular target of natural compounds used in obesity management[89,90].In combination with other molecules, including vitamin D, melatonin has a synergistic effect on inhibiting adipogenesis[91]. The active form of Vitamin D is calcitriol, which is naturally synthesized following sun exposure or taken as dietary supplements. It controls calcium metabolism, apoptosis, and stimulates macrophages and immune responses[92,93]. When ASCs are cultured in the presence of melatonin and vitamin D in adipogenic-conditioned medium, adipogenic differentiation is blocked. This inhibitory effect is through the downregulation of specific genes controlling adipogenesis, protein contents, and fat depots[91]. Moreover, the synergistic effect of these two molecules epigenetically modulates ASC commitment towards osteogenic differentiation through the activation of HDAC1 and SIRT1, even in the presence of adipogenic conditions[35]. Natural compounds can therefore be considered potent differentiating agents able to drive cell proliferation and apoptosis resistance by epigenetic regulations and post-transcriptional modifications[94,95]. At the same time, they can act as anti-proliferative agents against many tumor cells, including hepatocarcinoma cells, without affecting the cell cycle or viability of non-cancer cells, thus representing novel specific tools for cancer prevention[96,97] (Figure 2).

FROM BENCH TO BEDSIDE

MSCs have largely attracted the attention of clinicians in regenerative medicine for their easy expansion and differentiation potential, avoiding the ethical issues related to the use of ESCs[98,99]. Stem cells are currently applied in gene therapy and treatment of serious pathologies, sometimes representing the only alternative to conventional treatments, to slow down the progression of the disease and improve life qualities of the patients[100,101]. Moreover, when transplanted in both autologous and allogenic fashion, MSCs can migrate into the damaged tissue to control inflammation and immune responses[102]. The use of stem cells represents the most frequently applied cell therapy in hematological diseases[103], although with the risk of rejection and potential failure[104]. Starting with allogenic bone marrow transplantation in 1957[105], stem cell therapy nowadays represent the main actor in many different clinical trials for several diseases, such as neurological diseases like amyotrophic lateral sclerosis (commonly known as ALS)[106].

BONE MARROW HEMATOPOIETIC STEM CELLS IN

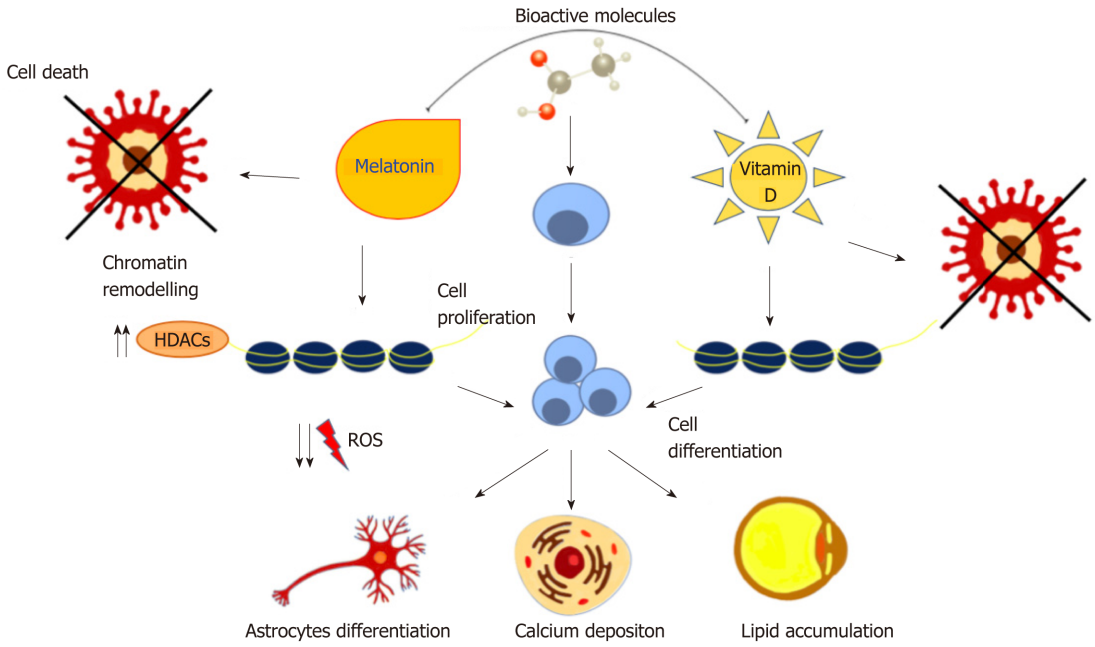

Figure 2 Natural molecules and stem cell fate. Bioactive molecules induce cell proliferation and differentiation, reducing ROS production and apoptosis, through chromatin remodeling and epigenetic modifications. ROS: Reactive oxygen species; HDACs: Histone deacetylases.

CLINICAL PRACTICE

MSCs are multipotent cells that are able to differentiate into different lineages, and they can also be easily expanded for clinical practice[107]. Bone marrow is a mesenchymal specialized connective tissue composed of progenitor cells that can undergo adipogenic, osteogenic, chondrogenic and myogenic differentiation[108]. Thus, bone represents a microenvironment in which hematopoietic stem cells (HSCs) can maintain their undifferentiated state and participate in hematopoiesis when exposed to different stimuli[109]. Hematopoiesis is a complex process, during which HSCs undergo asymmetric division to become progenitor blood and bone marrow cells, as erythrocytes, lymphocytes and monocytes[110]. HSC self-renewal potential is regulated by different signaling pathways. Among them, physiological Notch signaling is required for bone formation, regulates the HSC microenvironment and cell fate decisions, and is also associated with tumorigenic potential and leukemia when dysregulated[111]. Moreover, the crosstalk between Notch and Wnt signaling is crucial for tissue development and turnover[112]. Wnt/β-catenin signal is essential for HSC growth and homeostasis *in vitro* and *in vivo*, and its inhibition causes cell growth arrest with a related decline in self-renewal potential of stem cells. On the other hand, activation of Wnt patterning increases Notch expression and supports the self-renewal potential of progenitor cells from different tissues, suppressing differentiation[113,114]. Alterations in signaling pathways and normal microenvironment play a crucial role in the development of hematopoietic diseases, such as chronic and acute myeloid leukemia[115]. HSCs are employed as therapeutic tools in stem cell transplantations[116] due to their immunomodulatory properties, secretion of growth factors and regeneration of injured tissues, especially in patients refractory to conventional chemotherapy[117]. Autologous transplantations are used in leukemia, lymphomas, multiple myeloma and other hematological malignancies[118]. There are several retrospective studies in which patients were monitored after 10-12 years from the transplant to evaluate survival and transplant-related mortality[119-121]. HSC transplantation was shown to be effective in counteracting the progression of the disease, notably at the early stages of disease[122].

MSC TRANSPLANTATION FOR AMYOTROPHIC LATERAL SCLEROSIS

ALS is the most frequent neurodegenerative dysfunction of the midlife[123]. ALS is characterized by progressive degeneration of spinal cord motor neurons, muscle paralysis and death in 3-5 years due to respiratory failure. Degeneration involves

toxicity and inflammatory processes associated with proliferation of resident cellular populations[124]. Genetic and epigenetic risk factors are certainly the main causes related to progression of the disease. Superoxide dismutase 1 (SOD1), which encodes Cu/Zn superoxide dismutase 1, was the first gene whose alteration was associated with ALS. Its mutation is related to protein misfolding and loss-of-function, and it is found in many familiar forms[125,126]. Misfolded proteins have a central role in neurodegenerative disease, since in their abnormally aggregated forms, cellular proteins are prevented from exerting their essential roles in RNA binding/metabolism and cellular homeostasis[127]. MicroRNAs (miRNAs) are able to regulate gene expression and promote or repress mRNA stabilization through post-transcriptional modification and by binding specific targets[128]. MiRNAs are involved in different physiological mechanisms, such as cell growth and apoptotic processes, while orchestrating pluripotency and differentiation in stem cells[129]. Altered miRNA expression in the skeletal muscle is related to neurological symptoms and disease progression. Some *in vivo* and *in vitro* studies have described how MiR-206 is enrolled upon muscle denervation in the attempt to regenerate neuromuscular synapses, highlighting the role of this miRNA in different stages of ALS progression[130,131]. Actually, there are no curative therapies for ALS. While drugs that suppress oxidative stress can be used to try to maintain motor neuron function[132] to slightly increase patient survival, novel compounds are now being tested[133]. An alternative to conventional therapy may be autologous MSC transplantation. Stem cells, thanks to their immunomodulatory properties, secrete neurotrophic factors and other anti-inflammatory cytokines, thus supporting motor neuron survival and functionality[134,135]. Notwithstanding, bone marrow is the most common source for MSCs, Wharton jelly, umbilical cord blood and in particular ASCs, represent a valid alternative in ALS therapy[136], due to their efficient isolation and high toleration by the patients.

In several clinical studies, patients received intravenous injection of MSCs while being monitored at regular time intervals. In all trials, autologous cell therapy proved to be a safe procedure. The recipient tissues did not exhibit any structural changes, tumor formation or toxicity related to transplantation, while it was shown to be effective in counteracting disease progression, improving the quality of patient's life[137-139].

CONCLUSION

Epigenetic regulators were identified as new promising therapeutic targets in patients with hematological, breast cancer and other malignancies, as well as in neurodegenerative diseases[140,141]. The rescuing potential of stem cells is under control of different kinds of signals, including the environment, which epigenetically regulate their differentiation processes[142]. Understanding the molecular pathways involved in stem cell fate is critical to develop novel tools for both the prevention and treatment of a variety of diseases, with great impact in regenerative medicine, bioengineering and clinical transplantation.

REFERENCES

1 **Biehl JK**, Russell B. Introduction to stem cell therapy. *J Cardiovasc Nurs* 2009; **24**: 98-103; quiz 104-5 [PMID: 19242274 DOI: 10.1097/JCN.0b013e318197a6a5]

2 **Fischbach GD**, Fischbach RL. Stem cells: science, policy, and ethics. *J Clin Invest* 2004; **114**: 1364-1370 [PMID: 15545983 DOI: 10.1172/JCI23549]

3 **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]

4 **Wood A**. Ethics and embryonic stem cell research. *Stem Cell Rev* 2005; **1**: 317-324 [PMID: 17142874 DOI: 10.1385/SCR:1:4:317]

5 **Mizukami A**, Swiech K. Mesenchymal Stromal Cells: From Discovery to Manufacturing and Commercialization. *Stem Cells Int* 2018; **2018**: 4083921 [PMID: 30057622 DOI: 10.1155/2018/4083921]

6 **Kolf CM**, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther* 2007; **9**: 204 [PMID: 17316462 DOI: 10.1186/ar2116]

7 **Ding DC**, Shyu WC, Lin SZ. Mesenchymal stem cells. *Cell Transplant* 2011; **20**: 5-14 [PMID: 21396235 DOI: 10.3727/096368910X]

8 **Baghaei K**, Hashemi SM, Tokhanbigli S, Asadi Rad A, Assadzadeh-Aghdaei H, Sharifian A, Zali MR. Isolation, differentiation, and characterization of mesenchymal stem cells from human bone marrow. *Gastroenterol Hepatol Bed Bench* 2017; **10**: 208-213 [PMID: 29118937]

9 **Gronthos S**, Brahim 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]

Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. Adipose-derived stem cells: isolation, expansion and

10 differentiation. *Methods* 2008; **45**: 115-120 [PMID: 18593609 DOI: 10.1016/j.ymeth.2008.03.006]
11 **Lee OK**, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004; **103**: 1669-1675 [PMID: 14576065 DOI: 10.1182/blood-2003-05-1670]
12 **Ranjbaran H**, Abediankenari S, Mohammadi M, Jafari N, Khalilian A, Rahmani Z, Momeninezhad Amiri M, Ebrahimi P. Wharton's Jelly Derived-Mesenchymal Stem Cells: Isolation and Characterization. *Acta Med Iran* 2018; **56**: 28-33 [PMID: 29436792]
13 **Siegel G**, Kluba T, Hermanutz-Klein U, Bieback K, Northoff H, Schäfer R. Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. *BMC Med* 2013; **11**: 146 [PMID: 23758701 DOI: 10.1186/1741-7015-11-146]
14 **Balzano F**, Bellu E, Basoli V, Dei Giudici S, Santaniello S, Cruciani S, Facchin F, Oggiano A, Capobianco G, Dessole F, Ventura C, Dessole S, Maioli M. Lessons from human umbilical cord: gender differences in stem cells from Wharton's jelly. *Eur J Obstet Gynecol Reprod Biol* 2019; **234**: 143-148 [PMID: 30690190 DOI: 10.1016/j.ejogrb.2018.12.028]
15 **Chen Q**, Shou P, Zheng C, Jiang M, Cao G, Yang Q, Cao J, Xie N, Velletri T, Zhang X, Xu C, Zhang L, Yang H, Hou J, Wang Y, Shi Y. Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? *Cell Death Differ* 2016; **23**: 1128-1139 [PMID: 26868907 DOI: 10.1038/cdd.2015.168]
16 **Aronowitz JA**, Lockhart RA, Hakakian CS. Mechanical versus enzymatic isolation of stromal vascular fraction cells from adipose tissue. *Springerplus* 2015; **4**: 713 [PMID: 26636001 DOI: 10.1186/s40064-015-1509-2]
17 **Chaput B**, Bertheuil N, Escubes M, Grolleau JL, Garrido I, Laloze J, Espagnolle N, Casteilla L, Sensebé L, Varin A. Mechanically Isolated Stromal Vascular Fraction Provides a Valid and Useful Collagenase-Free Alternative Technique: A Comparative Study. *Plast Reconstr Surg* 2016; **138**: 807-819 [PMID: 27307342 DOI: 10.1097/PRS.0000000000002494]
18 **Bianchi F**, Maioli M, Leonardi E, Olivi E, Pasquinelli G, Valente S, Mendez AJ, Ricordi C, Raffaini M, Tremolada C, Ventura C. A new nonenzymatic method and device to obtain a fat tissue derivative highly enriched in pericyte-like elements by mild mechanical forces from human lipoaspirates. *Cell Transplant* 2013; **22**: 2063-2077 [PMID: 23051701 DOI: 10.3727/096368912X657855]
19 **Coccè V**, Brini A, Giannì AB, Sordi V, Berenzi A, Alessandri G, Tremolada C, Versari S, Bosetto A, Pessina A. A Nonenzymatic and Automated Closed-Cycle Process for the Isolation of Mesenchymal Stromal Cells in Drug Delivery Applications. *Stem Cells Int* 2018; **2018**: 4098140 [PMID: 29531535 DOI: 10.1155/2018/4098140]
20 **Tuan RS**, Boland G, Tuli R. Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther* 2003; **5**: 32-45 [PMID: 12716446 DOI: 10.1186/ar614]
21 **Nöth U**, Rackwitz L, Steinert AF, Tuan RS. Cell delivery therapeutics for musculoskeletal regeneration. *Adv Drug Deliv Rev* 2010; **62**: 765-783 [PMID: 20398712 DOI: 10.1016/j.addr.2010.04.004]
22 **Biteau B**, Hochmuth CE, Jasper H. Maintaining tissue homeostasis: dynamic control of somatic stem cell activity. *Cell Stem Cell* 2011; **9**: 402-411 [PMID: 22056138 DOI: 10.1016/j.stem.2011.10.004]
23 **Wang Z**, Oron E, Nelson B, Razis S, Ivanova N. Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. *Cell Stem Cell* 2012; **10**: 440-454 [PMID: 22482508 DOI: 10.1016/j.stem.2012.02.016]
24 **Gagliardi A**, Mullin NP, Ying Tan Z, Colby D, Kousa AI, Halbritter F, Weiss JT, Felker A, Bezstarosti K, Favaro R, Demmers J, Nicolis SK, Tomlinson SR, Poot RA, Chambers I. A direct physical interaction between Nanog and Sox2 regulates embryonic stem cell self-renewal. *EMBO J* 2013; **32**: 2231-2247 [PMID: 23892456 DOI: 10.1038/emboj.2013.161]
25 **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]
26 **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]
27 **Laird PW**. Principles and challenges of genomewide DNA methylation analysis. *Nat Rev Genet* 2010; **11**: 191-203 [PMID: 20125086 DOI: 10.1038/nrg2732]
28 **Boland MJ**, Nazor KL, Loring JF. Epigenetic regulation of pluripotency and differentiation. *Circ Res* 2014; **115**: 311-324 [PMID: 24989490 DOI: 10.1161/CIRCRESAHA.115.301517]
29 **Cheung P**, Allis CD, Sassone-Corsi P. Signaling to chromatin through histone modifications. *Cell* 2000; **103**: 263-271 [PMID: 11057899 DOI: 10.1016/S0092-8674(00)00118-5]
30 **Fischle W**, Wang Y, Allis CD. Histone and chromatin cross-talk. *Curr Opin Cell Biol* 2003; **15**: 172-183 [PMID: 12648673 DOI: 10.1016/S0955-0674(03)00013-9]
31 **Wu H**, Sun YE. Epigenetic regulation of stem cell differentiation. *Pediatr Res* 2006; **59**: 21R-25R [PMID: 16549544 DOI: 10.1203/01.pdr.0000203565.76028.2a]
32 **Rinaldi L**, Benitah SA. Epigenetic regulation of adult stem cell function. *FEBS J* 2015; **282**: 1589-1604 [PMID: 25060320 DOI: 10.1111/febs.12946]
33 **Wang Y**, Shi ZY, Feng J, Cao JK. HDAC6 regulates dental mesenchymal stem cells and osteoclast differentiation. *BMC Oral Health* 2018; **18**: 190 [PMID: 30463548 DOI: 10.1186/s12903-018-0624-1]
34 **Etchegaray JP**, Chavez L, Huang Y, Ross KN, Choi J, Martinez-Pastor B, Walsh RM, Sommer CA, Lienhard M, Gladden A, Kugel S, Silberman DM, Ramaswamy S, Mostoslavsky G, Hochedlinger K, Goren A, Rao A, Mostoslavsky R. The histone deacetylase SIRT6 controls embryonic stem cell fate via TET-mediated production of 5-hydroxymethylcytosine. *Nat Cell Biol* 2015; **17**: 545-557 [PMID: 25915124 DOI: 10.1038/ncb3147]
35 **Santaniello S**, Cruciani S, Basoli V, Balzano F, Bellu E, Garroni G, Ginesu GC, Cossu ML, Facchin F, Delitala AP, Ventura C, Maioli M. Melatonin and Vitamin D Orchestrate Adipose Derived Stem Cell Fate by Modulating Epigenetic Regulatory Genes. *Int J Med Sci* 2018; **15**: 1631-1639 [PMID: 30588186 DOI: 10.7150/ijms.27669]
36 **Gasiūnienė M**, Zubova A, Utkus A, Navakauskienė R. Epigenetic and metabolic alterations in human amniotic fluid stem cells induced to cardiomyogenic differentiation by DNA methyltransferases and p53 inhibitors. *J Cell Biochem* 2018 [PMID: 30485506 DOI: 10.1002/jcb.28092]
37 **Pathania R**, Ramachandran S, Mariappan G, Thakur P, Shi H, Choi JH, Manicassamy S, Kolhe R, Prasad PD, Sharma S, Lokeshwar BL, Ganapathy V, Thangaraju M. Combined Inhibition of DNMT and HDAC Blocks the Tumorigenicity of Cancer Stem-like Cells and Attenuates Mammary Tumor Growth. *Cancer Res* 2016; **76**: 3224-3235 [PMID: 27197203 DOI: 10.1158/0008-5472.CAN-15-2249]

38 **Khavari DA**, Sen GL, Rinn JL. DNA methylation and epigenetic control of cellular differentiation. *Cell Cycle* 2010; **9**: 3880-3883 [PMID: 20890116 DOI: 10.4161/cc.9.19.13385]
39 **Cheng Y**, Xie N, Jin P, Wang T. DNA methylation and hydroxymethylation in stem cells. *Cell Biochem Funct* 2015; **33**: 161-173 [PMID: 25776144 DOI: 10.1002/cbf.3101]
40 **Keenen B**, de la Serna IL. Chromatin remodeling in embryonic stem cells: regulating the balance between pluripotency and differentiation. *J Cell Physiol* 2009; **219**: 1-7 [PMID: 19097034 DOI: 10.1002/jcp.21654]
41 **Gangaraju VK**, Lin H. MicroRNAs: key regulators of stem cells. *Nat Rev Mol Cell Biol* 2009; **10**: 116-125 [PMID: 19165214 DOI: 10.1038/nrm2621]
42 **Li N**, Long B, Han W, Yuan S, Wang K. microRNAs: important regulators of stem cells. *Stem Cell Res Ther* 2017; **8**: 110 [PMID: 28494789 DOI: 10.1186/s13287-017-0551-0]
43 **Beerman I**, Rossi DJ. Epigenetic Control of Stem Cell Potential during Homeostasis, Aging, and Disease. *Cell Stem Cell* 2015; **16**: 613-625 [PMID: 26046761 DOI: 10.1016/j.stem.2015.05.009]
44 **Rodrigues M**, Griffith LG, Wells A. Growth factor regulation of proliferation and survival of multipotential stromal cells. *Stem Cell Res Ther* 2010; **1**: 32 [PMID: 20977782 DOI: 10.1186/scrt32]
45 **Hwang KC**, Kim JY, Chang W, Kim DS, Lim S, Kang SM, Song BW, Ha HY, Huh YJ, Choi IG, Hwang DY, Song H, Jang Y, Chung N, Kim SH, Kim DW. Chemicals that modulate stem cell differentiation. *Proc Natl Acad Sci U S A* 2008; **105**: 7467-7471 [PMID: 18480249 DOI: 10.1073/pnas.0802825105]
46 **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]
47 **Basoli V**, Santaniello S, Rinaldi S, Fontani V, Pigliaru G, Wieser M, Strajeriu A, Castagna A, Redl H, Ventura C, Grillari R, Maioli M. Physical stimulation by REAC and BMP4/WNT-1 inhibitor synergistically enhance cardiogenic commitment in iPSCs. *PLoS One* 2019; **14**: e0211188 [PMID: 30673752 DOI: 10.1371/journal.pone.0211188]
48 **Wang J**, Anguera MC. In Vitro Differentiation of Human Pluripotent Stem Cells into Trophoblastic Cells. *J Vis Exp* 2017 [PMID: 28362386 DOI: 10.3791/55268]
49 **Ventura C**, Maioli M, Asara Y, Santoni D, Scarlata I, Cantoni S, Perbellini A. Butyric and retinoic mixed ester of hyaluronan. A novel differentiating glycoconjugate affording a high throughput of cardiogenesis in embryonic stem cells. *J Biol Chem* 2004; **279**: 23574-23579 [PMID: 15044487 DOI: 10.1074/jbc.M401869200]
50 **Ventura C**, Cantoni S, Bianchi F, Lionetti V, Cavallini C, Scarlata I, Foroni L, Maioli M, Bonsi L, Alviano F, Fossati V, Bagnara GP, Pasquinelli G, Recchia FA, Perbellini A. Hyaluronan mixed esters of butyric and retinoic Acid drive cardiac and endothelial fate in term placenta human mesenchymal stem cells and enhance cardiac repair in infarcted rat hearts. *J Biol Chem* 2007; **282**: 14243-14252 [PMID: 17363374 DOI: 10.1074/jbc.M609350200]
51 **Lionetti V**, Cantoni S, Cavallini C, Bianchi F, Valente S, Frascari I, Olivi E, Aquaro GD, Bonavita F, Scarlata I, Maioli M, Vaccari V, Tassinari R, Bartoli A, Recchia FA, Pasquinelli G, Ventura C. Hyaluronan mixed esters of butyric and retinoic acid affording myocardial survival and repair without stem cell transplantation. *J Biol Chem* 2010; **285**: 9949-9961 [PMID: 20097747 DOI: 10.1074/jbc.M109.087254]
52 **Maioli M**, Santaniello S, Montella A, Bandiera P, Cantoni S, Cavallini C, Bianchi F, Lionetti V, Rizzolio F, Marchesi I, Bagella L, Ventura C. Hyaluronan esters drive Smad gene expression and signaling enhancing cardiogenesis in mouse embryonic and human mesenchymal stem cells. *PLoS One* 2010; **5**: e15151 [PMID: 21152044 DOI: 10.1371/journal.pone.0015151]
53 **Maioli M**, Basoli V, Santaniello S, Cruciani S, Delitala AP, Pinna R, Milia E, Grillari-Voglauer R, Fontani V, Rinaldi S, Muggironi R, Pigliaru G, Ventura C. Osteogenesis from Dental Pulp Derived Stem Cells: A Novel Conditioned Medium Including Melatonin within a Mixture of Hyaluronic, Butyric, and Retinoic Acids. *Stem Cells Int* 2016; **2016**: 2056416 [PMID: 26880937 DOI: 10.1155/2016/2056416]
54 **Santini MT**, Rainaldi G, Indovina PL. Cellular effects of extremely low frequency (ELF) electromagnetic fields. *Int J Radiat Biol* 2009; **85**: 294-313 [PMID: 19399675 DOI: 10.1080/09553000902781097]
55 **Collodel G**, Fioravanti A, Pascarelli NA, Lamboglia A, Fontani V, Maioli M, Santaniello S, Pigliaru G, Castagna A, Moretti E, Iacoponi F, Rinaldi S, Ventura C. Effects of regenerative radioelectric asymmetric conveyer treatment on human normal and osteoarthritic chondrocytes exposed to IL-1β. A biochemical and morphological study. *Clin Interv Aging* 2013; **8**: 309-316 [PMID: 23682210 DOI: 10.2147/CIA.S42229]
56 **Maziarz A**, Kocan B, Bester M, Budzik S, Cholewa M, Ochiya T, Banas A. How electromagnetic fields can influence adult stem cells: positive and negative impacts. *Stem Cell Res Ther* 2016; **7**: 54 [PMID: 27086866 DOI: 10.1186/s13287-016-0312-5]
57 **Ventura C**, Maioli M, Asara Y, Santoni D, Mesirca P, Remondini D, Bersani F. Turning on stem cell cardiogenesis with extremely low frequency magnetic fields. *FASEB J* 2005; **19**: 155-157 [PMID: 15507470 DOI: 10.1096/fj.04-2695fje]
58 **Thrivikraman G**, Madras G, Basu B. Intermittent electrical stimuli for guidance of human mesenchymal stem cell lineage commitment towards neural-like cells on electroconductive substrates. *Biomaterials* 2014; **35**: 6219-6235 [PMID: 24816362 DOI: 10.1016/j.biomaterials.2014.04.018]
59 **Vinatier C**, Mrugala D, Jorgensen C, Guicheux J, Noël D. Cartilage engineering: a crucial combination of cells, biomaterials and biofactors. *Trends Biotechnol* 2009; **27**: 307-314 [PMID: 19329205 DOI: 10.1016/j.tibtech.2009.02.005]
60 **Singh A**, Elisseeff J. Biomaterials for stem cell differentiation. *J Mater Chem* 2010; **20**: 8832-8847 [DOI: 10.1039/C0JM01613F]
61 **Ghasemi-Mobarakeh L**, Prabhakaran MP, Tian L, Shamirzaei-Jeshvaghani E, Dehghani L, Ramakrishna S. Structural properties of scaffolds: Crucial parameters towards stem cells differentiation. *World J Stem Cells* 2015; **7**: 728-744 [PMID: 26029344 DOI: 10.4252/wjsc.v7.i4.728]
62 **Nakayama KH**, Hou L, Huang NF. Role of extracellular matrix signaling cues in modulating cell fate commitment for cardiovascular tissue engineering. *Adv Healthc Mater* 2014; **3**: 628-641 [PMID: 24443420 DOI: 10.1002/adhm.201300620]
63 **Sun S**, Liu Y, Lipsky S, Cho M. Physical manipulation of calcium oscillations facilitates osteodifferentiation of human mesenchymal stem cells. *FASEB J* 2007; **21**: 1472-1480 [PMID: 17264165 DOI: 10.1096/fj.06-7153com]
64 **Fodor J**, Matta C, Oláh T, Juhász T, Takács R, Tóth A, Dienes B, Csernoch L, Zákány R. Store-operated calcium entry and calcium influx via voltage-operated calcium channels regulate intracellular calcium

oscillations in chondrogenic cells. *Cell Calcium* 2013; **54**: 1-16 [PMID: 23664335 DOI: 10.1016/j.ceca.2013.03.003]

65 **Haupt G**. Use of extracorporeal shock waves in the treatment of pseudarthrosis, tendinopathy and other orthopedic diseases. *J Urol* 1997; **158**: 4-11 [PMID: 9186313 DOI: 10.1097/00005392-199707000-00003]

66 **Mittermayr R**, Antonic V, Hartinger J, Kaufmann H, Redl H, Téot L, Stojadinovic A, Schaden W. Extracorporeal shock wave therapy (ESWT) for wound healing: technology, mechanisms, and clinical efficacy. *Wound Repair Regen* 2012; **20**: 456-465 [PMID: 22642362 DOI: 10.1111/j.1524-475X.2012.00796.x]

67 **Weihs AM**, Fuchs C, Teuschl AH, Hartinger J, Slezak P, Mittermayr R, Redl H, Junger WG, Sitte HH, Rünzler D. Shock wave treatment enhances cell proliferation and improves wound healing by ATP release-coupled extracellular signal-regulated kinase (ERK) activation. *J Biol Chem* 2014; **289**: 27090-27104 [PMID: 25118288 DOI: 10.1074/jbc.M114.580936]

68 **Schuh CM**, Heher P, Weihs AM, Banerjee A, Fuchs C, Gabriel C, Wolbank S, Mittermayr R, Redl H, Rünzler D, Teuschl AH. In vitro extracorporeal shock wave treatment enhances stemness and preserves multipotency of rat and human adipose-derived stem cells. *Cytotherapy* 2014; **16**: 1666-1678 [PMID: 25174738 DOI: 10.1016/j.jcyt.2014.07.005]

69 **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]

70 **Maioli M**, Rinaldi S, Santaniello S, Castagna A, Pigliaru G, Delitala A, Bianchi F, Tremolada C, Fontani V, Ventura C. Radioelectric asymmetric conveyed fields and human adipose-derived stem cells obtained with a nonenzymatic method and device: a novel approach to multipotency. *Cell Transplant* 2014; **23**: 1489-1500 [PMID: 24044359 DOI: 10.3727/096368913X672037]

71 **Maioli M**, Rinaldi S, Santaniello S, Castagna A, Pigliaru G, Delitala A, Lotti Margotti M, Bagella L, Fontani V, Ventura C. Anti-senescence efficacy of radio-electric asymmetric conveyer technology. *Age (Dordr)* 2014; **36**: 9-20 [PMID: 23653328 DOI: 10.1007/s11357-013-9537-8]

72 **Maioli M**, Rinaldi S, Pigliaru G, Santaniello S, Basoli V, Castagna A, Fontani V, Ventura C. REAC technology and hyaluron synthase 2, an interesting network to slow down stem cell senescence. *Sci Rep* 2016; **6**: 28682 [PMID: 27339908 DOI: 10.1038/srep28682]

73 **Rinaldi S**, Maioli M, Santaniello S, Castagna A, Pigliaru G, Gualini S, Margotti ML, Carta A, Fontani V, Ventura C. Regenerative treatment using a radioelectric asymmetric conveyor as a novel tool in antiaging medicine: an in vitro beta-galactosidase study. *Clin Interv Aging* 2012; **7**: 191-194 [PMID: 22807628 DOI: 10.2147/CIA.S33312]

74 **Rinaldi S**, Maioli M, Pigliaru G, Castagna A, Santaniello S, Basoli V, Fontani V, Ventura C. Stem cell senescence. Effects of REAC technology on telomerase-independent and telomerase-dependent pathways. *Sci Rep* 2014; **4**: 6373 [PMID: 25224681 DOI: 10.1038/srep06373]

75 **Maioli M**, Rinaldi S, Santaniello S, Castagna A, Pigliaru G, Gualini S, Cavallini C, Fontani V, Ventura C. Radio electric conveyed fields directly reprogram human dermal skin fibroblasts toward cardiac, neuronal, and skeletal muscle-like lineages. *Cell Transplant* 2013; **22**: 1227-1235 [PMID: 23057961 DOI: 10.3727/096368912X657297]

76 **Maioli M**, Rinaldi S, Migheli R, Pigliaru G, Rocchitta G, Santaniello S, Basoli V, Castagna A, Fontani V, Ventura C, Serra PA. Neurological morphofunctional differentiation induced by REAC technology in PC12. A neuro protective model for Parkinson's disease. *Sci Rep* 2015; **5**: 10439 [PMID: 25976344 DOI: 10.1038/srep10439]

77 **Kim YM**, Kang YG, Park SH, Han MK, Kim JH, Shin JW, Shin JW. Effects of mechanical stimulation on the reprogramming of somatic cells into human-induced pluripotent stem cells. *Stem Cell Res Ther* 2017; **8**: 139 [PMID: 28595633 DOI: 10.1186/s13287-017-0594-2]

78 **Baek S**, Choi H, Park H, Cho B, Kim S, Kim J. Effects of a hypomagnetic field on DNA methylation during the differentiation of embryonic stem cells. *Sci Rep* 2019; **9**: 1333 [PMID: 30718529 DOI: 10.1038/s41598-018-37372-2]

79 **Baek S**, Quan X, Kim S, Lengner C, Park JK, Kim J. Electromagnetic fields mediate efficient cell reprogramming into a pluripotent state. *ACS Nano* 2014; **8**: 10125-10138 [PMID: 25248035 DOI: 10.1021/nn502923s]

80 **Aronson JK**. Defining 'nutraceuticals': neither nutritious nor pharmaceutical. *Br J Clin Pharmacol* 2017; **83**: 8-19 [PMID: 26991455 DOI: 10.1111/bcp.12935]

81 **Cruciani S**, Santaniello S, Garroni G, Fadda A, Balzano F, Bellu E, Sarais G, Fais G, Mulas M, Maioli M. Myrtus Polyphenols, from Antioxidants to Anti-Inflammatory Molecules: Exploring a Network Involving Cytochromes P450 and Vitamin D. *Molecules* 2019; **24**: pii: E1515 [PMID: 30999678 DOI: 10.3390/molecules24081515]

82 **Claustrat B**, Leston J. Melatonin: Physiological effects in humans. *Neurochirurgie* 2015; **61**: 77-84 [PMID: 25908646 DOI: 10.1016/j.neuchi.2015.03.002]

83 **Claustrat B**, Brun J, Chazot G. The basic physiology and pathophysiology of melatonin. *Sleep Med Rev* 2005; **9**: 11-24 [PMID: 15649735 DOI: 10.1016/j.smrv.2004.08.001]

84 **Zhang S**, Chen S, Li Y, Liu Y. Melatonin as a promising agent of regulating stem cell biology and its application in disease therapy. *Pharmacol Res* 2017; **117**: 252-260 [PMID: 28042087 DOI: 10.1016/j.phrs.2016.12.035]

85 **Lee JH**, Yoon YM, Han YS, Jung SK, Lee SH. Melatonin protects mesenchymal stem cells from autophagy-mediated death under ischaemic ER-stress conditions by increasing prion protein expression. *Cell Prolif* 2019; **52**: e12545 [PMID: 30430685 DOI: 10.1111/cpr.12545]

86 **Mendivil-Perez M**, Soto-Mercado V, Guerra-Librero A, Fernandez-Gil BI, Florido J, Shen YQ, Tejada MA, Capilla-Gonzalez V, Rusanova I, Garcia-Verdugo JM, Acuña-Castroviejo D, López LC, Velez-Pardo C, Jimenez-Del-Rio M, Ferrer JM, Escames G. Melatonin enhances neural stem cell differentiation and engraftment by increasing mitochondrial function. *J Pineal Res* 2017; 63 [PMID: 28423196 DOI: 10.1111/jpi.12415]

87 **Domazetovic V**, Marcucci G, Iantomasi T, Brandi ML, Vincenzini MT. Oxidative stress in bone remodeling: role of antioxidants. *Clin Cases Miner Bone Metab* 2017; **14**: 209-216 [PMID: 29263736 DOI: 10.11138/ccmbm/2017.14.1.209]

88 **Lee S**, Le NH, Kang D. Melatonin alleviates oxidative stress-inhibited osteogenesis of human bone marrow-derived mesenchymal stem cells through AMPK activation. *Int J Med Sci* 2018; **15**: 1083-1091 [PMID: 30013450 DOI: 10.7150/ijms.26314]

89 **Feng S**, Reuss L, Wang Y. Potential of Natural Products in the Inhibition of Adipogenesis through Regulation of PPARγ Expression and/or Its Transcriptional Activity. *Molecules* 2016; **21**: pii: E1278 [PMID: 27669202 DOI: 10.3390/molecules21101278]
90 **Vingtdeux V**, Chandakkar P, Zhao H, Davies P, Marambaud P. Small-molecule activators of AMP-activated protein kinase (AMPK), RSVA314 and RSVA405, inhibit adipogenesis. *Mol Med* 2011; **17**: 1022-1030 [PMID: 21647536 DOI: 10.2119/molmed.2011.00163]
91 **Basoli V**, Santaniello S, Cruciani S, Ginesu GC, Cossu ML, Delitala AP, Serra PA, Ventura C, Maioli M. Melatonin and Vitamin D Interfere with the Adipogenic Fate of Adipose-Derived Stem Cells. *Int J Mol Sci* 2017; **18**: pii: E981 [PMID: 28475114 DOI: 10.3390/ijms18050981]
92 **Bikle DD**. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol* 2014; **21**: 319-329 [PMID: 24529992 DOI: 10.1016/j.chembiol.2013.12.016]
93 **Szymczak I**, Pawliczak R. The Active Metabolite of Vitamin D3 as a Potential Immunomodulator. *Scand J Immunol* 2016; **83**: 83-91 [PMID: 26678915 DOI: 10.1111/sji.12403]
94 **Morceau F**, Chateauvieux S, Orsini M, Trécul A, Dicato M, Diederich M. Natural compounds and pharmaceuticals reprogram leukemia cell differentiation pathways. *Biotechnol Adv* 2015; **33**: 785-797 [PMID: 25886879 DOI: 10.1016/j.biotechadv.2015.03.013]
95 **Nobili S**, Lippi D, Witort E, Donnini M, Bausi L, Mini E, Capaccioli S. Natural compounds for cancer treatment and prevention. *Pharmacol Res* 2009; **59**: 365-378 [PMID: 19429468 DOI: 10.1016/j.phrs.2009.01.017]
96 **Maioli M**, Basoli V, Carta P, Fabbri D, Dettori MA, Cruciani S, Serra PA, Delogu G. Synthesis of magnolol and honokiol derivatives and their effect against hepatocarcinoma cells. *PLoS One* 2018; **13**: e0192178 [PMID: 29415009 DOI: 10.1371/journal.pone.0192178]
97 **Ferhi S**, Santaniello S, Zerizer S, Cruciani S, Fadda A, Sanna D, Dore A, Maioli M, D'hallewin G. Total Phenols from Grape Leaves Counteract Cell Proliferation and Modulate Apoptosis-Related Gene Expression in MCF-7 and HepG2 Human Cancer Cell Lines. *Molecules* 2019; **24**: pii: E612 [PMID: 30744145 DOI: 10.3390/molecules24030612]
98 **Squillaro T**, Peluso G, Galderisi U. Clinical Trials With Mesenchymal Stem Cells: An Update. *Cell Transplant* 2016; **25**: 829-848 [PMID: 26423725 DOI: 10.3727/096368915X689622]
99 **Giordano A**, Galderisi U, Marino IR. From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. *J Cell Physiol* 2007; **211**: 27-35 [PMID: 17226788 DOI: 10.1002/jcp.20959]
100 **Lindvall O**, Kokaia Z. Stem cells for the treatment of neurological disorders. *Nature* 2006; **441**: 1094-1096 [PMID: 16810245 DOI: 10.1038/nature04960]
101 **Cohen JA**. Mesenchymal stem cell transplantation in multiple sclerosis. *J Neurol Sci* 2013; **333**: 43-49 [PMID: 23294498 DOI: 10.1016/j.jns.2012.12.009]
102 **Le Blanc K**, Mougiakakos D. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol* 2012; **12**: 383-396 [PMID: 22531326 DOI: 10.1038/nri3209]
103 **Esiashvili N**, Pulsipher MA. Hematopoietic stem cell transplantation. *Pediatric Oncology* 2018; 301-311 [DOI: 10.1007/978-3-319-43545-9_14]
104 **Robin M**, Porcher R, De Castro Araujo R, de Latour RP, Devergie A, Rocha V, Larghero J, Adès L, Ribaud P, Mary JY, Socié G. Risk factors for late infections after allogeneic hematopoietic stem cell transplantation from a matched related donor. *Biol Blood Marrow Transplant* 2007; **13**: 1304-1312 [PMID: 17950917 DOI: 10.1016/j.bbmt.2007.07.007]
105 **Henig I**, Zuckerman T. Hematopoietic stem cell transplantation-50 years of evolution and future perspectives. *Rambam Maimonides Med J* 2014; **5**: e0028 [PMID: 25386344 DOI: 10.5041/RMMJ.10162]
106 **Kim SU**, de Vellis J. Stem cell-based cell therapy in neurological diseases: a review. *J Neurosci Res* 2009; **87**: 2183-2200 [PMID: 19301431 DOI: 10.1002/jnr.22054]
107 **Kim N**, Cho SG. Clinical applications of mesenchymal stem cells. *Korean J Intern Med* 2013; **28**: 387-402 [PMID: 23864795 DOI: 10.3904/kjim.2013.28.4.387]
108 **Ogawa M**, Larue AC, Watson PM, Watson DK. Hematopoietic stem cell origin of connective tissues. *Exp Hematol* 2010; **38**: 540-547 [PMID: 20412832 DOI: 10.1016/j.exphem.2010.04.005]
109 **Kunisaki Y**. [The hematopoietic stem cell niche]. *Rinsho Ketsueki* 2015; **56**: 1888-1893 [PMID: 26458426 DOI: 10.11406/rinketsu.56.1888]
110 **Hoggatt J**, Kfoury Y, Scadden DT. Hematopoietic Stem Cell Niche in Health and Disease. *Annu Rev Pathol* 2016; **11**: 555-581 [PMID: 27193455 DOI: 10.1146/annurev-pathol-012615-044414]
111 **Guruharsha KG**, Kankel MW, Artavanis-Tsakonas S. The Notch signalling system: recent insights into the complexity of a conserved pathway. *Nat Rev Genet* 2012; **13**: 654-666 [PMID: 22868267 DOI: 10.1038/nrg3272]
112 **Collu GM**, Hidalgo-Sastre A, Brennan K. Wnt-Notch signalling crosstalk in development and disease. *Cell Mol Life Sci* 2014; **71**: 3553-3567 [PMID: 24942883 DOI: 10.1007/s00018-014-1644-x]
113 **Reya T**, Duncan AW, Ailles L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* 2003; **423**: 409-414 [PMID: 12717450 DOI: 10.1038/nature01593]
114 **Liu J**, Sato C, Cerletti M, Wagers A. Notch signaling in the regulation of stem cell self-renewal and differentiation. *Curr Top Dev Biol* 2010; **92**: 367-409 [PMID: 20816402 DOI: 10.1016/S0070-2153(10)92012-7]
115 **García-García A**, de Castillejo CL, Méndez-Ferrer S. BMSCs and hematopoiesis. *Immunol Lett* 2015; **168**: 129-135 [PMID: 26192443 DOI: 10.1016/j.imlet.2015.06.020]
116 **Nauta AJ**, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* 2007; **110**: 3499-3506 [PMID: 17664353 DOI: 10.1182/blood-2007-02-069716]
117 **Stiff PJ**, Unger JM, Cook JR, Constine LS, Couban S, Stewart DA, Shea TC, Porcu P, Winter JN, Kahl BS, Miller TP, Tubbs RR, Marcellus D, Friedberg JW, Barton KP, Mills GM, LeBlanc M, Rimsza LM, Forman SJ, Fisher RI. Autologous transplantation as consolidation for aggressive non-Hodgkin's lymphoma. *N Engl J Med* 2013; **369**: 1681-1690 [PMID: 24171516 DOI: 10.1056/NEJMoa1301077]
118 **Ali N**, Adil SN, Shaikh MU. Autologous Hematopoietic Stem Cell Transplantation-10 Years of Data From a Developing Country. *Stem Cells Transl Med* 2015; **4**: 873-877 [PMID: 26032748 DOI: 10.5966/sctm.2015-0015]
119 **Farge D**, Labopin M, Tyndall A, Fassas A, Mancardi GL, Van Laar J, Ouyang J, Kozak T, Moore J, Kötter I, Chesnel V, Marmont A, Gratwohl A, Saccardi R. Autologous hematopoietic stem cell transplantation for autoimmune diseases: an observational study on 12 years' experience from the European Group for Blood and Marrow Transplantation Working Party on Autoimmune Diseases.

Haematologica 2010; **95**: 284-292 [PMID: 19773265 DOI: 10.3324/haematol.2009.013458]
120 **Gratwohl A**, Baldomero H, Aljurf M, Pasquini MC, Bouzas LF, Yoshimi A, Szer J, Lipton J, Schwendener A, Gratwohl M, Frauendorfer K, Niederwieser D, Horowitz M, Kodera Y; Worldwide Network of Blood and Marrow Transplantation. Hematopoietic stem cell transplantation: a global perspective. *JAMA* 2010; **303**: 1617-1624 [PMID: 20424252 DOI: 10.1001/jama.2010.491]
121 **Annaloro C**, Onida F, Lambertenghi Deliliers G. Autologous hematopoietic stem cell transplantation in autoimmune diseases. *Expert Rev Hematol* 2009; **2**: 699-715 [PMID: 21082959 DOI: 10.1586/ehm.09.60]
122 **Klingemann HG**, Storb R, Fefer A, Deeg HJ, Appelbaum FR, Buckner CD, Cheever MA, Greenberg PD, Stewart PS, Sullivan KM. Bone marrow transplantation in patients aged 45 years and older. *Blood* 1986; **67**: 770-776 [PMID: 3511986 DOI: 10.1002/ajh.2830210314]
123 **Bourke SC**, Gibson GJ. Non-invasive ventilation in ALS: current practice and future role. *Amyotroph Lateral Scler Other Motor Neuron Disord* 2004; **5**: 67-71 [PMID: 15204008 DOI: 10.1080/14660820410020330]
124 **Morgan S**, Orrell RW. Pathogenesis of amyotrophic lateral sclerosis. *Br Med Bull* 2016; **119**: 87-98 [PMID: 27450455 DOI: 10.1093/bmb/ldw026]
125 **Brown RH**. Amyotrophic lateral sclerosis: recent insights from genetics and transgenic mice. *Cell* 1995; **80**: 687-692 [PMID: 7889564 DOI: 10.1016/0092-8674(95)90346-1]
126 **Grad LI**, Cashman NR. Prion-like activity of Cu/Zn superoxide dismutase: implications for amyotrophic lateral sclerosis. *Prion* 2014; **8**: 33-41 [PMID: 24394345 DOI: 10.4161/pri.27602]
127 **Sibilla C**, Bertolotti A. Prion Properties of SOD1 in Amyotrophic Lateral Sclerosis and Potential Therapy. *Cold Spring Harb Perspect Biol* 2017; **9**: pii: a024141 [PMID: 28096265 DOI: 10.1101/cshperspect.a024141]
128 **Catalanotto C**, Cogoni C, Zardo G. MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions. *Int J Mol Sci* 2016; **17**: pii: E1712 [PMID: 27754357 DOI: 10.3390/ijms17101712]
129 **Balzano F**, Cruciani S, Basoli V, Santaniello S, Facchin F, Ventura C, Maioli M. MiR200 and miR302: Two Big Families Influencing Stem Cell Behavior. *Molecules* 2018; **23**: pii: E282 [PMID: 29385685 DOI: 10.3390/molecules23020282]
130 **Di Pietro L**, Lattanzi W, Bernardini C. Skeletal Muscle MicroRNAs as Key Players in the Pathogenesis of Amyotrophic Lateral Sclerosis. *Int J Mol Sci* 2018; **19**: pii: E1534 [PMID: 29786645 DOI: 10.3390/ijms19051534]
131 **Williams AH**, Valdez G, Moresi V, Qi X, McAnally J, Elliott JL, Bassel-Duby R, Sanes JR, Olson EN. MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science* 2009; **326**: 1549-1554 [PMID: 20007902 DOI: 10.1126/science.1181046]
132 **Yacila G**, Sari Y. Potential therapeutic drugs and methods for the treatment of amyotrophic lateral sclerosis. *Curr Med Chem* 2014; **21**: 3583-3593 [PMID: 24934355 DOI: 10.2174/0929867321666140601162710]
133 **Martinez A**, Palomo Ruiz MD, Perez DI, Gil C. Drugs in clinical development for the treatment of amyotrophic lateral sclerosis. *Expert Opin Investig Drugs* 2017; **26**: 403-414 [PMID: 28277881 DOI: 10.1080/13543784.2017.1302426]
134 **Meamar R**, Nasr-Esfahani MH, Mousavi SA, Basiri K. Stem cell therapy in amyotrophic lateral sclerosis. *J Clin Neurosci* 2013; **20**: 1659-1663 [PMID: 24148693 DOI: 10.1016/j.jocn.2013.04.024]
135 **Gowing G**, Svendsen CN. Stem cell transplantation for motor neuron disease: current approaches and future perspectives. *Neurotherapeutics* 2011; **8**: 591-606 [PMID: 21904789 DOI: 10.1007/s13311-011-0068-7]
136 **Mazzini L**, Ferrari D, Andjus PR, Buzanska L, Cantello R, De Marchi F, Gelati M, Giniatullin R, Glover JC, Grilli M, Kozlova EN, Maioli M, Mitrečić D, Pivoriunas A, Sanchez-Pernaute R, Sarnowska A, Vescovi AL; BIONECA COST ACTION WG Neurology. Advances in stem cell therapy for amyotrophic lateral sclerosis. *Expert Opin Biol Ther* 2018; **18**: 865-881 [PMID: 30025485 DOI: 10.1080/14712598.2018.1503248]
137 **Rushkevich YN**, Kosmacheva SM, Zabrodets GV, Ignatenko SI, Goncharova NV, Severin IN, Likhachev SA, Potapnev MP. The Use of Autologous Mesenchymal Stem Cells for Cell Therapy of Patients with Amyotrophic Lateral Sclerosis in Belarus. *Bull Exp Biol Med* 2015; **159**: 576-581 [PMID: 26395626 DOI: 10.1007/s10517-015-3017-3]
138 **Mazzini L**, Gelati M, Profico DC, Sgaravizzi G, Projetti Pensi M, Muzi G, Ricciolini C, Rota Nodari L, Carletti S, Giorgi C, Spera C, Domenico F, Bersano E, Petruzzelli F, Cisari C, Maglione A, Sarnelli MF, Stecco A, Querin G, Masiero S, Cantello R, Ferrari D, Zalfa C, Binda E, Visioli A, Trombetta D, Novelli A, Torres B, Bernardini L, Carriero A, Prandi P, Servo S, Cerino A, Cima V, Gaiani A, Nasuelli N, Massara M, Glass J, Sorarù G, Boulis NM, Vescovi AL. Human neural stem cell transplantation in ALS: initial results from a phase I trial. *J Transl Med* 2015; **13**: 17 [PMID: 25889343 DOI: 10.1186/s12967-014-0371-2]
139 **Mazzini L**, Vercelli A, Mareschi K, Ferrero I, Testa L, Fagioli F. Mesenchymal stem cells for ALS patients. *Amyotroph Lateral Scler* 2009; **10**: 123-124 [PMID: 19085190 DOI: 10.1080/17482960802572707]
140 **Gallipoli P**, Giotopoulos G, Huntly BJ. Epigenetic regulators as promising therapeutic targets in acute myeloid leukemia. *Ther Adv Hematol* 2015; **6**: 103-119 [PMID: 26137202 DOI: 10.1177/2040620715577614]
141 **Chuang DM**, Leng Y, Marinova Z, Kim HJ, Chiu CT. Multiple roles of HDAC inhibition in neurodegenerative conditions. *Trends Neurosci* 2009; **32**: 591-601 [PMID: 19775759 DOI: 10.1016/j.tins.2009.06.002]
142 **Fagnocchi L**, Mazzoleni S, Zippo A. Integration of Signaling Pathways with the Epigenetic Machinery in the Maintenance of Stem Cells. *Stem Cells Int* 2016; **2016**: 8652748 [PMID: 26798364 DOI: 10.1155/2016/8652748]

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REVIEW

Bone marrow microenvironment: The guardian of leukemia stem cells

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Abstract

Bone marrow microenvironment (BMM) is the main sanctuary of leukemic stem cells (LSCs) and protects these cells against conventional therapies. However, it may open up an opportunity to target LSCs by breaking the close connection between LSCs and the BMM. The elimination of LSCs is of high importance, since they follow cancer stem cell theory as a part of this population. Based on cancer stem cell theory, a cell with stem cell-like features stands at the apex of the hierarchy and produces a heterogeneous population and governs the disease. Secretion of cytokines, chemokines, and extracellular vesicles, whether through autocrine or paracrine mechanisms by activation of downstream signaling pathways in LSCs, favors their persistence and makes the BMM less hospitable for normal stem cells. While all details about the interactions of the BMM and LSCs remain to be elucidated, some clinical trials have been designed to limit these reciprocal interactions to cure leukemia more effectively. In this review, we focus on chronic myeloid leukemia and acute myeloid leukemia LSCs and their milieu in the bone marrow, how to segregate them from the normal compartment, and finally the possible ways to eliminate these cells.

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Core tip: Chronic myeloid leukemia stem cells (LSCs) and acute myeloid LSCs are resistant to common therapies due to the activation of downstream signaling pathways that guarantee their survival. In addition, they are smart enough to escape immune surveillance. Bone marrow microenvironment underlies these phenomena by providing an environment that favors leukemia development. Recent studies confirm that targeting LSCs and their crosstalk with the bone marrow microenvironment significantly reduced residual disease burden and eventuated in LSCs removal.

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INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell (HSC) disorder, emanating from t(9;22)(q34;q11.2), a translocation that involves fusion of Abelson murine leukemia viral oncogene homolog 1 (*ABL1*) on chromosome 9 and breakpoint cluster region protein (*BCR*) on chromosome 22[1]. The encoded protein by constitutive tyrosine kinase activity stimulates downstream signaling pathways that lead to increased expansion of leukemic cells. Although the chronic phase of CML is concomitant with normal cell maturation, in the absence of appropriate treatment, a second mutation transforms the chronic phase into acute phase that mimics the same pattern as *de novo* acute leukemia[2,3].

Acute myeloid leukemia (AML) is the most common form of leukemia in adults and is characterized by perturbed proliferation, block of differentiation, and infiltration of leukemic cells into the bone marrow and blood[4]. Current therapies result in overall survival of about 40% in patients younger than 60 years of age, while this rate declines in older patients to 5%-15% and is associated with higher morbidity and mortality[5]. One major concern in the treatment of AML is drug resistance, and a promising approach such as targeted therapy for relapsed or refractory AML is of the essence. While in CML the introduction of tyrosine kinase inhibitors (TKIs) as a milestone in the treatment of CML results in overall survival of about 86% and attaining treatment-free remission (TFR) seems achievable[6].

Common treatment of AML and CML is based on the elimination of bulk disease population[7]. As propagation of resistant leukemic cells may continue after the treatment discontinuation, the concept of cancer stem cell (CSC) came to light. Based on this theory, a cell with the self-renewal capability and leukemic related genetic alterations, which stands at the apex of the hierarchy, may be able to resist to therapy and sustain the relapse of the disease later on[8] (Figure 1). The first approach that proved the existence of CSC was in AML, where the transplantation of a small cell population with stem cell-like properties into non-obese diabetic/severe combined immunodeficiency mice culminated in leukemia[9]. The fact that every cell in different stages of the maturation by gaining stem cell-like features has the potential to become CSC is of paramount importance and depicts that it is not crucial for CSC to have stem cell origin[10].

While both CML and AML leukemia stem cells (LSCs) have distinctive characteristics in case of the biology and immunophenotype, they share common properties such as drug resistance, quiescence, heterogeneity, and the microenvironment they reside. The bone marrow microenvironment (BMM) underpins normal hematopoiesis by secreting various growth factors and physical interactions with HSCs and progenitor cells[11]. In AML and CML, the BMM boosts leukemogenesis through an interaction with LSCs, and in turn, LSCs change the BMM based on their requirements and make it less hospitable for normal stem/progenitor cells[12]. Considering BMM as the main sanctuary for LSCs, targeting these interactions may

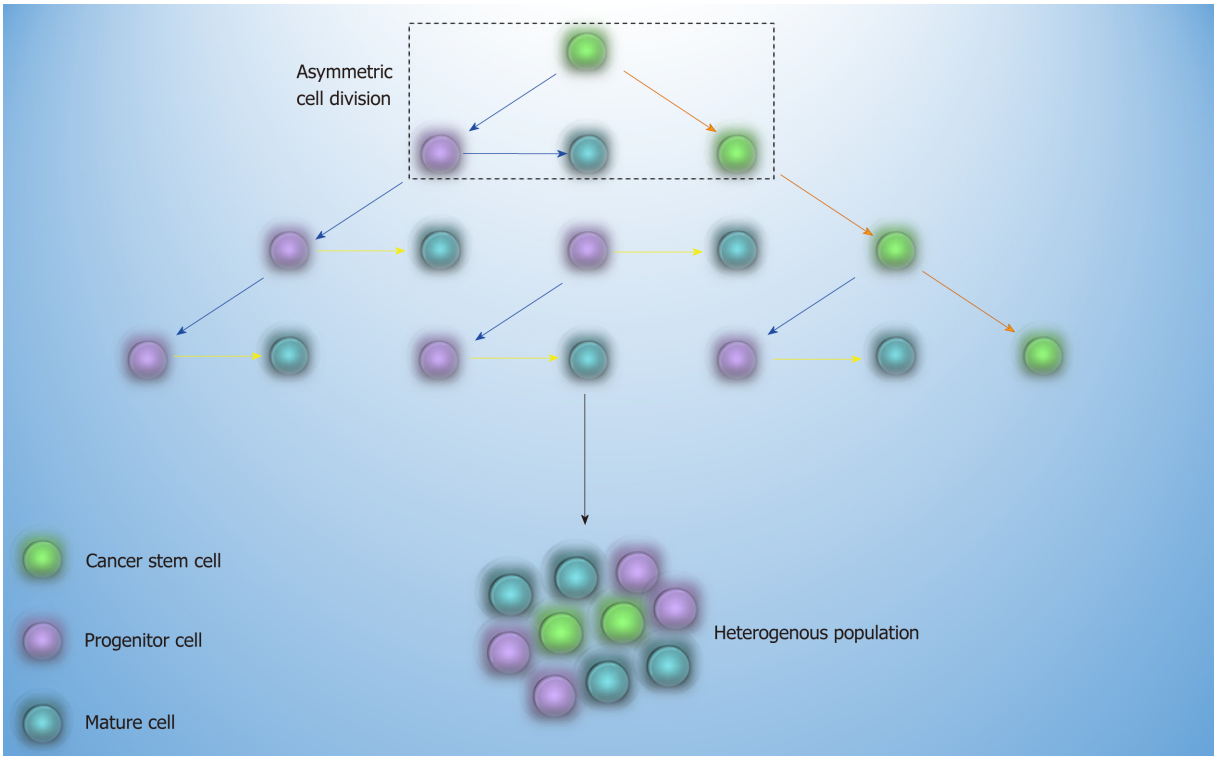

Figure 1 Cancer stem cell theory.

provide an ample opportunity to treat leukemia more effectively. In this review paper, we focus on the protective role of the BMM in the survival of CML and AML LSCs. We then move toward specific markers to identify these cells and put forward possible ways to target them within the BMM.

CML LSCs AND BONE MARROW MICROENVIRONMENT

CML LSCs, due to their resemblance to normal stem cells, reside in the same microenvironment in which a reciprocal relationship between these cells and components of the BMM is linked with enhanced proliferation, quiescence, and drug resistance. All of these mechanisms are conducted by sets of adhesion molecules or secretion of cytokines, chemokines, and growth factors *via* paracrine or autocrine mechanisms.

C-X-C motif chemokine ligand 12 (CXCL12), a known chemoattractant for the homing process, is secreted by mesenchymal stromal cells and osteoblastic cells and has a role in the localization of CML LSC and normal HSC in the BMM[13]. However, perturbed expression of C-X-C chemokine receptor type 4 (CXCR4) by CML LSCs or CXCL12 targeting by CML LSCs impacts the homing process. Kinase activity of P210*BCRABL1* and activation of downstream signaling pathways, such as phosphoinositide 3-kinases/protein kinase B [PI3K/PKB(AKT)], result in downregulation of CXCR4 by CML cells[14]. Moreover, increased secretion of granulocyte-colony stimulating factor (G-CSF) as an antagonist of CXCL12 by CML LSCs[15] and aberrant expression of surface marker dipeptidyl peptidase 4 (CD26) on CML LSCs with a chemokine cleavage activity favor mobilization of CML LSCs into the blood[16]. However, TKIs, by inhibiting P210*BCRABL1*, contribute to the upregulation of CXCR4 and migration of CML LSCs to the BMM[14].

The homing process for normal HSCs initiates with tethering and rolling of HSCs on endothelial cells *via* interaction with P and E-selectin. Then, a strong attachment through very late antigen-4 (VLA-4) and VLA-5 with vascular cell adhesion molecule 1 (VCAM-1) and fibronectin on endothelial cells and extracellular matrix supports the trafficking toward the BMM[17,18]. While CML LSCs have normal expression patterns of VLA-4 and VLA-5, their impaired function demonstrates that these cells are not entirely contingent on β1-integrins for the homing[19]. Simultaneously, it has been reported that E and L-selectin and related ligands such as CD44 seem to be closely involved in the bone marrow lodgment of CML LSCs and are considered as the compensatory mechanisms as opposed to normal stem cells[20]. Meanwhile, imatinib,

which is in first-line therapy for CML, increases another adhesion molecule N-cadherin in CML LSCs. Enhancement of N-cadherin promotes attachment to mesenchymal stromal cells and leads to N-cadherin-β catenin interaction[21]. Also, secretion of exogenous WNT by mesenchymal stromal cells activates WNT-β catenin pathway in CML LSCs[21]. WNT-β catenin is the leading signaling cascade in self-renewal and maintenance of normal HSCs and also CML LSCs, and it is important in leukemogenesis and drug resistance[22,23]. Although TKIs may attenuate the constative activity of this cascade by targeting P210*BCRABL1* and destabilize β catenin[24], activation *via* the BMM may negate this inhibitory effect.

Apart from direct contacts of CML LSCs with the BMM, secretion of some soluble factors prepares a proper context for the growth of CML LSCs and confers a number of disadvantages for the growth of the normal compartment. It has been reported that enhanced secretion of some chemokines and cytokines, such as macrophage inflammatory protein 1 alpha (MIP-1α) , MIP-1β, interleukin- 1 alpha (IL-1α), IL-1β, and tumor necrosis factor alpha (TNFα) in the CML BMM, selectively impedes growth of normal HSCs and supports the growth of CML LSCs[15]. Furthermore, secretion of IL-10, transforming growth factor beta (TGF-β), and IL-4 by the BMM or by CML LSCs in an autocrine manner downregulates expression of major histocompatibility complex-II (MHC-II) and helps CML LSCs to evade from the immune system and subsequent eradication[25].

A study reported that the higher expression of bone morphogenetic protein receptor type 1b in TKI resistant CML LSCs is activated by bone morphogenetic protein 4 *via* paracrine and autocrine loops and triggers upregulation of twist family BHLH transcription factor 1, which promotes TKI resistance[26,27]. Moreover, paracrine secretion of fibroblast growth factor 2 (FGF2) by mesenchymal stromal cells can provoke imatinib-resistance in CML patients[28]. Direct contact of CML cells with mesenchymal stromal cells stimulates secretion of placental growth factor, which in turn increases proliferation and metabolism of leukemic cells and promotes angiogenesis within the BMM[29].

Another secretory factor that reinforces quiescence and resistance of CML LSCs is germane to miR-126. miR-126 is considered to be the regulator of dormancy of CML LSCs as well as of normal HSCs[30]. P210*BCRABL1* kinase activity induces phosphorylation of Sprouty-related, EVH1 domain-containing protein 1, which causes reduction of mature miR-126 in CML LSCs. This depletion should be compensated by an external resource to keep up stemness features[30]. In the BMM, endosteal Sca-1+ endothelial cells are the credible alternative by providing a high amount of miR-126 possibly through extracellular vesicles[30]. Considering this, constraining the activity of miR-126 sensitizes LSCs to TKI and may expedite their removal[30].

Another experiment highlighted the role of the hypoxic BMM in favor of p210*BCRABL1* independent mechanisms in the survival of CML LSCs. In this milieu, a specific selection of LSC population occurs following the suppression of mature cells and stimulates TKI resistance. Sensitivity of leukemic cells to TKI is rescued by enhanced protein levels of BCRABL1 when LSCs migrate to normoxic condition[31,32]. As HSCs reside in the hypoxic endosteal niche, enhancement of low oxygen area in the bone marrow of leukemia patients coincides with resistance and presence of minimal residual disease[33,34]. Furthermore, it was demonstrated that hypoxia stabilizes hypoxia-inducible factor1 (HIF1), a transcription factor with a vital role in regulating proliferation, maintenance, and survival of CML LSCs[15]. Our knowledge about the interactions of CML LSC with the putative BMM is limited and much remains to be elucidated. Interaction of CML LSCs with their environment through different molecules is described in Table 1 and Figure 2.

AML LSCs AND BONE MARROW MICROENVIRONMENT

Recent studies reported that AML LSCs are highly dependent to the leukemic BMM. *In vivo* cell tracking has specified the anatomical adjacent of these cells to the trabecular osteoblasts *via* cell adhesion molecules[35]. Upregulation of VLA-4 in AML LSCs and its interaction with fibronectin that is distributed abundantly in endosteum facilitates AML LSCs homing to the niche. VLA-4 also has an integrity to VCAM-1 that is expressed by most of the niche cells, particularly endothelial cells[36]. These interactions promote drug resistance in LSCs, so that the combination of cytarabine with the antibody against VLA-4 in non-obese diabetic/severe combined immunodeficiency mice prevents AML LSC lodgment to the niche and makes them an easy target[37]. Meanwhile, similar to CML LSCs, elevated expression of CD44 on AML LSCs and high hyaluronic acid as its ligand on endosteal niche shift LSCs toward the BMM and chemoresistance state. Furthermore, this interaction promotes

Table 1 Possible molecules and their role in chronic myeloid leukemia stem cells-bone marrow microenvironment interaction

Target	Source	Role	Ref.
G-CSF	CML LSC	Mobilization	[15]
CD26	CML LSC	Mobilization	[16]
β1-integrins	CML LSC	Homing	[19]
Selectins	CML LSC, endothelial cells	Homing	[20]
CD44	CML LSC	Homing	[20]
Chemokines (MIP-1α, MIP-1β, *etc*)	BMM, CML LSC	Growth of CML LSC	[15,25]
Cytokines (IL-1α, IL-1β, TNFα, *etc*)	BMM, CML LSC	Growth of CML LSC	[15,25]
BMP2/4	MSC, CML LSC	Drug resistance	[26]
FGF2	MSC	Drug resistance	[28]
PIGF	MSC	Proliferation, metabolism	[29]
miR-126	CML LSC, endothelial cells	Dormancy	[30]
HIF-1	CML LSC	Growth of CML LSC	[15]
Jagged-1	Osteoblast	Dormancy	[94]
Parathyroid hormone	BMM	CML LSC removal	[95]
WNT	BMM	Growth of CML LSC	[21]
N-cadherin	CML LSC	Drug resistance	[21]

CML LSC: chronic myeloid leukemia stem cell; BMM: bone marrow microenvironment; MSC: mesenchymal stromal cell; G-CSF: granulocyte-colony stimulating factor; MIP-1α: macrophage inflammatory protein 1 alpha; MIP-1β: macrophage inflammatory protein 1 beta; IL-1α: Interleukin-1 alpha; IL-1β: Interleukin-1 beta; TNFα: tumor necrosis factor alpha; BMP2/4: bone morphogenetic protein 2/4; FGF2: fibroblast growth factor 2; PIGF: placental growth factor; HIF-1: hypoxia-inducible factor1.

activation of tyrosine kinases and proto-oncogenic signals in leukemic cells including human epidermal growth factor receptor 2, non-receptor kinase Src, Rho-associated protein kinase, and Rac family small GTPase 1[38]. While several adhesion molecules and stromal factors are involved in leukemic cell protection in the BMM, the principal mediator is related to the CXCL12-CXCR4 axis[39]. Elevated CXCR4 level in AML cells is concomitant with a poor prognosis and causes strong adhesion of AML LSCs to the BMM[40,41]. These cells play a bidirectional role by remarked Jagged1 expression that commences Notch1 pathway in neighbor leukemic cells and promotes autocrine signals in Jagged1 expressed stromal cells within the niche. Activation of Notch1 pathway accelerates self-renewal capacity of LSCs[42,43].

When AML LSCs reside in this supportive milieu, secretion of some growth factors, cytokines, and chemokines is considerably important to keep leukemogenesis up in the BMM. Secretion of IL-8 in an autocrine mechanism and its receptor CXCR2 by AML LSCs supports IL8-CXCR2 interaction and triggers activation of multiple pathways, including PI3K/AKT, phospholipase C/protein kinase C, mitogen-activated protein kinase, β catenin, HIF-1, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) in AML LSCs that brings about tumor progression and survival[44]. Moreover, CXCR2 inhibition reverses the growth of AML LSCs and enhances their removal[44].

Another study reported that elevated parathyroid hormone signaling in osteoblastic cells controls HSC pool. While parathyroid administration increases the number of AML LSCs, it decreases the number of CML LSCs and reflects the distinct role of the BMM components in different hematologic malignancies[45]. Activation of angiopoietin-Tie2 signaling in the osteoblastic niche preserves AML LSCs in dormancy and stimulates drug resistance[46]. Meanwhile, release of pro-angiogenesis factors, such as vascular endothelial growth factor, hepatocyte growth factor, basic fibroblast growth factor, TNFα, and vascular endothelial growth factor receptor by LSCs increases neoangiogenesis. On the other hand, secretion of inflammatory and proliferative cytokines like TNFα, IL-6, IL-1β, and G-CSF by leukemic cells and granulocyte-monocyte CSF by endothelial cells shares in niche neo vasculature that is considered as the major foundation of leukemia progression by providing metabolites and oxygen for AML LSC[47-51]. In some conditions, human AML LSCs increase vascular permeability to reduce nitric oxide levels produced during the anaerobic glycolytic pathway[52]. In a close relationship, endothelial cells also mediate proliferation and survival of LSCs by elevating the expression of CXCR4[53].

AML LSCs are capable of maintaining long term reconstitution in the hypoxia

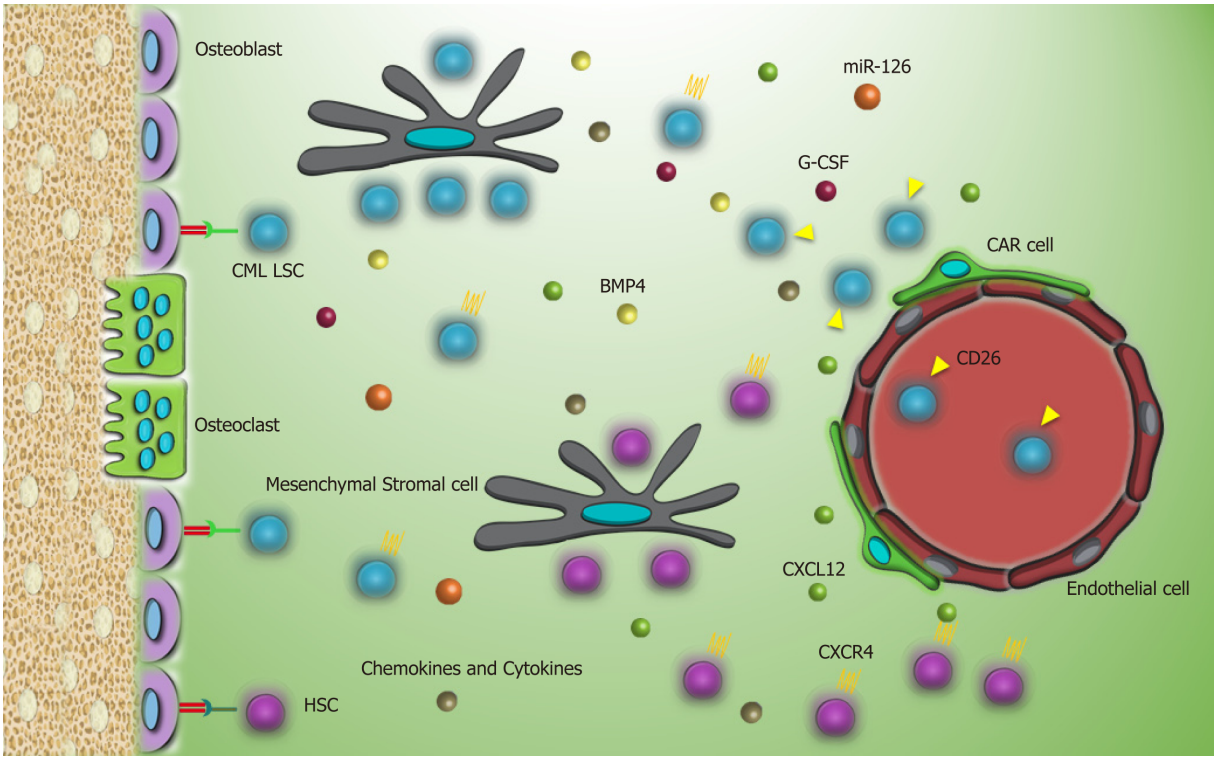

Figure 2 CML LSCs and their interaction with the bone marrow microenvironment. Expression of CXCR4 is downregulated by kinase activity of P210*BCRABL1*, and secretion of G-CSF and expression of CD26 by CML LSCs altogether lead to mobilization of CML LSCs into the blood. At the same time, secretion of some proteins such as bone morphogenetic protein 4, miR-126, and other chemokines and cytokines through autocrine or paracrine mechanisms may support dormancy, growth, and drug resistance of CML LSCs. CML LSC: Chronic myeloid leukemia stem cell; HSC: Hematopoietic stem cell; CAR cell: CXCL12-abundant reticular cell; G-CSF: Granulocyte-colony stimulating factor; CXCL12: C-X-C motif chemokine ligand 12; CXCR4: C-X-C chemokine receptor type 4; BMP4: Bone morphogenetic protein 4.

environment and modulate the differentiation process[54]. This finding is in agreement with low metabolism and energy status of AML LSCs in the BMM. However, during stresses and apoptosis, high expression of CD36, a fatty acid transporter, and enhanced lipolysis by leukemic stem cells provide a compensatory source of energy that underlies their persistence[55,56]. More investigations in LSCs and BMM crosstalk are needed to provide new insights to leukemogenesis biology and effective strategies for leukemia treatment. Interaction of AML LSCs with their environment through different molecules is summarized in Table 2 and Figure 3.

SPECIFIC MARKERS OF CML AND AML STEM CELLS

As CML LSCs reside in the CD34+/CD38- cell fraction, finding specific markers is one step ahead for recognizing and selectively targeting these cells and to discriminate from normal HSCs. A useful CD marker should first distinguish between normal and leukemic stem cells, and, second, show lack or limited expression on the more mature population.

Many markers, such as CD44 and CD117[57,58], have been recommended for detection of CML LSC, but shared expression with normal HSC has limited their application. On the other hand, surface markers such as CD25, IL-1 receptor accessory protein (IL-1RAP), and CD26 may offer a viable alternative in segregating CML LSCs[16,59,60]. CD25 (IL2Rα), which is expressed by CML LSCs, is regulated by signal transducer and activator of transcription 5 activity and serves as the suppressor of cell growth in CML LSCs. However, expression on the surface of progenitor cells might also be detectable[59]. IL-1RAP as a co-receptor of IL-1 receptor participates in activation of NF-kB and AKT signaling pathways that promote the growth of CML LSCs. As IL-1RAP expression increases with the disease progression, it seems that it may be a diagnostic marker for the advanced phase of the CML[60]. CD26, with a chemokine cleavage activity, has a role in the mobilization of the CML LSCs into the blood by cleaving CXCL12[16,61]. Expression of this marker in CML is just limited to CML LSCs in the chronic phase and is not expressed by normal HSCs, more mature population, and acute phase of the disease. So, CD26 may be regarded as a target

Table 2 Possible molecules and their role in acute myeloid leukemia stem cells-bone marrow microenvironment interaction

Target	Source	Role	Ref.
VLA-4	AML LSCs	Homing	[37]
CD44	AML LSCs	Homing	[38,96]
CXCR4	AML LSCs	Adhesion	[40]
Jagged-1	Osteoblast	Proliferation	[42]
CXCR2	AML LSCs	Proliferation, survival	[44]
Parathyroid hormone	BMM	OB proliferation, LSCs growth	[97]
Proangiogenesis factors (VEGF, HGF, BFGF, VEGFR)	AML LSCs, BMM	Endothelial and LSC proliferation	[47,48]
Cytokines (IL-6, IL1β, TNFα, G-CSF, GM-CSF)	AML LSCs, BMM	Angiogenesis, LSC proliferation	[51]
Tie-2	Osteoblast	LSCs quiescent	[46]
CD36	AML LSCs	Energy source provider	[56]

AML LSC: Acute myeloid leukemia stem cell; BMM: Bone marrow microenvironment; VLA-4: Very late antigen-4; CXCR4: C-X-C chemokine receptor type 4; CXCR2: C-X-C chemokine receptor type 2; VEGF: Vascular endothelial growth factor; HGF: Hepatocyte growth factor; BFGF: Basic fibroblast growth factor; VEGFR: Vascular endothelial growth factor receptor; IL-6: Interleukin-6; IL-1β: Interleukin-1 beta, TNFα: Tumor necrosis factor alpha; G-CSF: Granulocyte-colony stimulating factor; GM-CSF: Granulocyte-monocyte colony stimulating factor.

marker for detection of CML LSCs in newly diagnosed patients[16]. While acute lymphoblastic leukemia LSCs with P210*BCRABL1* also express CD26[62], whether its expression in acute lymphoblastic leukemia and CML LSC is P210*BCRABL1* dependent or independent remains to be discovered.

In contrast to the chronic phase of CML in which CML LSCs are defined in the CD34+/CD38- fraction, AML LSCs are composed of heterogenous populations and except the CD34+/CD38- fraction, they also reside in CD34+/CD38+ and CD34- fractions[63,64]. While the preleukemic state in AML initiates in HSC, they are considered non-leukemic, and progenitors are responsible for leukemia development. It has been reported that lymphoid primed multipotent progenitor cells in CD34+/CD38- fraction and granulocyte-macrophage progenitors in CD34+/CD38+ fraction are major AML LSC populations and that lymphoid primed multipotent progenitor cell like cells give rise to granulocyte-macrophage progenitor like cells (not vice versa) and show a higher self-renewal capability[63]. However, based on the engraftment potential and transcriptomic analysis, CD34 is not a determinant marker of AML LSCs, and other markers are needed for the identification of these cells. Meanwhile, CML acute phase mimics the same pattern as acute leukemia, and LSC populations in acute phase of CML are extended to different types of progenitor cells that reflect LSCs heterogeneity[65]. So, considering these, finding a proper marker to differentiate normal and leukemic stem cells in AML seems rather difficult and applying different markers is indispensable. For instance, some markers, such as CD96[66], C-type lectin-like molecule-1[67], CD123[68], CD25[69], CD47[70], T-cell immunoglobulin and mucin domain-3[71], *etc*, have been proposed for AML LSCs and are variably expressed by AML patients. In this case, a panel of markers might be helpful in dealing with AML LSCs. Apart from diagnosis, targeting of CML and AML LSCs based on these markers is already well underway, which may open up an opportunity to eliminate selectively LSCs and spare normal stem/progenitor cells. Different markers proposed for CML and AML LSCs are summarized in Table 3 and Figure 4.

TARGETING LEUKEMIC STEM CELLS AND THEIR ENVIRONMENT

Clinical trials have been reported that about 40%-60% of CML patients are eligible for treatment discontinuation[72,73]. While losing MR^3 in CML patients is considered the sign of TFR failure, almost all of them achieve major molecular response and deeper molecular responses after resuming the treatment[74,75]. Identification of the minimal residual disease is dependent on the application of quantitative real-time polymerase chain reaction, and subsequently it has been confirmed that CML LSCs are present from diagnosis, during the treatment and also in patients who are in TFR. These cells may be considered BCRABL1 negative due to undetectable transcript level of BCRABL1 in CML LSCs[76]. Furthermore, an inverse correlation between the number of residual CD26+ CML LSCs and the probability of remaining in TFR has been

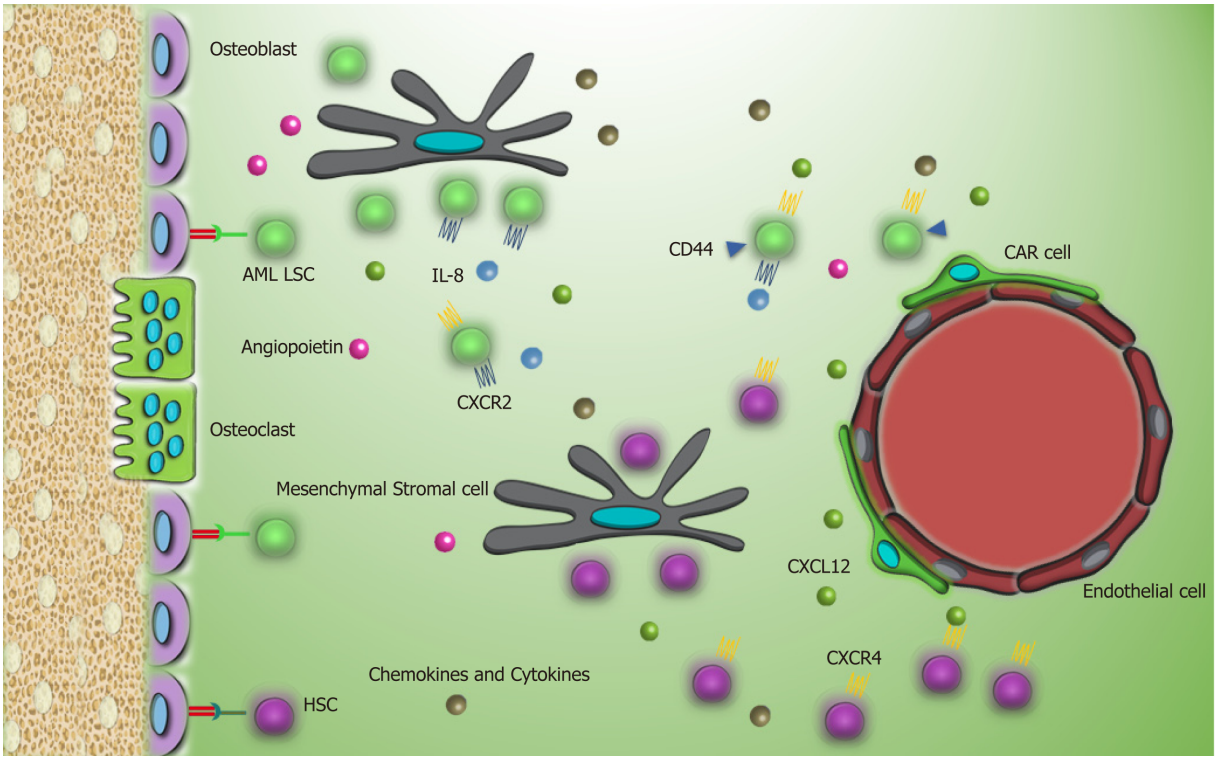

Figure 3 AML LSCs and their interaction with the bone marrow microenvironment in contrast to chronic myeloid leukemia stem cells, AML LSCs have high expression of CXCR4 that help them to reside in the bone marrow microenvironment. Meanwhile, autocrine secretion of IL-8 by AML LSCs increases their survival. Enhanced secretion of pro-angiogenesis factors *via* autocrine and paracrine mechanism extends angiogenesis, which by providing metabolites and oxygen for AML LSCs leads to leukemia progression. AML LSCs: Acute myeloid leukemia stem cell; HSC: Hematopoietic stem cell; CAR cell: CXCL12-abundant reticular cell; CXCL12: C-X-C motif chemokine ligand 12; CXCR4: C-X-C chemokine receptor type 4; CXCR2: C-X-C chemokine receptor type 2; IL-8: Interleukin-8.

reported[76]. Whereas CML LSCs are insensitive to common TKI therapy, targeting the BMM and breaking the close intimacy between CML LSCs and the BMM may help more patients achieve TFR and sustain it for a longer period.

A promising option in targeting CML LSCs is to disrupt the connection between these cells and the BMM, making them more sensitized to conventional therapy. Since the presence of CXCR4-CXCL12 axis enhances proliferation and survival of CML cells by upregulation of different signaling pathways, such as extracellular signal-regulated protein kinases 1 and 2, AKT, and Janus kinase (JAK)/STAT, interrupting this axis may dwindle the protective role of the BMM[77,78]. It has been reported that plerixafor (AMD31000), a CXCR4 antagonist, in combination with different generations of TKIs failed to reduce residual disease burden[79]. However, another experiment proved the potent role of BKT140, another antagonist of CXCR4, in declining the growth of leukemic cells both *in vitro* and *in vivo*[77].

IL-1RAP is a good marker for targeting CML LSCs in a selective manner due to its specific expression on CML LSCs. It was reported using an antibody against IL-1RAP that IL-1RAP potentially targets CML LSCs while normal stem cells remain untouched[80]. This killing effect was increased when TKIs were used in combination[80]. However, the limitation of therapeutic antibodies led to the introduction of IL1RAP CAR T cell, which is a prominent approach in dealing with resistant CML LSCs[81].

As mentioned above, secretion of some cytokines *via* autocrine or paracrine mechanisms helps CML LSC to escape from the immune system. These cytokines proceed through activation of JAK, which may activate in a P210*BCRABL1* independent fashion. So, applying ruxolitinib, a JAK inhibitor, might help upregulate MHC-II expression in CML LSCs and increase their immunogenicity for the detection and targeting by the immune system[25,82].

While targeting AML LSCs as leukemia-initiating cells may guarantee duration of the remission, eradication of these cells seems difficult because of their heterogeneity. In targeting AML LSCs, we have a vast variety of options considering cell cycle, surface markers that are useful for the segregation from normal HSCs, oncoproteins, and epigenetic participants[83]. However, the supportive role of the BMM is an undeniable fact and affects all pathways related to cell protection. Therefore, combination therapy with specific targets in the BMM is a promising approach to

Table 3 Chronic myeloid leukemia and acute myeloid leukemia stem cell markers for detection and selective targeting

Target	CD	CML LSC	AML LSC	Normal HSC	Normal progenitor	Ref.
IL-2Rα	CD25	+	+	-	+/-	[59,69]
DPP4	CD26	+	-	-	-	[16]
Siglec-3	CD33	+	+	+	+	[98]
SCARB3	CD36	+	+	+/-	+	[99]
Pgp-1	CD44	+	+	+	+	[38]
IAP	CD47	+	+	+	+	[70]
Campath-1	CD52	+	+	+	+	[100]
C1qR1	CD93	+	+	+/-	+/-	[101]
Tactile	CD96	-	+	-	-	[66]
MIC2	CD99	-	+	+	+	[102]
SCFR	CD117	+	+	+/-	+/-	[64]
IL-3Rα	CD123	+	+	+/-	+/-	[68]
CLL-1	-	+/-	+	+/-	+	[67]
TIM-3	-	-	+	+/-	+/-	[71]
IL-1RAP	-	+	+	-	+	[60,103]

CML LSC: Chronic myeloid leukemia stem cells; AML LSC: Acute myeloid leukemia stem cell; HSC: Hematopoietic stem cell; IL-2Rα: Interleukin-2 receptor alpha; DPP4: Dipeptidyl peptidase 4; Siglec-3: Sialic acid-binding immunoglobulin-type lectin-3; SCARB3: Mast/stem cell growth factor receptor; IL-3Rα: Interleukin receptor subunit α; CLL-1: C-type lectin-like molecule-1; TIM-3: T-cell immunoglobulin mucin-3; IL-1RAP: Interleukin-1 receptor accessory protein.

overcome resistance and to eradicate LSCs more effectively[10,84].

It was reported that blocking CXCR4 by plerixafor suppresses CXCL12-CXCR4 axis and increases the release of AML LSCs from the bone marrow to the blood[85]. AMD3465, another CXCR4 antagonist, in combination with G-CSF and bortezomib, a proteasome inhibitor, prevents AML LSC migration toward the BMM and consequently makes them more accessible to chemotherapy agents[86,87]. Meanwhile, in the leukemic BMM, HIF1-α and vascular endothelial growth factor modulate expression of CXCR4 and CXCL12, and targeting of these two in combination with CXCR4 antagonists significantly reduces homing of myeloid leukemia cells and reflects inducing mobilization of these cells to the blood might suppress leukemia development[88].

On the other hand, upregulation of CD44, VLA-4, and Tie2 on AML LSCs is considered a putative target. Anti-CD44 therapy in AMLs prevents LSCs homing. Also, neutralizing VLA-4 antibody together with cytarabine treatment hampers AML development in a patient-derived xenograft mouse model[38,89]. Adhesion of LSCs to mesenchymal stromal cells *via* VLA-4/VCAM-1 axis triggers NF-kB activation as an anti-apoptotic factor in AML LSCs and stromal cells. AS101, a VLA-4 inhibitor that is in Phase II of a clinical trial, prevents NF-kB activation and renders LSCs to chemotherapy[90]. Whilst interaction of Tie2 with Ang-1 concludes LSCs quiescent, disruption of Ang-1/Tie2 interaction makes cells to cycle and recover LSCs sensitivity to cell cycle targeting agents. Ang1/2 neutralizing peptibody Trebananib (AMG 386), a combination of a peptide with an antibody, demonstrated promising results in a monotherapy program in a clinical trial[91]. Another putative marker in AML LSCs is CD47 (SIRPα ligand), which is highly expressed by these cells. Interaction of CD47 with its ligand blocks phagocytosis, while blockade of this molecule leads to tumor cell phagocytosis and AML LSCs elimination in an efficient manner[70]. Direct contact of AML LSCs with the BMM *via* Notch1-Jagged interaction initiates Notch signaling by intracellular domain cleavage of Notch1 following -secretase activation. Application of -secretase inhibitors like dibenzazepine in order to inhibit Notch signaling culminates in the suppression of LSC cell growth[83]. However, in Kannan *et al*[92], a pan-Notch inhibitor could not affect LSC proliferation, which confirms further study is needed to consider Notch signaling for targeting AML LSCs.

Inducing apoptosis also is a common approach in AML targeted therapy. O' Reilly *et al*[93] reported that microenvironment mediated drug resistance in AML might occur following overexpression of myeloid cell leukemia 1, a BCL-2 family protein, in mesenchymal stromal cells. They confirmed that inhibition of myeloid cell leukemia 1 reverts the BMM mediated resistance against cytarabine and daunorubicin, prevents

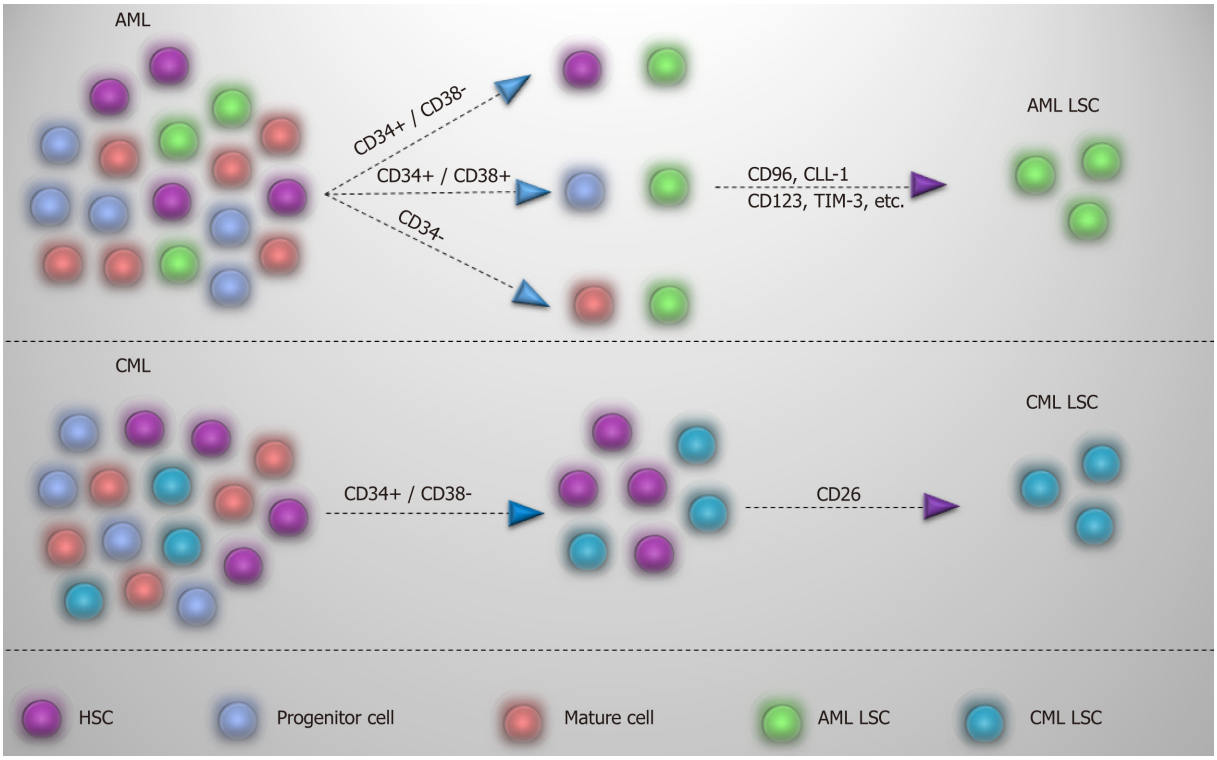

Figure 4 Detection of AML and CML LSCs. While CML LSCs in chronic phase are in CD34+/CD38-, using CD26 helps to segregate them from normal hematopoietic stem cells. In AML, CD34 is not a fixed marker for detection of AML LSCs, and due to the heterogeneity of AML LSC populations, other markers are needed to identify these cells. CLL-1: C-type lectin-like molecule-1; TIM-3: T-cell immunoglobulin and mucin domain-3; AML LSCs: Acute myeloid leukemia stem cell; HSC: Hematopoietic stem cell; CML LSCs: Chronic myeloid leukemia stem cells.

disease relapse, and ultimately improves patient survival. The proposed compounds under clinical trials related to targeting CML and AML LSCs interaction with BMM are summarized in Table 4. Other studies reported another possible target for elimination of AML LSCs by inhibiting the IL8-CXCR2 axis. This approach selectively eliminates AML LSCs while sparing normal HSCs[44].

CONCLUSION

The therapeutic approaches that we listed above are in most cases already the object of investigational clinical trials. Many others will certainly follow, and, as far as our knowledge about the biology, the phenotypical appearance and the biochemical pathways typical of the leukemic stem cells will be better understood. It is unlikely that a single agent will be able to eliminate the leukemic stem cells. Targeted therapy will most likely be a combination of new drugs and more conventional therapeutic agents, ranging from traditional chemotherapy to new molecularly targeted agents or immune modulating agents. The final goal that we hope to achieve is to cure the vast majority of our patients and to improve their quality of their life.

Table 4 A draft of compounds under clinical trial in leukemic stem cell bone marrow microenvironment target therapy

Disease	Target	Compound	Clinical trial ID
CML	CXCR4	BL-8040	NCT02115672
CML	IL-1RAP	CAR-LMC	NCT02842320
CML	JAK-inhibitor	Ruxolitinib	NCT01702064, NCT03654768, NCT01751425, NCT03610971
AML	CXCR4	Plerixafor (AMD3100)	NCT01455025
AML	Hypoxia	TH-302	NCT01149915
AML	VEGF	Aflibercept	NCT00601991
AML	VLA-4	AS101	NCT01010373
AML	Ang-1/2	Trebananib (AMG 386)	NCT01555268
AML	CD47	SRF231, TTI-621, CC90002, Hu5F9-G4	NCT03512340, NCT02663518, NCT02367196, NCT02678338, NCT03248479
AML	Notch	LY3039478, MK0752	NCT01695005, NCT00100152
AML	XIAP	AEG35156	NCT00363974
AML	BH3	ABT-199	NCT01994837
AML	Pan FGFR	LY274455	NCT01212107

CML: Chronic myeloid leukemia; AML: Acute myeloid leukemia; CXCR4: C-X-C chemokine receptor type 4; IL-1RAP: Interleukin-1 receptor accessory protein; JAK: Janus kinase; VEGF: Vascular endothelial growth factor; VLA-4: Very late antigen-4; Ang-1/2: angiopoietin-1/2; XIAP: X-Linked inhibitor of apoptosis; FGFR: Fibroblast growth factor receptor.

REFERENCES

1 **Goldman JM**, Melo JV. Targeting the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001; **344**: 1084-1086 [PMID: 11287980 DOI: 10.1056/NEJM200104053441409]
2 **Ren R**. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nat Rev Cancer* 2005; **5**: 172-183 [PMID: 15719031 DOI: 10.1038/nrc1567]
3 **Hehlmann R**. How I treat CML blast crisis. *Blood* 2012; **120**: 737-747 [PMID: 22653972 DOI: 10.1182/blood-2012-03-380147]
4 **Thomas J**, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: Implications for drug resistance. *Blood* 2004; **104**: 3739-3745 [PMID: 15315971 DOI: 10.1182/blood-2003-12-4276]
5 **De Kouchkovsky I**, Abdul-Hay M. 'Acute myeloid leukemia: A comprehensive review and 2016 update'. *Blood Cancer J* 2016; **6**: e441 [PMID: 27367478 DOI: 10.1038/bcj.2016.50]
6 **Hochhaus A**, Larson RA, Guilhot F, Radich JP, Branford S, Hughes TP, Baccarani M, Deininger MW, Cervantes F, Fujihara S, Ortmann CE, Menssen HD, Kantarjian H, O'Brien SG, Druker BJ; IRIS Investigators. Long-Term Outcomes of Imatinib Treatment for Chronic Myeloid Leukemia. *N Engl J Med* 2017; **376**: 917-927 [PMID: 28273028 DOI: 10.1056/NEJMoa1609324]
7 **Pollyea DA**, Gutman JA, Gore L, Smith CA, Jordan CT. Targeting acute myeloid leukemia stem cells: A review and principles for the development of clinical trials. *Haematologica* 2014; **99**: 1277-1284 [PMID: 25082785 DOI: 10.3324/haematol.2013.085209]
8 **Fulawka L**, Donizy P, Halon A. Cancer stem cells--the current status of an old concept: Literature review and clinical approaches. *Biol Res* 2014; **47**: 66 [PMID: 25723910 DOI: 10.1186/0717-6287-47-66]
9 **Lapidot T**, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**: 645-648 [PMID: 7509044 DOI: 10.1038/367645a0]
10 **Lane SW**, Scadden DT, Gilliland DG. The leukemic stem cell niche: Current concepts and therapeutic opportunities. *Blood* 2009; **114**: 1150-1157 [PMID: 19401558 DOI: 10.1182/blood-2009-01-202606]
11 **Bianco P**. Bone and the hematopoietic niche: A tale of two stem cells. *Blood* 2011; **117**: 5281-5288 [PMID: 21406722 DOI: 10.1182/blood-2011-01-315069]
12 **Krause DS**, Scadden DT. A hostel for the hostile: The bone marrow niche in hematologic neoplasms. *Haematologica* 2015; **100**: 1376-1387 [PMID: 26521296 DOI: 10.3324/haematol.2014.113852]
13 **Kim JA**, Shim JS, Lee GY, Yim HW, Kim TM, Kim M, Leem SH, Lee JW, Min CK, Oh IH. Microenvironmental remodeling as a parameter and prognostic factor of heterogeneous leukemogenesis in acute myelogenous leukemia. *Cancer Res* 2015; **75**: 2222-2231 [PMID: 25791383 DOI: 10.1158/0008-5472.CAN-14-3379]
14 **Jin L**, Tabe Y, Konoplev S, Xu Y, Leysath CE, Lu H, Kimura S, Ohsaka A, Rios MB, Calvert L, Kantarjian H, Andreeff M, Konopleva M. CXCR4 up-regulation by imatinib induces chronic myelogenous leukemia (CML) cell migration to bone marrow stroma and promotes survival of quiescent CML cells. *Mol Cancer Ther* 2008; **7**: 48-58 [PMID: 18202009 DOI: 10.1158/1535-7163.MCT-07-0042]
15 **Zhang H**, Li H, Xi HS, Li S. HIF1α is required for survival maintenance of chronic myeloid leukemia stem cells. *Blood* 2012; **119**: 2595-2607 [PMID: 22275380 DOI: 10.1182/blood-2011-10-387381]
16 **Herrmann H**, Sadovnik I, Cerny-Reiterer S, Rülicke T, Stefanzl G, Willmann M, Hoermann G, Bilban M, Blatt K, Herndlhofer S, Mayerhofer M, Streubel B, Sperr WR, Holyoake TL, Mannhalter C, Valent P. Dipeptidylpeptidase IV (CD26) defines leukemic stem cells (LSC) in chronic myeloid leukemia. *Blood* 2014; **123**: 3951-3962 [PMID: 24778155 DOI: 10.1182/blood-2013-10-536078]
17 **Papayannopoulou T**, Craddock C, Nakamoto B, Priestley GV, Wolf NS. The VLA4/VCAM-1 adhesion pathway defines contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. *Proc Natl Acad Sci U S A* 1995; **92**: 9647-9651 [PMID: 7568190 DOI: 10.1073/pnas.92.21.9647]

18 **Kim S**, Lin L, Brown GAJ, Hosaka K, Scott EW. Extended time-lapse in vivo imaging of tibia bone marrow to visualize dynamic hematopoietic stem cell engraftment. *Leukemia* 2017; **31**: 1582-1592 [PMID: 27890929 DOI: 10.1038/leu.2016.354]

19 **Bhatia R**, McCarthy JB, Verfaillie CM. Interferon-alpha restores normal beta 1 integrin-mediated inhibition of hematopoietic progenitor proliferation by the marrow microenvironment in chronic myelogenous leukemia. *Blood* 1996; **87**: 3883-3891 [PMID: 8611716 DOI: 10.1016/0006-355X(96)00021-2]

20 **Krause DS**, Lazarides K, Lewis JB, von Andrian UH, Van Etten RA. Selectins and their ligands are required for homing and engraftment of BCR-ABL1+ leukemic stem cells in the bone marrow niche. *Blood* 2014; **123**: 1361-1371 [PMID: 24394666 DOI: 10.1182/blood-2013-11-538694]

21 **Zhang B**, Li M, McDonald T, Holyoake TL, Moon RT, Campana D, Shultz L, Bhatia R. Microenvironmental protection of CML stem and progenitor cells from tyrosine kinase inhibitors through N-cadherin and Wnt-β-catenin signaling. *Blood* 2013; **121**: 1824-1838 [PMID: 23299311 DOI: 10.1182/blood-2012-02-412890]

22 **Zhao C**, Blum J, Chen A, Kwon HY, Jung SH, Cook JM, Lagoo A, Reya T. Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. *Cancer Cell* 2007; **12**: 528-541 [PMID: 18068630 DOI: 10.1016/j.ccr.2007.11.003]

23 **Riether C**, Schürch CM, Flury C, Hinterbrandner M, Drück L, Huguenin AL, Baerlocher GM, Radpour R, Ochsenbein AF. Tyrosine kinase inhibitor-induced CD70 expression mediates drug resistance in leukemia stem cells by activating Wnt signaling. *Sci Transl Med* 2015; **7**: 298ra119 [PMID: 26223302 DOI: 10.1126/scitranslmed.aab1740]

24 **Coluccia AM**, Vacca A, Duñach M, Mologni L, Redaelli S, Bustos VH, Benati D, Pinna LA, Gambacorti-Passerini C. Bcr-Abl stabilizes beta-catenin in chronic myeloid leukemia through its tyrosine phosphorylation. *EMBO J* 2007; **26**: 1456-1466 [PMID: 17318191 DOI: 10.1038/sj.emboj.7601485]

25 **Tarafdar A**, Hopcroft LE, Gallipoli P, Pellicano F, Cassels J, Hair A, Korfi K, Jørgensen HG, Vetrie D, Holyoake TL, Michie AM. CML cells actively evade host immune surveillance through cytokine-mediated downregulation of MHC-II expression. *Blood* 2017; **129**: 199-208 [PMID: 27793879 DOI: 10.1182/blood-2016-09-742049]

26 **Grockowiak E**, Laperrousaz B, Jeanpierre S, Voeltzel T, Guyot B, Gobert S, Nicolini FE, Maguer-Satta V. Immature CML cells implement a BMP autocrine loop to escape TKI treatment. *Blood* 2017; **130**: 2860-2871 [PMID: 29138221 DOI: 10.1182/blood-2017-08-801019]

27 **Houshmand M**, Simonetti G, Circosta P, Gaidano V, Cignetti A, Martinelli G, Saglio G, Gale RP. Chronic myeloid leukemia stem cells. *Leukemia* 2019 [PMID: 31127148 DOI: 10.1038/s41375-019-0490-0]

28 **Traer E**, Javidi-Sharifi N, Agarwal A, Dunlap J, English I, Martinez J, Tyner JW, Wong M, Druker BJ. Ponatinib overcomes FGF2-mediated resistance in CML patients without kinase domain mutations. *Blood* 2014; **123**: 1516-1524 [PMID: 24408322 DOI: 10.1182/blood-2013-07-518381]

29 **Schmidt T**, Kharabi Masouleh B, Loges S, Cauwenberghs S, Fraisl P, Maes C, Jonckx B, De Keersmaecker K, Kleppe M, Tjwa M, Schenk T, Vinckier S, Fragoso R, De Mol M, Beel K, Dias S, Verfaillie C, Clark RE, Brümmendorf TH, Vandenberghe P, Rafii S, Holyoake T, Hochhaus A, Cools J, Karin M, Carmeliet G, Dewerchin M, Carmeliet P. Loss or inhibition of stromal-derived PlGF prolongs survival of mice with imatinib-resistant Bcr-Abl1(+) leukemia. *Cancer Cell* 2011; **19**: 740-753 [PMID: 21665148 DOI: 10.1016/j.ccr.2011.05.007]

30 **Zhang B**, Nguyen LXT, Li L, Zhao D, Kumar B, Wu H, Lin A, Pellicano F, Hopcroft L, Su YL, Copland M, Holyoake TL, Kuo CJ, Bhatia R, Snyder DS, Ali H, Stein AS, Brewer C, Wang H, McDonald T, Swiderski P, Troadec E, Chen CC, Dorrance A, Pullarkat V, Yuan YC, Perrotti D, Carlesso N, Forman SJ, Kortylewski M, Kuo YH, Marcucci G. Bone marrow niche trafficking of miR-126 controls the self-renewal of leukemia stem cells in chronic myelogenous leukemia. *Nat Med* 2018; **24**: 450-462 [PMID: 29505034 DOI: 10.1038/nm.4499]

31 **Giuntoli S**, Rovida E, Barbetti V, Cipolleschi MG, Olivotto M, Dello Sbarba P. Hypoxia suppresses BCR/Abl and selects imatinib-insensitive progenitors within clonal CML populations. *Leukemia* 2006; **20**: 1291-1293 [PMID: 16710305 DOI: 10.1038/sj.leu.2404224]

32 **Tanturli M**, Giuntoli S, Barbetti V, Rovida E, Dello Sbarba P. Hypoxia selects bortezomib-resistant stem cells of chronic myeloid leukemia. *PLoS One* 2011; **6**: e17008 [PMID: 21347297 DOI: 10.1371/journal.pone.0017008]

33 **Benito J**, Zeng Z, Konopleva M, Wilson WR. Targeting hypoxia in the leukemia microenvironment. *Int J Hematol Oncol* 2013; **2**: 279-288 [PMID: 24490034 DOI: 10.2217/IJH.13.32]

34 **Petit C**, Gouel F, Dubus I, Heuclin C, Roget K, Vannier JP. Hypoxia promotes chemoresistance in acute lymphoblastic leukemia cell lines by modulating death signaling pathways. *BMC Cancer* 2016; **16**: 746 [PMID: 27658583 DOI: 10.1186/s12885-016-2776-1]

35 **Ishikawa F**, Yoshida S, Saito Y, Hijikata A, Kitamura H, Tanaka S, Nakamura R, Tanaka T, Tomiyama H, Saito N, Fukata M, Miyamoto T, Lyons B, Ohshima K, Uchida N, Taniguchi S, Ohara O, Akashi K, Harada M, Shultz LD. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol* 2007; **25**: 1315-1321 [PMID: 17952057 DOI: 10.1038/nbt1350]

36 **Ulyanova T**, Scott LM, Priestley GV, Jiang Y, Nakamoto B, Koni PA, Papayannopoulou T. VCAM-1 expression in adult hematopoietic and nonhematopoietic cells is controlled by tissue-inductive signals and reflects their developmental origin. *Blood* 2005; **106**: 86-94 [PMID: 15769895 DOI: 10.1182/blood-2004-09-3417]

37 **Matsunaga T**, Takemoto N, Sato T, Takimoto R, Tanaka I, Fujimi A, Akiyama T, Kuroda H, Kawano Y, Kobune M, Kato J, Hirayama Y, Sakamaki S, Kohda K, Miyake K, Niitsu Y. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. *Nat Med* 2003; **9**: 1158-1165 [PMID: 12897778 DOI: 10.1038/nm909]

38 **Jin L**, Hope KJ, Zhai Q, Smadja-Joffe F, Dick JE. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med* 2006; **12**: 1167-1174 [PMID: 16998484 DOI: 10.1038/nm1483]

39 **Zeng Z**, Samudio IJ, Munsell M, An J, Huang Z, Estey E, Andreeff M, Konopleva M. Inhibition of CXCR4 with the novel RCP168 peptide overcomes stroma-mediated chemoresistance in chronic and acute leukemias. *Mol Cancer Ther* 2006; **5**: 3113-3121 [PMID: 17172414 DOI: 10.1158/1535-7163.MCT-06-0228]

40 **Rombouts EJ**, Pavic B, Löwenberg B, Ploemacher RE. Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia. *Blood* 2004; **104**: 550-557 [PMID:

15054042 DOI: 10.1182/blood-2004-02-0566]
41 **Spoo AC**, Lübbert M, Wierda WG, Burger JA. CXCR4 is a prognostic marker in acute myelogenous leukemia. *Blood* 2007; **109**: 786-791 [PMID: 16888090 DOI: 10.1182/blood-2006-05-024844]
42 **Czemerska M**, Pluta A, Szmigielska-Kaplon A, Wawrzyniak E, Cebula-Obrzut B, Medra A, Smolewski P, Robak T, Wierzbowska A. Jagged-1: A new promising factor associated with favorable prognosis in patients with acute myeloid leukemia. *Leuk Lymphoma* 2015; **56**: 401-406 [PMID: 24844362 DOI: 10.3109/10428194.2014.917638]
43 **Liu N**, Zhang J, Ji C. The emerging roles of Notch signaling in leukemia and stem cells. *Biomark Res* 2013; **1**: 23 [PMID: 24252593 DOI: 10.1186/2050-7771-1-23]
44 **Schinke C**, Giricz O, Li W, Shastri A, Gordon S, Barreyro L, Bhagat T, Bhattacharyya S, Ramachandra N, Bartenstein M, Pellagatti A, Boultwood J, Wickrema A, Yu Y, Will B, Wei S, Steidl U, Verma A. IL8-CXCR2 pathway inhibition as a therapeutic strategy against MDS and AML stem cells. *Blood* 2015; **125**: 3144-3152 [PMID: 25810490 DOI: 10.1182/blood-2015-01-621631]
45 **Calvi LM**, Sims NA, Hunzelman JL, Knight MC, Giovannetti A, Saxton JM, Kronenberg HM, Baron R, Schipani E. Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. *J Clin Invest* 2001; **107**: 277-286 [PMID: 11160151 DOI: 10.1172/JCI11296]
46 **Arai F**, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 2004; **118**: 149-161 [PMID: 15260986 DOI: 10.1016/j.cell.2004.07.004]
47 **Aguayo A**, Kantarjian H, Manshouri T, Gidel C, Estey E, Thomas D, Koller C, Estrov Z, O'Brien S, Keating M, Freireich E, Albitar M. Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. *Blood* 2000; **96**: 2240-2245 [PMID: 10979972 DOI: 10.1016/S0955-3886(00)00083-7]
48 **Bellamy WT**, Richter L, Sirjani D, Roxas C, Glinsmann-Gibson B, Frutiger Y, Grogan TM, List AF. Vascular endothelial cell growth factor is an autocrine promoter of abnormal localized immature myeloid precursors and leukemia progenitor formation in myelodysplastic syndromes. *Blood* 2001; **97**: 1427-1434 [PMID: 11222390 DOI: 10.1182/blood.v97.5.1427]
49 **Janowska-Wieczorek A**, Majka M, Marquez-Curtis L, Wertheim JA, Turner AR, Ratajczak MZ. Bcr-abl-positive cells secrete angiogenic factors including matrix metalloproteinases and stimulate angiogenesis in vivo in Matrigel implants. *Leukemia* 2002; **16**: 1160-1166 [PMID: 12040448 DOI: 10.1038/sj.leu.2402486]
50 **Sainson RC**, Johnston DA, Chu HC, Holderfield MT, Nakatsu MN, Crampton SP, Davis J, Conn E, Hughes CC. TNF primes endothelial cells for angiogenic sprouting by inducing a tip cell phenotype. *Blood* 2008; **111**: 4997-5007 [PMID: 18337563 DOI: 10.1182/blood-2007-08-108597]
51 **Mirantes C**, Passegué E, Pietras EM. Pro-inflammatory cytokines: Emerging players regulating HSC function in normal and diseased hematopoiesis. *Exp Cell Res* 2014; **329**: 248-254 [PMID: 25149680 DOI: 10.1016/j.yexcr.2014.08.017]
52 **Passaro D**, Di Tullio A, Abarrategi A, Rouault-Pierre K, Foster K, Ariza-McNaughton L, Montaner B, Chakravarty P, Bhaw L, Diana G, Lassailly F, Gribben J, Bonnet D. Increased Vascular Permeability in the Bone Marrow Microenvironment Contributes to Disease Progression and Drug Response in Acute Myeloid Leukemia. *Cancer Cell* 2017; **32**: 324-341.e6 [PMID: 28870739 DOI: 10.1016/j.ccell.2017.08.001]
53 **Veiga JP**, Costa LF, Sallan SE, Nadler LM, Cardoso AA. Leukemia-stimulated bone marrow endothelium promotes leukemia cell survival. *Exp Hematol* 2006; **34**: 610-621 [PMID: 16647567 DOI: 10.1016/j.exphem.2006.01.013]
54 **Cheloni G**, Poteti M, Bono S, Masala E, Mazure NM, Rovida E, Lulli M, Dello Sbarba P. The Leukemic Stem Cell Niche: Adaptation to "Hypoxia" versus Oncogene Addiction. *Stem Cells Int* 2017; **2017**: 4979474 [PMID: 29118813 DOI: 10.1155/2017/4979474]
55 **Ito K**, Suda T. Metabolic requirements for the maintenance of self-renewing stem cells. *Nat Rev Mol Cell Biol* 2014; **15**: 243-256 [PMID: 24651542 DOI: 10.1038/nrm3772]
56 **Ye H**, Adane B, Khan N, Sullivan T, Minhajuddin M, Gasparetto M, Stevens B, Pei S, Balys M, Ashton JM, Klemm DJ, Woolthuis CM, Stranahan AW, Park CY, Jordan CT. Leukemic Stem Cells Evade Chemotherapy by Metabolic Adaptation to an Adipose Tissue Niche. *Cell Stem Cell* 2016; **19**: 23-37 [PMID: 27374788 DOI: 10.1016/j.stem.2016.06.001]
57 **Florian S**, Sonneck K, Hauswirth AW, Krauth MT, Schernthaner GH, Sperr WR, Valent P. Detection of molecular targets on the surface of CD34+/CD38-- stem cells in various myeloid malignancies. *Leuk Lymphoma* 2006; **47**: 207-222 [PMID: 16321850 DOI: 10.1080/10428190500272507]
58 **Foster BM**, Zaidi D, Young TR, Mobley ME, Kerr BA. CD117/c-kit in Cancer Stem Cell-Mediated Progression and Therapeutic Resistance. *Biomedicines* 2018; **6**: pii: E31 [PMID: 29518044 DOI: 10.3390/biomedicines6010031]
59 **Sadovnik I**, Herrmann H, Eisenwort G, Blatt K, Hoermann G, Mueller N, Sperr WR, Valent P. Expression of CD25 on leukemic stem cells in BCR-ABL1+CML: Potential diagnostic value and functional implications. *Exp Hematol* 2017; **51**: 17-24 [PMID: 28457753 DOI: 10.1016/j.exphem.2017.04.003]
60 **Zhao K**, Yin LL, Zhao DM, Pan B, Chen W, Cao J, Cheng H, Li ZY, Li DP, Sang W, Zeng LY, Xu KL. IL1RAP as a surface marker for leukemia stem cells is related to clinical phase of chronic myeloid leukemia patients. *Int J Clin Exp Med* 2014; **7**: 4787-4798 [PMID: 25663975]
61 **Christopherson KW 2nd**, Cooper S, Broxmeyer HE. Cell surface peptidase CD26/DPPIV mediates G-CSF mobilization of mouse progenitor cells. *Blood* 2003; **101**: 4680-4686 [PMID: 12576320 DOI: 10.1182/blood-2002-12-3893]
62 **Blatt K**, Menzl I, Eisenwort G, Cerny-Reiterer S, Herrmann H, Herndlhofer S, Stefanzl G, Sadovnik I, Berger D, Keller A, Hauswirth A, Hoermann G, Willmann M, Rülicke T, Sill H, Sperr WR, Mannhalter C, Melo JV, Jäger U, Sexl V, Valent P. Phenotyping and Target Expression Profiling of CD34+/CD38- and CD34+/CD38+ Stem- and Progenitor cells in Acute Lymphoblastic Leukemia. *Neoplasia* 2018; **20**: 632-642 [PMID: 29772458 DOI: 10.1016/j.neo.2018.04.004]
63 **Goardon N**, Marchi E, Atzberger A, Quek L, Schuh A, Soneji S, Woll P, Mead A, Alford KA, Rout R, Chaudhury S, Gilkes A, Knapper S, Beldjord K, Begum S, Rose S, Geddes N, Griffiths M, Standen G, Sternberg A, Cavenagh J, Hunter H, Bowen D, Killick S, Robinson L, Price A, Macintyre E, Virgo P, Burnett A, Craddock C, Enver T, Jacobsen SE, Porcher C, Vyas P. Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. *Cancer Cell* 2011; **19**: 138-152 [PMID: 21251617 DOI: 10.1016/j.ccr.2010.12.012]
64 **Quek L**, Otto GW, Garnett C, Lhermitte L, Karamitros D, Stoilova B, Lau IJ, Doondeea J, Usukhbayar B,

Kennedy A, Metzner M, Goardon N, Ivey A, Allen C, Gale R, Davies B, Sternberg A, Killick S, Hunter H, Cahalin P, Price A, Carr A, Griffiths M, Virgo P, Mackinnon S, Grimwade D, Freeman S, Russell N, Craddock C, Mead A, Peniket A, Porcher C, Vyas P. Genetically distinct leukemic stem cells in human CD34- acute myeloid leukemia are arrested at a hemopoietic precursor-like stage. *J Exp Med* 2016; **213**: 1513-1535 [PMID: 27377587 DOI: 10.1084/jem.20151775]

65 **Ding Y**, Gao H, Zhang Q. The biomarkers of leukemia stem cells in acute myeloid leukemia. *Stem Cell Investig* 2017; **4**: 19 [PMID: 28447034 DOI: 10.21037/sci.2017.02.10]

66 **Hosen N**, Park CY, Tatsumi N, Oji Y, Sugiyama H, Gramatzki M, Krensky AM, Weissman IL. CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proc Natl Acad Sci U S A* 2007; **104**: 11008-11013 [PMID: 17576927 DOI: 10.1073/pnas.0704271104]

67 **van Rhenen A**, van Dongen GA, Kelder A, Rombouts EJ, Feller N, Moshaver B, Stigter-van Walsum M, Zweegman S, Ossenkoppele GJ, Jan Schuurhuis G. The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. *Blood* 2007; **110**: 2659-2666 [PMID: 17609428 DOI: 10.1182/blood-2007-03-083048]

68 **Jordan CT**, Upchurch D, Szilvassy SJ, Guzman ML, Howard DS, Pettigrew AL, Meyerrose T, Rossi R, Grimes B, Rizzieri DA, Luger SM, Phillips GL. The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* 2000; **14**: 1777-1784 [PMID: 11021753 DOI: 10.1038/sj.leu.2401903]

69 **Gönen M**, Sun Z, Figueroa ME, Patel JP, Abdel-Wahab O, Racevskis J, Ketterling RP, Fernandez H, Rowe JM, Tallman MS, Melnick A, Levine RL, Paietta E. CD25 expression status improves prognostic risk classification in AML independent of established biomarkers: ECOG phase 3 trial, E1900. *Blood* 2012; **120**: 2297-2306 [PMID: 22855599 DOI: 10.1182/blood-2012-02-414425]

70 **Majeti R**, Chao MP, Alizadeh AA, Pang WW, Jaiswal S, Gibbs KD, van Rooijen N, Weissman IL. CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell* 2009; **138**: 286-299 [PMID: 19632179 DOI: 10.1016/j.cell.2009.05.045]

71 **Kikushige Y**, Shima T, Takayanagi S, Urata S, Miyamoto T, Iwasaki H, Takenaka K, Teshima T, Tanaka T, Inagaki Y, Akashi K. TIM-3 is a promising target to selectively kill acute myeloid leukemia stem cells. *Cell Stem Cell* 2010; **7**: 708-717 [PMID: 21112565 DOI: 10.1016/j.stem.2010.11.014]

72 **Mahon FX**, Réa D, Guilhot J, Guilhot F, Huguet F, Nicolini F, Legros L, Charbonnier A, Guerci A, Varet B, Etienne G, Reiffers J, Rousselot P; Intergroupe Français des Leucémies Myéloïdes Chroniques. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: The prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol* 2010; **11**: 1029-1035 [PMID: 20965785 DOI: 10.1016/S1470-2045(10)70233-3]

73 **Hughes TP**, Ross DM. Moving treatment-free remission into mainstream clinical practice in CML. *Blood* 2016; **128**: 17-23 [PMID: 27013442 DOI: 10.1182/blood-2016-01-694265]

74 **Ross DM**, Branford S, Seymour JF, Schwarer AP, Arthur C, Yeung DT, Dang P, Goyne JM, Slader C, Filshie RJ, Mills AK, Melo JV, White DL, Grigg AP, Hughes TP. Safety and efficacy of imatinib cessation for CML patients with stable undetectable minimal residual disease: Results from the TWISTER study. *Blood* 2013; **122**: 515-522 [PMID: 23704092 DOI: 10.1182/blood-2013-02-483750]

75 **Rousselot P**, Charbonnier A, Cony-Makhoul P, Agape P, Nicolini FE, Varet B, Gardembas M, Etienne G, Réa D, Roy L, Escoffre-Barbe M, Guerci-Bresler A, Tulliez M, Prost S, Spentchian M, Cayuela JM, Reiffers J, Chomel JC, Turhan A, Guilhot J, Guilhot F, Mahon FX. Loss of major molecular response as a trigger for restarting tyrosine kinase inhibitor therapy in patients with chronic-phase chronic myelogenous leukemia who have stopped imatinib after durable undetectable disease. *J Clin Oncol* 2014; **32**: 424-430 [PMID: 24323036 DOI: 10.1200/JCO.2012.48.5797]

76 **Bocchia M**, Sicuranza A, Abruzzese E, Iurlo A, Sirianni S, Gozzini A, Galimberti S, Aprile L, Martino B, Pregno P, Sorà F, Alunni G, Fava C, Castagnetti F, Puccetti L, Breccia M, Cattaneo D, Defina M, Mulas O, Baratè C, Caocci G, Sica S, Gozzetti A, Luciano L, Crugnola M, Annunziata M, Tiribelli M, Pacelli P, Ferrigno I, Usala E, Sgherza N, Rosti G, Bosi A, Raspadori D. Residual Peripheral Blood CD26+ Leukemic Stem Cells in Chronic Myeloid Leukemia Patients During TKI Therapy and During Treatment-Free Remission. *Front Oncol* 2018; **8**: 194 [PMID: 29900128 DOI: 10.3389/fonc.2018.00194]

77 **Beider K**, Darash-Yahana M, Blaier O, Koren-Michowitz M, Abraham M, Wald H, Wald O, Galun E, Eizenberg O, Peled A, Nagler A. Combination of imatinib with CXCR4 antagonist BKT140 overcomes the protective effect of stroma and targets CML in vitro and in vivo. *Mol Cancer Ther* 2014; **13**: 1155-1169 [PMID: 24502926 DOI: 10.1158/1535-7163.MCT-13-0410]

78 **Wang Y**, Miao H, Li W, Yao J, Sun Y, Li Z, Zhao L, Guo Q. CXCL12/CXCR4 axis confers adriamycin resistance to human chronic myelogenous leukemia and oroxylin A improves the sensitivity of K562/ADM cells. *Biochem Pharmacol* 2014; **90**: 212-225 [PMID: 24858801 DOI: 10.1016/j.bcp.2014.05.007]

79 **Agarwal A**, Fleischman AG, Petersen CL, MacKenzie R, Luty S, Loriaux M, Druker BJ, Woltjer RL, Deininger MW. Effects of plerixafor in combination with BCR-ABL kinase inhibition in a murine model of CML. *Blood* 2012; **120**: 2658-2668 [PMID: 22889761 DOI: 10.1182/blood-2011-05-355396]

80 **Ågerstam H**, Hansen N, von Palffy S, Sandén C, Reckzeh K, Karlsson C, Lilljebjörn H, Landberg N, Askmyr M, Högberg C, Rissler M, Porkka K, Wadenvik H, Mustjoki S, Richter J, Järås M, Fioretos T. IL1RAP antibodies block IL-1-induced expansion of candidate CML stem cells and mediate cell killing in xenograft models. *Blood* 2016; **128**: 2683-2693 [PMID: 27621309 DOI: 10.1182/blood-2015-11-679985]

81 **Warda W**, Larosa F, Neto Da Rocha M, Trad R, Deconinck E, Fajloun Z, Faure C, Caillot D, Moldovan M, Valmary-Degano S, Biichle S, Daguindau E, Garnache-Ottou F, Tabruyn S, Adotevi O, Deschamps M, Ferrand C. CML Hematopoietic Stem Cells Expressing IL1RAP Can Be Targeted by Chimeric Antigen Receptor-Engineered T Cells. *Cancer Res* 2019; **79**: 663-675 [PMID: 30514753 DOI: 10.1158/0008-5472.CAN-18-1078]

82 **Gallipoli P**, Cook A, Rhodes S, Hopcroft L, Wheadon H, Whetton AD, Jørgensen HG, Bhatia R, Holyoake TL. JAK2/STAT5 inhibition by nilotinib with ruxolitinib contributes to the elimination of CML CD34+ cells in vitro and in vivo. *Blood* 2014; **124**: 1492-1501 [PMID: 24957147 DOI: 10.1182/blood-2013-12-545640]

83 **Hira VVV**, Van Noorden CJF, Carraway HE, Maciejewski JP, Molenaar RJ. Novel therapeutic strategies to target leukemic cells that hijack compartmentalized continuous hematopoietic stem cell niches. *Biochim Biophys Acta Rev Cancer* 2017; **1868**: 183-198 [PMID: 28363872 DOI: 10.1016/j.bbcan.2017.03.010]

84 **Houshmand M**, Soleimani M, Atashi A, Saglio G, Abdollahi M, Nikougoftar Zarif M. Mimicking the Acute Myeloid Leukemia Niche for Molecular Study and Drug Screening. *Tissue Eng Part C Methods* 2017; **23**: 72-85 [PMID: 28007011 DOI: 10.1089/ten.TEC.2016.0404]

85 **Liu T**, Li X, You S, Bhuyan SS, Dong L. Effectiveness of AMD3100 in treatment of leukemia and solid

tumors: From original discovery to use in current clinical practice. *Exp Hematol Oncol* 2016; **5**: 19 [PMID: 27429863 DOI: 10.1186/s40164-016-0050-5]

86 **Liesveld JL**, Rosell KE, Lu C, Bechelli J, Phillips G, Lancet JE, Abboud CN. Acute myelogenous leukemia--microenvironment interactions: Role of endothelial cells and proteasome inhibition. *Hematology* 2005; **10**: 483-494 [PMID: 16321813 DOI: 10.1080/10245330500233452]

87 **Uy GL**, Rettig MP, Stone RM, Konopleva MY, Andreeff M, McFarland K, Shannon W, Fletcher TR, Reineck T, Eades W, Stockerl-Goldstein K, Abboud CN, Jacoby MA, Westervelt P, DiPersio JF. A phase 1/2 study of chemosensitization with plerixafor plus G-CSF in relapsed or refractory acute myeloid leukemia. *Blood Cancer J* 2017; **7**: e542 [PMID: 28282031 DOI: 10.1038/bcj.2017.21]

88 **Wigerup C**, Påhlman S, Bexell D. Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer. *Pharmacol Ther* 2016; **164**: 152-169 [PMID: 27139518 DOI: 10.1016/j.pharmthera.2016.04.009]

89 **Krause DS**, Lazarides K, von Andrian UH, Van Etten RA. Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. *Nat Med* 2006; **12**: 1175-1180 [PMID: 16998483 DOI: 10.1038/nm1489]

90 **Jacamo R**, Chen Y, Wang Z, Ma W, Zhang M, Spaeth EL, Wang Y, Battula VL, Mak PY, Schallmoser K, Ruvolo P, Schober WD, Shpall EJ, Nguyen MH, Strunk D, Bueso-Ramos CE, Konoplev S, Davis RE, Konopleva M, Andreeff M. Reciprocal leukemia-stroma VCAM-1/VLA-4-dependent activation of NF-κB mediates chemoresistance. *Blood* 2014; **123**: 2691-2702 [PMID: 24599548 DOI: 10.1182/blood-2013-06-511527]

91 **Reikvam H**, Hatfield KJ, Lassalle P, Kittang AO, Ersvaer E, Bruserud Ø. Targeting the angiopoietin (Ang)/Tie-2 pathway in the crosstalk between acute myeloid leukaemia and endothelial cells: Studies of Tie-2 blocking antibodies, exogenous Ang-2 and inhibition of constitutive agonistic Ang-1 release. *Expert Opin Investig Drugs* 2010; **19**: 169-183 [PMID: 20050812 DOI: 10.1517/13543780903485659]

92 **Kannan S**, Sutphin RM, Hall MG, Golfman LS, Fang W, Nolo RM, Akers LJ, Hammitt RA, McMurray JS, Kornblau SM, Melnick AM, Figueroa ME, Zweidler-McKay PA. Notch activation inhibits AML growth and survival: A potential therapeutic approach. *J Exp Med* 2013; **210**: 321-337 [PMID: 23359069 DOI: 10.1084/jem.20121527]

93 **O' Reilly E**, Dhami SPS, Baev DV, Ortutay C, Halpin-McCormick A, Morrell R, Santocanale C, Samali A, Quinn J, O'Dwyer ME, Szegezdi E. Repression of Mcl-1 expression by the CDC7/CDK9 inhibitor PHA-767491 overcomes bone marrow stroma-mediated drug resistance in AML. *Sci Rep* 2018; **8**: 15752 [PMID: 30361682 DOI: 10.1038/s41598-018-33982-y]

94 **Bowers M**, Zhang B, Ho Y, Agarwal P, Chen CC, Bhatia R. Osteoblast ablation reduces normal long-term hematopoietic stem cell self-renewal but accelerates leukemia development. *Blood* 2015; **125**: 2678-2688 [PMID: 25742698 DOI: 10.1182/blood-2014-06-582924]

95 **Krause DS**, Fulzele K, Catic A, Sun CC, Dombkowski D, Hurley MP, Lezeau S, Attar E, Wu JY, Lin HY, Divieti-Pajevic P, Hasserjian RP, Schipani E, Van Etten RA, Scadden DT. Differential regulation of myeloid leukemias by the bone marrow microenvironment. *Nat Med* 2013; **19**: 1513-1517 [PMID: 24162813 DOI: 10.1038/nm.3364]

96 **Zhou HS**, Carter BZ, Andreeff M. Bone marrow niche-mediated survival of leukemia stem cells in acute myeloid leukemia: Yin and Yang. *Cancer Biol Med* 2016; **13**: 248-259 [PMID: 27458532 DOI: 10.20892/j.issn.2095-3941.2016.0023]

97 **Calvi LM**, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 2003; **425**: 841-846 [PMID: 14574413 DOI: 10.1038/nature02040]

98 **Herrmann H**, Cerny-Reiterer S, Gleixner KV, Blatt K, Herndlhofer S, Rabitsch W, Jäger E, Mitterbauer-Hohendanner G, Streubel B, Selzer E, Schwarzinger I, Sperr WR, Valent P. CD34(+)/CD38(-) stem cells in chronic myeloid leukemia express Siglec-3 (CD33) and are responsive to the CD33-targeting drug gemtuzumab/ozogamicin. *Haematologica* 2012; **97**: 219-226 [PMID: 21993666 DOI: 10.3324/haematol.2010.035006]

99 **Landberg N**, von Palffy S, Askmyr M, Lilljebjörn H, Sandén C, Rissler M, Mustjoki S, Hjorth-Hansen H, Richter J, Ågerstam H, Järås M, Fioretos T. CD36 defines primitive chronic myeloid leukemia cells less responsive to imatinib but vulnerable to antibody-based therapeutic targeting. *Haematologica* 2018; **103**: 447-455 [PMID: 29284680 DOI: 10.3324/haematol.2017.169946]

100 **Blatt K**, Herrmann H, Hoermann G, Willmann M, Cerny-Reiterer S, Sadovnik I, Herndlhofer S, Streubel B, Rabitsch W, Sperr WR, Mayerhofer M, Rülicke T, Valent P. Identification of campath-1 (CD52) as novel drug target in neoplastic stem cells in 5q-patients with MDS and AML. *Clin Cancer Res* 2014; **20**: 3589-3602 [PMID: 24799522 DOI: 10.1158/1078-0432.CCR-13-2811]

101 **Iwasaki M**, Liedtke M, Gentles AJ, Cleary ML. CD93 Marks a Non-Quiescent Human Leukemia Stem Cell Population and Is Required for Development of MLL-Rearranged Acute Myeloid Leukemia. *Cell Stem Cell* 2015; **17**: 412-421 [PMID: 26387756 DOI: 10.1016/j.stem.2015.08.008]

102 **Chung SS**, Eng WS, Hu W, Khalaj M, Garrett-Bakelman FE, Tavakkoli M, Levine RL, Carroll M, Klimek VM, Melnick AM, Park CY. CD99 is a therapeutic target on disease stem cells in myeloid malignancies. *Sci Transl Med* 2017; **9**: pii: eaaj2025 [PMID: 28123069 DOI: 10.1126/scitranslmed.aaj2025]

103 **Barreyro L**, Will B, Bartholdy B, Zhou L, Todorova TI, Stanley RF, Ben-Neriah S, Montagna C, Parekh S, Pellagatti A, Boultwood J, Paietta E, Ketterling RP, Cripe L, Fernandez HF, Greenberg PL, Tallman MS, Steidl C, Mitsiades CS, Verma A, Steidl U. Overexpression of IL-1 receptor accessory protein in stem and progenitor cells and outcome correlation in AML and MDS. *Blood* 2012; **120**: 1290-1298 [PMID: 22723552 DOI: 10.1182/blood-2012-01-404699]

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REVIEW

Induced pluripotent stem cells throughout the animal kingdom: Availability and applications

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Abstract

Up until the mid 2000s, the capacity to generate every cell of an organism was exclusive to embryonic stem cells. In 2006, researchers Takahashi and Yamanaka developed an alternative method of generating embryonic-like stem cells from adult cells, which they coined induced pluripotent stem cells (iPSCs). Such iPSCs possess most of the advantages of embryonic stem cells without the ethical stigma associated with derivation of the latter. The possibility of generating "custom-made" pluripotent cells, ideal for patient-specific disease models, alongside their possible applications in regenerative medicine and reproduction, has drawn a lot of attention to the field with numbers of iPSC studies published growing exponentially. IPSCs have now been generated for a wide variety of species, including but not limited to, mouse, human, primate, wild felines, bovines, equines, birds and rodents, some of which still lack well-established embryonic stem cell lines. The paucity of robust characterization of some of these iPSC lines as well as the residual expression of transgenes involved in the reprogramming process still hampers the use of such cells in species preservation or medical research, underscoring the requirement for further investigations. Here, we provide an extensive overview of iPSC generated from a broad range of animal species including their potential applications and limitations.

Key words: Pluripotency; Embryonic; Stem cell; Reprogramming; Animal; Wild; Induced pluripotency

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Core tip: Induced pluripotent stem cells (iPSC) have opened up the possibility of converting literally any mature cell type into an embryonic like pluripotent state. This procedure has had a large impact on biomedical sciences for patient specific disease modeling, cell-type specific differentiation and regenerative medicine with or without gene editing. These advances are clearly not restricted to human iPSCs, and indeed it was mouse iPSCs that were derived first. In this review we will provide a comprehensive overview of iPSC generated throughout the animal kingdom as well as an elaboration on their possible applications and limitations.

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INTRODUCTION

The ability to differentiate into any given cell type within an organism was limited solely to embryonic stem cells (ESC) until 2006. ESCs possess the capacity to proliferate indefinitely without differentiation, form chimeras and display germline transmission. Currently, ESCs with these characteristics have only been confirmed in mice and rats[1]. In regard to culturing ESC of other species, besides human, mice and rats, undefined culture conditions already present the first hurdle.

In 2006, an alternative means was developed to generate embryonic-like stem cells whereby differentiated adult cells are reprogrammed into induced pluripotent stem cells (iPSC)[2]. This reprogramming process entails the delivery of so-called pluripotency factors to mature cells to induce their conversion into ESC-like cells, which subsequently triggers the transcriptional and translational activation of endogenous pluripotency factors. These pluripotency factors are all regulators of ESC proliferation, renewal and pluripotency.

The most commonly used genes for achieving such conversion are: *Homeobox protein* (*NANOG*); *octamer-binding transcription factor 4* (*OCT4/POU5F1*); *SRY-Box 2* (*SOX2*); *Kruppel Like Factor 4* (*KLF4*); *proto-oncogene MYC* (*c-MYC*) and *Lin-28 Homolog A* (*LIN28*). NANOG is a key transcription factor inhibiting differentiation towards extraembryonic endoderm and trophectoderm lineages. Moreover, by directly inhibiting SMAD Family Member 1, NANOG prevents bone morphogenetic protein-induced mesoderm differentiation[3]. NANOG also plays an important role in binding and activating the OCT4 promoter and is consequently a transcriptional activator of OCT4[4]. OCT4 is required for naïve epiblast formation, and *OCT4*-null embryos lack pluripotent characteristics within their inner cell mass[5]. Furthermore, abrogation of OCT4 expression in ESCs results in trophoblast differentiation of the inner cell mass[6]. OCT4 therefore plays prominent roles in pluripotency maintenance in ESCs and during the reprogramming of mature cells to iPSC. SOX2 forms a complex with OCT4 to bind DNA and govern the expression of several genes required for embryonic development[7].

Consequently, it has been claimed that NANOG, OCT4 and SOX2 act as master regulators of ESC pluripotency[8] at least for the generation of mouse, human and rat iPSC. However, less is known about gene expression requirements for achieving pluripotency in other species. Therefore, several additional genes have been tested for their ability to generate iPSC. These include KLF4, which regulates the expression of key transcription factors during embryonic development, including NANOG[9]. MYC has been shown to play a role in maintaining the glycolytic energy metabolism in stem cells[10]. Additionally, while not essential, MYC has been shown to promote the generation of iPSC from human and mouse skin fibroblasts[11]. Finally, LIN28 is an RNA-binding protein and regulates gene expression at a post-transcriptional level. The products of genes regulated by LIN28 function in developmental timing and self-renewal in ESCs[12].

Other less commonly used genes involved in achieving and/or maintaining the undifferentiated state will be discussed directly in the following description of the various iPSCs generated from various species. In addition to the plethora of pluripotency factors applied for reprogramming, several methods exist with which to deliver them into adult cells. Both early protocols and reprogramming efforts in notoriously challenging species use viral approaches, such as retrovirus and

lentivirus[13,14]. Whilst the human and mouse fields have progressed to employing non-integrative methods such as Sendai-viral[15], episomal[16] and mRNA transfection[17], very few attempts have been made to apply these to other species. In the following sections we will provide a comprehensive overview of the iPSCs available from various species, their method of generation, their pluripotent characteristics and their applications in science.

DOMESTIC RUMINANTS

The establishment of iPSC from domestic ruminants was reported for human[13], mouse[2], monkey[18], rat[19,20], pig[21-24], dog[25] and rabbit[26]. These species, from which iPSCs have already been generated, unsurprisingly comprise commonly used animal models in the field of regenerative medicine. However, ruminants, especially small ruminants, are equally attractive for biomedical research. For example, sheep are often employed as the preferred model for human pregnancy and perinatal-related studies[27], as models for rare or degenerative diseases[28,29], for several chirurgic procedures[30,31] and cancer[32]. This is predominantly based on the fact that ruminants share more phylogenetic characteristics, similar size and longevity with humans than do rodents[33].

Aside from being explored as biomedical models, small ruminants, cattle and buffalo present significant commercial value and agricultural importance, being raised for meat or milk production and for wool and other animal-derived products. In this context, the generation of genetically modified animals for the production of therapeutic proteins in milk (bioreactors), with increased resistance to diseases or selection for other valuable traits is highly desirable. To date, robust pluripotent stem cells derived from these species are still lacking[34-36] despite very recent encouraging efforts[37]. The production of genetically modified livestock is usually accomplished through somatic cell nuclear transfer (SCNT) after genetic modification of donor cells[38-40].

The generation of iPSCs from these species presents a major objective to facilitate the application of advanced reproductive technologies[41] including allowing easier genetic manipulation (knock-ins or knock-outs) in pluripotent cells used for chimera generation, improving SCNT efficiency by using iPSCs as nuclei donors[42,43] or producing functional gametes *in vitro*[44-46]. The first studies in cattle reported that the four Yamanaka factors were insufficient for inducing pluripotency, and that NANOG or NANOG plus LIN28 were additionally required[47,48]. In 2012, Cao *et al*[49] reported bovine iPSCs derived from buffalo defined factors [OCT4, SOX2, KLF4, and MYC (OSKM)] and fetal fibroblasts that could be differentiated into putative female germ cells, a first step towards future use in reproductive sciences. Subsequently, Kawaguchi *et al*[50] contributed to chimera production (90 d of gestation) (Supplemental material 1).

In cattle, different cell types such as adult or fetal fibroblasts, amniotic, mammary and retina-derived cells have been used in conjunction with integrative vectors[47-54]. Testicular cells were induced into pluripotency after electroporation of OCT4 alone[55]. However, silencing of exogenous factors when integrated was not reported, and some studies were unable to characterize bovine iPSCs (biPSCs) after culture due to characteristics related to quiescence *in vitro*[56,57]. It has been shown that buffalo fetal fibroblasts can be retrovirally reprogrammed into iPSC by buffalo OSKM, and that the generation efficiency of biPSCs can be increased by inhibiting p53 expression[58] (Supplemental material 1).

In small ruminants, both ovine and caprine iPSCs were reported only from fibroblasts, either embryonic, fetal or adult[59-69]. Although integrative methodologies (retro- and lentiviral) are still most commonly used, silencing of exogenous factors has already been reported[63,64] (Supplemental material 2). This is a significant achievement considering the objective of producing new organisms from pluripotent cells. Recently, induction of pluripotency using an mRNA approach with OSKM transcription factors was achieved in goats[69]. Regarding generation of animals from iPSCs, ovine iPSCs were already reported contributing to the inner cell mass of blastocysts[63] and live born chimeras[62]. BiPSCs were used as donor cells in SCNT, and cloned embryos were generated in cattle. Despite initial beliefs that the use of pluripotent cells might enhance cloning success, low rates of embryonic development were observed. No live-born animals have yet been reported probably due to persistent expression of transgenes and increased numbers of aneuploidies in iPSC donor cells[67,68].

SWINE

The generation of pluripotent cell lines from swine has very clear and common objectives even between different research groups, being either the use of these cells for regenerative medicine or to preserve and/or augment agriculturally important traits in this species. The pig is considered the most attractive non-primate animal model for biomedical purposes due to its similarities to human immunology, genome organization, aging and whole animal physiology[70-72]. The use of this large domestic animal (or also its miniature version-the minipig) enables long-term studies on tissue or organ transplantation or for modeling specific diseases[73-75] in a more ethically acceptable environment when compared to the use of non-human primates or domestic pet animals (dogs and cats). It is envisaged that pluripotent cells will facilitate the generation of transgenic animals for use as preclinical models and production of animals with valuable traits through the use of chimeric or nuclear transfer technologies.

The swine, however, is considered one of the "non-permissive" species meaning that bona fide robust pluripotent stem cells derived from blastocysts-ESCs have not yet been successfully generated[41,76,77]. The generation of iPSCs is of great importance. The pig was the first domesticated species from which iPSCs were derived, which was after ESCs had already been established for mouse, human, rat and monkey[78-82].

At least 25 studies have already described porcine iPSC (piPSC) production *via* various reprogramming and characterization protocols in the last decade (Supplemental material 3). The first three reports date from 2009 and describe human ESC-like cells dependent or not on basic fibroblast growth factor (bFGF) supplementation[21-23]. Most of the subsequent studies focused upon dissecting the differences between naïve or primed cell generation, especially attempting to obtain naïve cells in order to produce chimeric offspring through the use of leukemia inhibitory factor (LIF) supplementation with or without other inhibitors such as CHIR99021, PD0325901, 5-AZA and others[83-92].

Contribution to embryo development at short term (embryos and/or fetuses) was reported by several groups, even though the status of exogenous gene silencing was not described and/or teratoma formation was not robust in some lineages[24,85,87,90-93]. In contrast, contribution to live chimeric offspring and germline contribution has been proven by only one group thus far[24,94], with piPSCs resembling primed, human ES-like cells. The study reports[24] contribution of piPSCs to both embryo and placenta during gestation and 85.3% efficiency of chimerism in live-born piglets. As only naïve, but not primed pluripotent cells are believed to support chimerism, this suggests that the classical definitions differentiating between the two types of pluripotent cells may be a lot more complex and still poorly-defined in other species compared to mouse and human.

PiPSCs have also been tested for specific *in vitro* differentiation potential; for example, they were able to differentiate into beating cardiomyocyte-like cells[95,96] and neuronal lineage[97]. PiPSCs have also been used as donor cells for nuclear transfer experiments. Although blastocysts were produced, the efficiency rate did not significantly increase when compared to blastocyst developmental rate achieved using embryonic fibroblasts as nuclei donors, and no born piglets were reported[85].

In summary, the production of piPSCs until now has predominantly relied upon the use of integrative vectors, lenti- or retrovirus-carrying human or mouse OSKM, including some variations such as NANOG, LIN-28 or the absence of OCT4 or SOX2 and KLF4. Few studies have described the use of porcine or monkey factors. Even when episomal non-integrative approaches have been used, persistence or integration of plasmids, and therefore silencing of the transgenes, was reported (please refer to Supplemental material 3 for details). Failure to inactivate the exogenous factors is considered a major flaw in the generation of bona fide iPSCs. Defining proper culture conditions and reprogramming protocols is still the major objective of most of the reported studies, even though differentiation is possible in this sub-optimal condition. Ji *et al*[89] reported that two cell lines transduced with lentivirus containing monkey OSKM and cultured with LIF, bFGF and inhibitors presented silencing of exogenous factors. Using episomal vectors, Li *et al*[93] were the first to report the generation of cell lines able to maintain pluripotent characteristics for 20 passages and absence of integration at this time. This represents a great advance in the generation of pluripotent cells from pig, which arguably remains the most desirable model for both human and veterinary medicine.

HORSES

According to the latest report from The American Horse Council Foundation, the United States horse industry has an economic impact of United States $122 billion with 74% of horses participating in the sports sector (racing and competition). Sports horses are constantly exposed to risks of career-ending or even life-threatening musculoskeletal injuries[98]. Besides the magnitude of the horse industry, the possibility of using these animals as models for human musculoskeletal injuries or diseases[99] has contributed to intensify the stem cell and regenerative research in the last few years.

During the past decade, equine iPSCs (eiPSCs) have been produced using both integrative and non-integrative systems carrying mouse or human reprogramming factors in conjunction with multiple cell sources, including adult or fetal fibroblasts, adipose tissue mesenchymal cells, keratinocytes, myogenic mangioblast, peripheral mesenchymal stem cells and umbilical cord cells (Supplemental material 4)[100-108]. Although subsequent attempts were made using episomal vectors[107], only the initial eiPSCs report succeeded in producing equine pluripotent stem cells with a non-integrative PiggyBac transposon system, which is considered safer for clinical uses but allegedly with lower reprogramming efficiency[100,109]. Of the eiPSCs generated *via* integrative systems, only one group has reported transgene silencing[106] with others reporting partial silencing[102,104] and others conversely showing transgenes to still be activated[101,103,108]. Other studies do not mention the state of transgene expression[105,107]. Regarding further characterization procedures, eiPSCs generated in the above studies show *in vitro* or *in vivo* potential to generate cells of all three germ layers as well as expression of multiple pluripotency markers (Supplemental material 4).

The safety of clinical and reproductive applications of iPSCs remains a concern, especially regarding tumorigenesis, epigenetic abnormalities and eventual immune rejection[110-112]. Fittingly, some reports do address these issues. Aguiar *et al*[113] analyzed the immunogenicity of allogenic eiPSCs intradermally transplanted into immunosuppressant-free horses and observed moderate cellular response but not acute rejection. This suggests that allogenic eiPSC banking might serve as a future possibility for cell therapy. In the reproductive field, eiPSCs have been used as donor cells in an attempt to improve SCNT efficiency, but blastocysts were not successfully produced[114].

The possibilities of applying eiPSCs in tissue engineering and regenerative medicine are also being actively explored. While some have reported failure of eiPSCs to generate artificial tendons after induced differentiation[115], others have demonstrated eiPSCs to be capable of inducing muscle regeneration in immunodeficient mice with dystrophin deficiency[105]. Furthermore, Aguiar *et al*[116] showed that eiPSCs could be differentiated into keratinocytes focusing on skin trauma and wound management. Other research groups have studied eventual uses for mesenchymal-like progenitors capable of chondrogenesis and adipogenesis[117] or even induction into functional osteoblasts[118] and transgenic induced myocytes[119], thus providing extra cell sources for regenerative veterinary medicine. Although some studies have already tested the potential and applicability of eiPSCs as seen above, there is still a long road ahead until eiPSCs and their derivatives are completely understood and deemed safe to use in disease models and regenerative veterinary medicine.

DOGS

Dogs play multiple roles in modern society, ranging from livestock management, rescue and security services and emotional and disability assistance besides their major role as companion and best friend[120]. Every year, the number of households with pets increases, having reached 68% in the United States during 2017-2018 with the majority of people owning dogs , and secondly cats. This represented an expenditure of around United States $70 billion during that period of which a little over United States $17 billion was spent on veterinary services. If that alone was not ample reason to increase dog-related research on innovative therapies like regenerative medicine and stem cells, dogs are also considered physiologically relevant model of human diseases. In addition to hundreds of canine hereditary diseases having equivalent human disorders, humans also share a similar physiology and environment with their canine companions[121]. The first canine iPSC (ciPSC) report was published in 2010[25], and since then around ten reports on new ciPSCs lineages have been published. The main cell source used for reprogramming was adult fibroblasts[122-126] followed by fetal or embryonic fibroblasts[25,127-129] and adipose tissue mesenchymal cells[123,130].

As seen in Supplemental material 5, with the exception of studies using non-

integrative Sendai viruses[126,129] the majority of the ciPSCs reported were generated using retroviral or lentiviral systems. Although they are considered more efficient than non-integrative systems, their use in research with clinical applications raises concerns, specifically through potentially dangerous viral integration in the cell genome, transgene reactivation and epigenetic changes[131]. Transgene expression on these ciPSC was reported in variable levels from silenced[128,130] to low levels of expression[124,125] to expression in different states[122,127,128] and not reported[25]. These ciPSCs also vary widely on the pluripotency markers used for characterization purposes (Supplemental material 5).

Further studies on ciPSC applications have been performed by few research groups. Lee *et al*[123] generated endothelial cells from ciPSCs and tested their therapeutic potential in mouse models of myocardial infarction and hindlimb ischemia, besides transplanting labeled ciPSCs autologously into dogs' hearts to monitor cell fate in large animal models of cardiac delivery. Others derived mesenchymal stem cells from ciPSCs that exhibited high proliferative potential, capacity to differentiate into mesodermal-derived tissues and both mesenchymal and pluripotent markers but did not form teratoma-like tissues, a desired characteristic for stem cell therapy[132]. Chow *et al*[126] compared ciPSC-derived mesenchymal cells with adipose tissue and bone marrow mesenchymal cells with regard to their surface markers, gene expression profiles and immune modulation potency. The results showed ciPSCs-derived mesenchymal cells to present a slightly different surface phenotype than regular mesenchymal cells but to be capable of inducing suppression of *in vitro* immune function much like the other analyzed cells. Taken together, these studies demonstrate the continued efforts of the veterinary and research fields, not only in order to pursue longer and healthier lives for our pets, but also to develop disease and therapeutic models for human disorders.

RABBITS

Rabbits have long been used as animal models in research. They are considered highly physiologically relevant even for some human pathology such as heart diseases[133]. Their larger size compared with mice and rats enables their use in surgical procedures, they possess a longer life span and rabbits are phylogenetically more similar to humans. When compared to other suitable larger animal models such as pigs and dogs, rabbits are easier to handle and maintain, are more economical to keep and have shorter reproductive cycles, which facilitates breeding and long-term research analyses[26,134]. It has been reported that rabbit ESCs are very similar to human ESCs in regard to their morphology as well as biochemical and pluripotency features[26].

Rabbit iPSCs (rbiPSCs) have been described in few reports. Honda *et al*[26] generated rbiPSCs from adult liver and stomach cells using lentivirus and human OSKM. These rbiPSCs were silenced after about 18 passages. Interestingly, the authors were not successful in reprogramming fibroblasts using the same methodology. The rbiPSCs produced in this report were LIF- and bFGF-dependent and expressed the same pluripotency markers as rabbit ESCs (Supplemental material 6). In a follow-up study, the rbiPSCs generated were converted to a naïve-like state *via* forced expression of human OCT3/4 increasing these cells' potential for *in vitro* neural differentiation[135].

Using a retroviral system also containing human OSKM, Osteil *et al*[136] compared adult fibroblast-derived rbiPSCs with ESCs. Theses rbiPSCs showed transgene silencing at passage 25 and expressed the pluripotency markers OCT4 and NANOG. Later, the same group showed that *via* expression of KLF2 and KLF4, rbiPSCs could be converted into epiblast-like cells, capable of colonizing pre-implantation rabbit embryos[137].

Finally, the most recently published study on rbiPSCs also employed human OSKM in a retroviral system to reprogram embryonic fibroblasts. The cells generated were dependent upon LIF and bFGF, expressed key pluripotency markers and showed no transgene expression. Focusing on the use of rabbits as heart models, the authors showed these cells to be capable of successful differentiation into cardiac cells, underscoring a possible future application as a disease model[133].

AVIAN

According to the United States Department of Agricultureâs Production and Value Summary, the combined value of avian products reached United States $42.7 billion in 2017, besides the over 20 million birds kept as pets currently in the United States

(APPA, 2018), denoting the significance of these animals to modern society. In research, avian models are considered extremely important because they permit easy monitoring of embryonic development[138] and can also be used as disease models[139].

Avian iPSCs (aiPSCs) were the first non-mammalian iPSCs to be derived[140] and were reported for quail, chicken and zebra finch (Supplemental material 7). The majority of the reports published used embryonic fibroblasts[140-145], but they were also isolated from adult feather follicles[146]. Although non-viral approaches have been applied[142,144], retroviral and lentiviral methodologies have mostly been used and transgene expression was either detected[140,145,146] or not discussed[142-144] with the exception of the chicken iPSCs reported by Rosselló *et al*[141] where transgenes were silenced after five passages.

Further characterization of aiPSCs was performed by Dai *et al*[138], who were able to produce aiPSCs-derived neurons. So far, perhaps the most exciting potential application of these cells is vaccine production and related research[147,148]. In those reports, it is shown that aiPSCs grown in modified conditions possess great potential as candidates for Newcastle disease virus production serving as a suitable replacement for the embryonating eggs currently used for vaccine generation[147]. It should be possible to generate aiPSCs more tolerant to the Newcastle disease virus, which might eventually also be employed in disease-resistant poultry studies[148]. These newly developed methodologies represent a great potential application of aiPSCs to future livestock, health and food security.

EXOTIC ANIMALS

The most obvious reason for generating iPSC from exotic animals is species conservation. Genetic material can be stored and expanded on demand in the form of viable and proliferating iPSCs. Ideally, protocols would be developed to differentiate such iPSCs into primordial germ cells and subsequently generate egg and sperm cells to facilitate *in vitro* fertilization. Such efforts form the basis for the generation of iPSC from *Madrillus leucophaeus* (primate/drill) and *Ceratotherium simum cottoni* (northern white rhinoceros). Both species teeter on the brink of extinction, and the generation of iPSCs might be beneficial for species conservation. Drill iPSC lines were generated using retroviral vectors containing the human sequences for *OCT4*, *SOX2*, *KLF4* and *c-MYC*. The fibroblast source originated from a 15-year-old drill. All factors integrated successfully into the drill genome and the authors were able to show that exogenous transcription factors ceased to be expressed whilst the endogenous drill transcription factors became activated. Drill iPSC were karyotypically normal and exhibited the potential to form teratomas containing all three lineages (ectoderm, endoderm and mesoderm)[149].

The same report describes the generation of iPSC from northern white rhinoceros. Here, the same human genes for *OCT4*, *SOX2*, *KLF4* and *c-MYC* were delivered using the retro VSV-G virus system. These also integrated successfully with the exception of *KLF4*. Similar to the drill iPSC, exogenous gene expression was silenced and endogenous gene expression initiated. Northern white rhinoceros iPSCs were karyotypically normal and gave rise to teratomas[149] (Supplemental material 8). These are extremely promising results for species conservation, but it remains to be seen whether these iPSCs can be used for SCNT or for the generation of *in vitro* germ cells.

Most efforts have centered upon the generation of iPSC from monkeys. Again, species conservation forms one aspect of such efforts, but another facet is the possibility of applying these monkeys and their iPSCs in biomedical research. Monkeys share a high degree of genetic, anatomical, physiological and cardiological similarities with humans[150-152]. Consequently, monkey iPSCs and monkey models represent powerful models for drug development. To date, iPSCs have been generated from rhesus monkeys[18,153,154]. Whilst the first two of these studies[18,154] used retroviral approaches, the more recent report[153] generated rhesus monkey iPSCs using non-integrative episomal plasmids (Supplemental material 8). In a further notable study[155], the retroviral method was employed to derive iPSCs from rhesus monkeys with Huntington's disease (Supplemental material 8). These monkeys and their iPSCs were not only very valuable for testing potential drug candidates but could also be used to investigate autologous and allogenic cell transplantations and graft incorporations as well as safety assessment of CRISPR/Cas9 gene-edited transplants.

Further iPSCs have been generated from cynomolgus monkeys[156,157]. Cynomolgus monkeys are commonly used in biomedical research and the described iPSCs have been derived using both retroviral approaches[156] and non-integrative Sendai virus[157] approaches. Whilst in the retroviral approach pluripotency was confirmed *via*

teratoma assays, the Sendai virus reprogrammed cells were not subjected to pluripotency assays and were directly differentiated into the cell type of interest (Supplemental material 8). Marmoset iPSCs have been generated from fetal liver cells *via* retroviral-mediated transduction with the six human pluripotency factors *OCT4*, *SOX2*, *KLF4*, *c-MYC*, *NANOG* and *LIN28*. These cells displayed a normal karyotype and pluripotency capacity as tested by embryonic body formation and teratoma assays (Supplemental material 8).

Lastly, even iPSCs of great apes such as orangutans have been derived using the retroviral approach with the classical four human pluripotency factors *OCT4*, *SOX2*, *KLF4* and *c-MYC* and pluripotency potential confirmed *via* teratoma assays[158]. Common amongst all of these studies is the lack of *in vivo* chimeric analyses using monkey-derived iPSCs. Only upon demonstration of bona fide germline chimeras will we be able to confirm the pluripotent status of monkey-derived iPSCs.

With the ultimate goal of species conservation, efforts have been made to generate iPSCs from wild feline species such as snow leopards[159] and Bengal tigers, servals and jaguars[160]. For all of these feline species, iPSC retroviral reprogramming was applied using *OCT4*, *KLF4*, *SOX2*, *c-MYC* and *NANOG* (Supplemental material 8). Similar to the iPSCs from monkeys, only teratoma assays were performed. Thus it cannot be excluded that germline transmission for actual cloning of these animals may prove challenging.

Likewise, it is hoped that iPSC generation will safeguard the future of Tasmanian devils, which are currently precariously close to extinction. For their generation, *OCT4*, *KLF4*, *SOX2*, *c-MYC, LIN28* and *NANOG* were transduced *via* lentiviral approaches into dermal skin fibroblasts[161]. It is to be hoped that these iPSCs will provide excellent tools with which to develop strategies to treat Tasmanian devil facial tumor disease, which is desperately needed to halt the extinction of devils.

Other exotic animals from which iPSCs have been generated include the prairie vole using PiggyBac delivery of mouse *Oct4*, *Klf4*, *Sox2*, *c-Myc, Lin28* and *Nanog*[162]. The authors proposed that studying oxytocin and vasopressin effects on neurons derived from these iPSCs might be of benefit in dissecting the functional roles and effects (including on gene expression) of these factors in social animals. Another intriguing application would be bat iPSCs. Bats are considered long-lived animals in relation to their body size and thus might hold some interesting answers on how to extend lifespan in other species including humans. Moreover, bats possess an immune system, which allows them to carry viruses in high titers without deleterious effects. Studying specific immune cells *in vitro* might prove a possibility with successfully derived iPSCs. Bat iPSCs have been generated using the PiggyBac system delivering human *OCT4*, *SOX2*, *KLF4*, *c-MYC*, *NANOG*, *LIN28*, *NR5A2* and bat *MIR302/367*[163].

Additional exotic animals for which iPSCs have been generated include platypus[164] and mink[165] (Supplemental material 8). In contrast, it has proven challenging to generate iPSCs from more common but non-mammals model species such as drosophila and zebrafish. These attempts used mouse *Oct4*, *Sox2*, *Klf4* and *c-Myc* lentiviral delivery and resulted in only partially reprogrammed iPSCs[141].

CONCLUSION AND FUTURE DIRECTIONS

Taken together, the data reviewed here highlights some interesting and conflicting aspects of iPSC research throughout the animal kingdom. Although there exist a reasonable number of well-established animal iPSCs, the lack of description for some species, for example the domestic cat, draws attention to the fact that a global mechanism of cellular reprogramming has certainly not yet been unraveled. The non-standardized reports in most species hamper the comparison of some features, such as reprogramming efficiency. This information was estimated in some of the studies as the ratio between emerging colonies and seeded cells, and in others as the ratio between Alkaline Phosphatase (AP)-positive colonies and seeded cells. In some cases, reprogramming efficiency was not reported at all or was even reported as transduction efficiency through evaluation of fluorochrome-labeled reprogramming vectors. Another important matter is the lack of proper and robust characterization of some of the generated cell lines. Regarding *in vitro* characterization, perhaps the lack of criteria, based on the fact that no ES-derived cell lines exist, makes it difficult to define whether a given cell line is truly pluripotent or not.

Furthermore, residual expression of transgenes, even in high passage cells, is still observed in most of the cell lines derived, a flawed hallmark of true reprogramming. Alongside this, many of the animal iPSC lines established were not tested or even failed to produce viable chimeras, the golden standard validation *in vivo* of these cells'

ability to give rise to cells from all three germ layers. This introduces a veil of doubt regarding the actual reprogramming state of those cells.

Regardless, even if the generated cells lines are incompletely reprogrammed, there is no doubt that the production of iPSCs is a major breakthrough, especially for those "non-permissive" species. However, more comprehensive studies are still very much required to elucidate pluripotency acquisition mechanisms for each of them, once it is already known that they differ from human and mouse. Perhaps a deep dive into genomics or proteomics can enlighten us regarding the roles of specific pathways involved in those reprogramming processes and bring us closer to the practical application of iPSCs in such fields as stem cell research, regenerative medicine and reproduction.

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REFERENCES

1 **Gafni O**, Weinberger L, Mansour AA, Manor YS, Chomsky E, Ben-Yosef D, Kalma Y, Viukov S, Maza I, Zviran A, Rais Y, Shipony Z, Mukamel Z, Krupalnik V, Zerbib M, Geula S, Caspi I, Schneir D, Shwartz T, Gilad S, Amann-Zalcenstein D, Benjamin S, Amit I, Tanay A, Massarwa R, Novershtern N, Hanna JH. Derivation of novel human ground state naive pluripotent stem cells. *Nature* 2013; **504**: 282-286 [PMID: 24172903 DOI: 10.1038/nature12745]

2 **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]

3 **Suzuki A**, Raya Á, Kawakami Y, Morita M, Matsui T, Nakashima K, Gage FH, Rodríguez-Esteban C, Izpisúa Belmonte JC. Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. *Proc Natl Acad Sci U S A* 2006; **103**: 10294-10299 [PMID: 16801560 DOI: 10.1073/pnas.0506945103]

4 **Hayashi Y**, Caboni L, Das D, Yumoto F, Clayton T, Deller MC, Nguyen P, Farr CL, Chiu HJ, Miller MD, Elsliger MA, Deacon AM, Godzik A, Lesley SA, Tomoda K, Conklin BR, Wilson IA, Yamanaka S, Fletterick RJ. Structure-based discovery of NANOG variant with enhanced properties to promote self-renewal and reprogramming of pluripotent stem cells. *Proc Natl Acad Sci U S A* 2015; **112**: 4666-4671 [PMID: 25825768 DOI: 10.1073/pnas.1502855112]

5 **Nichols J**, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 1998; **95**: 379-391 [PMID: 9814708 DOI: 10.1016/S0092-8674(00)81769-9]

6 **Niwa H**, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 2000; **24**: 372-376 [PMID: 10742100 DOI: 10.1038/74199]

7 **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]

8 **Kashyap V**, Rezende NC, Scotland KB, Shaffer SM, Persson JL, Gudas LJ, Mongan NP. Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the NANOG, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell microRNAs. *Stem Cells Dev* 2009; **18**: 1093-1108 [PMID: 19480567 DOI: 10.1089/scd.2009.0113]

9 **Zhang P**, Andrianakos R, Yang Y, Liu C, Lu W. Kruppel-like factor 4 (Klf4) prevents embryonic stem (ES) cell differentiation by regulating Nanog gene expression. *J Biol Chem* 2010; **285**: 9180-9189 [PMID: 20071344 DOI: 10.1074/jbc.M109.077958]

10 **Cliff TS**, Wu T, Boward BR, Yin A, Yin H, Glushka JN, Prestegaard JH, Dalton S. MYC Controls Human Pluripotent Stem Cell Fate Decisions through Regulation of Metabolic Flux. *Cell Stem Cell* 2017; **21**: 502-516.e9 [PMID: 28965765 DOI: 10.1016/j.stem.2017.08.018]

11 **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]

12 **Viswanathan SR**, Daley GQ. Lin28: A microRNA regulator with a macro role. *Cell* 2010; **140**: 445-449 [PMID: 20178735 DOI: 10.1016/j.cell.2010.02.007]

13 **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]

14 **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]

15 **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]

16 **Okita K**, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M, Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H, Yamanaka S. A more efficient method to generate integration-free human iPS cells. *Nat Methods* 2011; **8**: 409-412 [PMID: 21460823

DOI: 10.1038/nmeth.1591]
17 **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, Schlaeger 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]
18 **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]
19 **Li W**, Wei W, Zhu S, Zhu J, Shi Y, Lin T, Hao E, Hayek A, Deng H, Ding S. Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell* 2009; **4**: 16-19 [PMID: 19097958 DOI: 10.1016/j.stem.2008.11.014]
20 **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]
21 **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]
22 **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 U S A* 2009; **106**: 10993-10998 [PMID: 19541600 DOI: 10.1073/pnas.0905284106]
23 **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]
24 **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]
25 **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]
26 **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]
27 **Morrison JL**, Berry MJ, Botting KJ, Darby JRT, Frasch MG, Gatford KL, Giussani DA, Gray CL, Harding R, Herrera EA, Kemp MW, Lock MC, McMillen IC, Moss TJ, Musk GC, Oliver MH, Regnault TRH, Roberts CT, Soo JY, Tellam RL. Improving pregnancy outcomes in humans through studies in sheep. *Am J Physiol Regul Integr Comp Physiol* 2018; **315**: R1123-R1153 [PMID: 30325659 DOI: 10.1152/ajpregu.00391.2017]
28 **Pinnapureddy AR**, Stayner C, McEwan J, Baddeley O, Forman J, Eccles MR. Large animal models of rare genetic disorders: Sheep as phenotypically relevant models of human genetic disease. *Orphanet J Rare Dis* 2015; **10**: 107 [PMID: 26329332 DOI: 10.1186/s13023-015-0327-5]
29 **McBride SD**, Perentos N, Morton AJ. A mobile, high-throughput semi-automated system for testing cognition in large non-primate animal models of Huntington disease. *J Neurosci Methods* 2016; **265**: 25-33 [PMID: 26327320 DOI: 10.1016/j.jneumeth.2015.08.025]
30 **DiVincenti L**, Westcott R, Lee C. Sheep (Ovis aries) as a model for cardiovascular surgery and management before, during, and after cardiopulmonary bypass. *J Am Assoc Lab Anim Sci* 2014; **53**: 439-448 [PMID: 25255065 DOI: 10.1002/clc.21952]
31 **Katz MG**, Kendle AP, Fargnoli AS, Mihalko KL, Bridges CR. Sheep (Ovis aries) as a model for cardiovascular surgery and management before, during, and after cardiopulmonary bypass. *J Am Assoc Lab Anim Sci* 2015; **54**: 7-8 [PMID: 25651084]
32 **Youssef G**, Wallace WA, Dagleish MP, Cousens C, Griffiths DJ. Ovine pulmonary adenocarcinoma: A large animal model for human lung cancer. *ILAR J* 2015; **56**: 99-115 [PMID: 25991702 DOI: 10.1093/ilar/ilv014]
33 **Harding J**, Roberts RM, Mirochnitchenko O. Large animal models for stem cell therapy. *Stem Cell Res Ther* 2013; **4**: 23 [PMID: 23672797 DOI: 10.1186/scrt171]
34 **Gong G**, Roach ML, Jiang L, Yang X, Tian XC. Culture conditions and enzymatic passaging of bovine ESC-like cells. *Cell Reprogram* 2010; **12**: 151-160 [PMID: 20677930 DOI: 10.1089/cell.2009.0049]
35 **Maruotti J**, Muñoz M, Degrelle SA, Gómez E, Louet C, Díez C, de Longchamp PH, Brochard V, Hue I, Caamaño JN, Jouneau A. Efficient derivation of bovine embryonic stem cells needs more than active core pluripotency factors. *Mol Reprod Dev* 2012; **79**: 461-477 [PMID: 22573702 DOI: 10.1002/mrd.22051]
36 **Muñoz M**, Rodríguez A, De Frutos C, Caamaño JN, Díez C, Facal N, Gómez E. Conventional pluripotency markers are unspecific for bovine embryonic-derived cell-lines. *Theriogenology* 2008; **69**: 1159-1164 [PMID: 18420262 DOI: 10.1016/j.theriogenology.2008.02.014]
37 **Bogliotti YS**, Wu J, Vilarino M, Okamura D, Soto DA, Zhong C, Sakurai M, Sampaio RV, Suzuki K, Izpisua Belmonte JC, Ross PJ. Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proc Natl Acad Sci U S A* 2018; **115**: 2090-2095 [PMID: 29440377 DOI: 10.1073/pnas.1716161115]
38 **Murray JD**, Maga EA. Genetically engineered livestock for agriculture: A generation after the first transgenic animal research conference. *Transgenic Res* 2016; **25**: 321-327 [PMID: 26820413 DOI: 10.1007/s11248-016-9927-7]
39 **Rogers CS**. Genetically engineered livestock for biomedical models. *Transgenic Res* 2016; **25**: 345-359 [PMID: 26820410 DOI: 10.1007/s11248-016-9928-6]
40 **Colman A**. Dolly, Polly and other 'ollys': Likely impact of cloning technology on biomedical uses of livestock. *Genet Anal* 1999; **15**: 167-173 [PMID: 10596758 DOI: 10.1016/S1050-3862(99)00022-4]
41 **Pieri NCG**, de Souza AF, Botigelli RC, Machado LS, Ambrosio CE, Dos Santos Martins D, de Andrade AFC, Meirelles FV, Hyttel P, Bressan FF. Stem cells on regenerative and reproductive science in domestic animals. *Vet Res Commun* 2019; **43**: 7-16 [PMID: 30656543 DOI: 10.1007/s11259-019-9744-6]
42 **Gurdon J**, Murdoch A. Nuclear transfer and iPS may work best together. *Cell Stem Cell* 2008; **2**: 135-138 [PMID: 18371434 DOI: 10.1016/j.stem.2008.01.009]
43 **Kou Z**, Kang L, Yuan Y, Tao Y, Zhang Y, Wu T, He J, Wang J, Liu Z, Gao S. Mice cloned from induced pluripotent stem cells (iPSCs). *Biol Reprod* 2010; **83**: 238-243 [PMID: 20427755 DOI: 10.1095/biolreprod.110.084731]

44 **Hayashi K**, Ohta H, Kurimoto K, Aramaki S, Saitou M. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell* 2011; **146**: 519-532 [PMID: 21820164 DOI: 10.1016/j.cell.2011.06.052]

45 **Hayashi K**, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science* 2012; **338**: 971-975 [PMID: 23042295 DOI: 10.1126/science.1226889]

46 **Hikabe O**, Hamazaki N, Nagamatsu G, Obata Y, Hirao Y, Hamada N, Shimamoto S, Imamura T, Nakashima K, Saitou M, Hayashi K. Reconstitution in vitro of the entire cycle of the mouse female germ line. *Nature* 2016; **539**: 299-303 [PMID: 27750280 DOI: 10.1038/nature20104]

47 **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]

48 **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]

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 **Kawaguchi T**, Tsukiyama T, Kimura K, Matsuyama S, Minami N, Yamada M, Imai H. Generation of Naïve Bovine Induced Pluripotent Stem Cells Using PiggyBac Transposition of Doxycycline-Inducible Transcription Factors. *PLoS One* 2015; **10**: e0135403 [PMID: 26287611 DOI: 10.1371/journal.pone.0135403]

51 **Talluri TR**, Kumar D, Glage S, Garrels W, Ivics Z, Debowski K, Behr R, Niemann H, Kues WA. Derivation and characterization of bovine induced pluripotent stem cells by transposon-mediated reprogramming. *Cell Reprogram* 2015; **17**: 131-140 [PMID: 25826726 DOI: 10.1089/cell.2014.0080]

52 **Cravero D**, Martignani E, Miretti S, Accornero P, Pauciullo A, Sharma R, Donadeu FX, Baratta M. Generation of Induced Pluripotent Stem Cells from Bovine Epithelial Cells and Partial Redirection Toward a Mammary Phenotype In Vitro. *Cell Reprogram* 2015; **17**: 211-220 [PMID: 26053520 DOI: 10.1089/cell.2014.0087]

53 **Bai C**, Li X, Gao Y, Yuan Z, Hu P, Wang H, Liu C, Guan W, Ma Y. Melatonin improves reprogramming efficiency and proliferation of bovine-induced pluripotent stem cells. *J Pineal Res* 2016; **61**: 154-167 [PMID: 27090494 DOI: 10.1111/jpi.12334]

54 **Zhao L**, Wang Z, Zhang J, Yang J, Gao X, Wu B, Zhao G, Bao S, Hu S, Liu P, Li X. Characterization of the single-cell derived bovine induced pluripotent stem cells. *Tissue Cell* 2017; **49**: 521-527 [PMID: 28720304 DOI: 10.1016/j.tice.2017.05.005]

55 **Wang SW**, Wang SS, Wu DC, Lin YC, Ku CC, Wu CC, Chai CY, Lee JN, Tsai EM, Lin CL, Yang RC, Ko YC, Yu HS, Huo C, Chuu CP, Murayama Y, Nakamura Y, Hashimoto S, Matsushima K, Jin C, Eckner R, Lin CS, Saito S, Yokoyama KK. Androgen receptor-mediated apoptosis in bovine testicular induced pluripotent stem cells in response to phthalate esters. *Cell Death Dis* 2013; **4**: e907 [PMID: 24201806 DOI: 10.1038/cddis.2013.420]

56 **Huang B**, Li T, Alonso-Gonzalez L, Gorre R, Keatley S, Green A, Turner P, Kallingappa PK, Verma V, Oback 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]

57 **Canizo JR**, Vazquez Echegaray C, Klisch D, Aller JF, Paz DA, Alberio RH, Alberio R, Guberman AS. Exogenous human OKSM factors maintain pluripotency gene expression of bovine and porcine iPS-like cells obtained with STEMCCA delivery system. *BMC Res Notes* 2018; **11**: 509 [PMID: 30053877 DOI: 10.1186/s13104-018-3627-8]

58 **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]

59 **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]

60 **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]

61 **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]

62 **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]

63 **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]

64 **Sandmaier SE**, Nandal A, Powell A, Garrett W, Blomberg L, Donovan DM, Talbot N, Telugu BP. Generation of induced pluripotent stem cells from domestic goats. *Mol Reprod Dev* 2015; **82**: 709-721 [PMID: 26118622 DOI: 10.1002/mrd.22512]

65 **Chu Z**, Niu B, Zhu H, He X, Bai C, Li G, Hua J. PRMT5 enhances generation of induced pluripotent stem cells from dairy goat embryonic fibroblasts via down-regulation of p53. *Cell Prolif* 2015; **48**: 29-38 [PMID: 25424361 DOI: 10.1111/cpr.12150]

66 **Tai D**, Liu P, Gao J, Jin M, Xu T, Zuo Y, Liang H, Liu D. Generation of Arbas Cashmere Goat Induced Pluripotent Stem Cells Through Fibroblast Reprogramming. *Cell Reprogram* 2015; **17**: 297-305 [PMID: 26731591 DOI: 10.1089/cell.2014.0107]

67 **German SD**, Campbell KH, Thornton E, McLachlan G, Sweetman D, Alberio R. Ovine induced pluripotent stem cells are resistant to reprogramming after nuclear transfer. *Cell Reprogram* 2015; **17**: 19-27 [PMID: 25513856 DOI: 10.1089/cell.2014.0071]

68 **Song H**, Li H, Huang M, Xu D, Wang Z, Wang F. Big Animal Cloning Using Transgenic Induced Pluripotent Stem Cells: A Case Study of Goat Transgenic Induced Pluripotent Stem Cells. *Cell Reprogram* 2016; **18**: 37-47 [PMID: 26836033 DOI: 10.1089/cell.2015.0035]

69 **Chen H**, Zuo Q, Wang Y, Song J, Yang H, Zhang Y, Li B. Inducing goat pluripotent stem cells with four transcription factor mRNAs that activate endogenous promoters. *BMC Biotechnol* 2017; **17**: 11 [PMID: 28193206 DOI: 10.1186/s12896-017-0336-7]

70 **Gün G**, Kues WA. Current progress of genetically engineered pig models for biomedical research. *Biores Open Access* 2014; **3**: 255-264 [PMID: 25469311 DOI: 10.1089/biores.2014.0039]

71 **Walters EM**, Wells KD, Bryda EC, Schommer S, Prather RS. Swine models, genomic tools and services to enhance our understanding of human health and diseases. *Lab Anim (NY)* 2017; **46**: 167-172 [PMID: 28328880 DOI: 10.1038/laban.1215]

72 **Stricker-Krongrad A**, Shoemake CR, Bouchard GF. The Miniature Swine as a Model in Experimental and Translational Medicine. *Toxicol Pathol* 2016; **44**: 612-623 [PMID: 27073085 DOI: 10.1177/0192623316641784]

73 **Askeland G**, Rodinova M, Štufková H, Dosoudilova Z, Baxa M, Smatlikova P, Bohuslavova B, Klempir J, Nguyen TD, Kuśnierczyk A, Bjørås M, Klungland A, Hansikova H, Elledérova Z, Eide L. A transgenic minipig model of Huntington's disease shows early signs of behavioral and molecular pathologies. *Dis Model Mech* 2018; **11**: pii: dmm035949 [PMID: 30254085 DOI: 10.1242/dmm.035949]

74 **Baxa M**, Hruska-Plochan M, Juhas S, Vodicka P, Pavlok A, Juhasova J, Miyanohara A, Nejime T, Klima J, Macakova M, Marsala S, Weiss A, Kubickova S, Musilova P, Vrtel R, Sontag EM, Thompson LM, Schier J, Hansikova H, Howland DS, Cattaneo E, DiFiglia M, Marsala M, Motlik J. A transgenic minipig model of Huntington's Disease. *J Huntingtons Dis* 2013; **2**: 47-68 [PMID: 25063429 DOI: 10.3233/JHD-130001]

75 **Duran-Struuck R**, Huang CA, Orf K, Bronson RT, Sachs DH, Spitzer TR. Miniature Swine as a Clinically Relevant Model of Graft-Versus-Host Disease. *Comp Med* 2015; **65**: 429-443 [PMID: 26473348]

76 **Ezashi T**, Yuan Y, Roberts RM. Pluripotent Stem Cells from Domesticated Mammals. *Annu Rev Anim Biosci* 2016; **4**: 223-253 [PMID: 26566158 DOI: 10.1146/annurev-animal-021815-111202]

77 **Gandolfi F**, Pennarossa G, Maffei S, Brevini T. Why is it so difficult to derive pluripotent stem cells in domestic ungulates? *Reprod Domest Anim* 2012; **47** Suppl 5: 11-17 [PMID: 22913556 DOI: 10.1111/j.1439-0531.2012.02106.x]

78 **Evans MJ**, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; **292**: 154-156 [PMID: 7242681 DOI: 10.1038/292154a0]

79 **Martin GR**. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 1981; **78**: 7634-7638 [PMID: 6950406 DOI: 10.1073/pnas.78.12.7634]

80 **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]

81 **Thomson JA**, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, Hearn JP. Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci U S A* 1995; **92**: 7844-7848 [PMID: 7544005 DOI: 10.1073/pnas.92.17.7844]

82 **Ueda S**, Kawamata M, Teratani T, Shimizu T, Tamai Y, Ogawa H, Hayashi K, Tsuda H, Ochiya T. Establishment of rat embryonic stem cells and making of chimera rats. *PLoS One* 2008; **3**: e2800 [PMID: 18665239 DOI: 10.1371/journal.pone.0002800]

83 **Telugu BP**, Ezashi T, Roberts RM. Porcine induced pluripotent stem cells analogous to naïve and primed embryonic stem cells of the mouse. *Int J Dev Biol* 2010; **54**: 1703-1711 [PMID: 21305472 DOI: 10.1387/ijdb.103200bt]

84 **Thomson AJ**, Pierart H, Meek S, Bogerman A, Sutherland L, Murray H, Mountjoy E, Downing A, Talbot R, Sartori C, Whitelaw CB, Freeman TC, Archibald AL, Burdon T. Reprogramming pig fetal fibroblasts reveals a functional LIF signaling pathway. *Cell Reprogram* 2012; **14**: 112-122 [PMID: 22339199 DOI: 10.1089/cell.2011.0078]

85 **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]

86 **Rodríguez A**, Allegrucci C, Alberio R. Modulation of pluripotency in the porcine embryo and iPS cells. *PLoS One* 2012; **7**: e49079 [PMID: 23145076 DOI: 10.1371/journal.pone.0049079]

87 **Fujishiro SH**, Nakano K, Mizukami Y, Azami T, Arai Y, Matsunari H, Ishino R, Nishimura T, Watanabe M, Abe T, Furukawa Y, Umeyama K, Yamanaka S, Ema M, Nagashima H, Hanazono Y. Generation of naive-like porcine-induced pluripotent stem cells capable of contributing to embryonic and fetal development. *Stem Cells Dev* 2013; **22**: 473-482 [PMID: 22889279 DOI: 10.1089/scd.2012.0173]

88 **Kwon DJ**, Jeon H, Oh KB, Ock SA, Im GS, Lee SS, Im SK, Lee JW, Oh SJ, Park JK, Hwang S. Generation of leukemia inhibitory factor-dependent induced pluripotent stem cells from the Massachusetts General Hospital miniature pig. *Biomed Res Int* 2013; **2013**: 140639 [PMID: 24371815 DOI: 10.1155/2013/140639]

89 **Ji G**, Ruan W, Liu K, Wang F, Sakellariou D, Chen J, Yang Y, Okuka M, Han J, Liu Z, Lai L, Gagos S, Xiao L, Deng H, Li N, Liu L. Telomere reprogramming and maintenance in porcine iPS cells. *PLoS One* 2013; **8**: e74202 [PMID: 24098638 DOI: 10.1371/journal.pone.0074202]

90 **Petkov S**, Glage S, Nowak-Imialek M, Niemann H. Long-Term Culture of Porcine Induced Pluripotent Stem-Like Cells Under Feeder-Free Conditions in the Presence of Histone Deacetylase Inhibitors. *Stem Cells Dev* 2016; **25**: 386-394 [PMID: 26691930 DOI: 10.1089/scd.2015.0317]

91 **Secher JO**, Ceylan A, Mazzoni G, Mashayekhi K, Li T, Muenthaisong S, Nielsen TT, Li D, Li S, Petkov S, Cirera S, Luo Y, Thombs L, Kadarmideen HN, Dinnyes A, Bolund L, Roelen BA, Schmidt M, Callesen H, Hyttel P, Freude KK. Systematic in vitro and in vivo characterization of Leukemia-inhibiting factor- and Fibroblast growth factor-derived porcine induced pluripotent stem cells. *Mol Reprod Dev* 2017; **84**: 229-245 [PMID: 28044390 DOI: 10.1002/mrd.22771]

92 **Zhang W**, Wang H, Zhang S, Zhong L, Wang Y, Pei Y, Han J, Cao S. Lipid Supplement in the Cultural Condition Facilitates the Porcine iPSC Derivation through cAMP/PKA/CREB Signal Pathway. *Int J Mol Sci* 2018; **19**: pii: E509 [PMID: 29419748 DOI: 10.3390/ijms19020509]

93 **Li D**, Secher J, Hyttel P, Ivask M, Kolko M, Hall VJ, Freude KK. Generation of transgene-free porcine intermediate type induced pluripotent stem cells. *Cell Cycle* 2018; **17**: 2547-2563 [PMID: 30457474 DOI: 10.1080/15384101.2018.1548790]

94 **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]

95 **Montserrat N**, Bahima EG, Batlle L, Häfner S, Rodrigues AM, González F, Izpisúa Belmonte JC. Generation of pig iPS cells: A model for cell therapy. *J Cardiovasc Transl Res* 2011; **4**: 121-130 [PMID: 21088946 DOI: 10.1007/s12265-010-9233-3]

96 **Chakritbudsabong W**, Sariya L, Pamonsupornvichit S, Pronarkngver R, Chaiwattanarungruengpaisan S, Ferreira JN, Setthawong P, Phakdeedindan P, Techakumphu M, Tharasanit T, Rungarunlert S. Generation of a pig induced pluripotent stem cell (piPSC) line from embryonic fibroblasts by incorporating LIN28 to the four transcriptional factor-mediated reprogramming: VSMUi001-D. *Stem Cell Res* 2017; **24**: 21-24 [PMID: 29034889 DOI: 10.1016/j.scr.2017.08.005]

97 **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]

98 **Lopez MJ**, Jarazo J. State of the art: Stem cells in equine regenerative medicine. *Equine Vet J* 2015; **47**: 145-154 [PMID: 24957845 DOI: 10.1111/evj.12311]

99 **Smith RK**, Garvican ER, Fortier LA. The current 'state of play' of regenerative medicine in horses: What the horse can tell the human. *Regen Med* 2014; **9**: 673-685 [PMID: 25372081 DOI: 10.2217/rme.14.42]

100 **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]

101 **Khodadadi K**, Sumer H, Pashaiasl M, Lim S, Williamson M, Verma PJ. Induction of pluripotency in adult equine fibroblasts without c-MYC. *Stem Cells Int* 2012; **2012**: 429160 [PMID: 22550508 DOI: 10.1155/2012/429160]

102 **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]

103 **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 DOI: 10.1089/scd.2013.0565]

104 **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 DOI: 10.1089/scd.2013.0461]

105 **Lee EM**, Kim AY, Lee EJ, Park JK, Park SI, Cho SG, Kim HK, Kim SY, Jeong KS. Generation of Equine-Induced Pluripotent Stem Cells and Analysis of Their Therapeutic Potential for Muscle Injuries. *Cell Transplant* 2016; **25**: 2003-2016 [PMID: 27226077 DOI: 10.3727/096368916X691691]

106 **Quattrocelli M**, Giacomazzi G, Broeckx SY, Ceelen L, Bolca S, Spaas JH, Sampaolesi M. Equine-Induced Pluripotent Stem Cells Retain Lineage Commitment Toward Myogenic and Chondrogenic Fates. *Stem Cell Reports* 2016; **6**: 55-63 [PMID: 26771353 DOI: 10.1016/j.stemcr.2015.12.005]

107 **Moro LN**, Amin G, Furmento V, Waisman A, Garate X, Neiman G, La Greca A, Santín Velazque NL, Luzzani C, Sevlever GE, Vichera G, Miriuka SG. MicroRNA characterization in equine induced pluripotent stem cells. *PLoS One* 2018; **13**: e0207074 [PMID: 30507934 DOI: 10.1371/journal.pone.0207074]

108 **Pessôa LVF**, Pires PRL, Del Collado M, Pieri NCG, Recchia K, Souza AF, Perecin F, da Silveira JC, de Andrade AFC, Ambrosio CE, Bressan FF, Meirelles FV. Generation and miRNA Characterization of Equine Induced Pluripotent Stem Cells Derived from Fetal and Adult Multipotent Tissues. *Stem Cells Int* 2019; **2019**: 1393791 [PMID: 31191664 DOI: 10.1155/2019/1393791]

109 **Donadeu FX**, Esteves CL. Prospects and Challenges of Induced Pluripotent Stem Cells in Equine Health. *Front Vet Sci* 2015; **2**: 59 [PMID: 26664986 DOI: 10.3389/fvets.2015.00059]

110 **Yamanaka S**. A fresh look at iPS cells. *Cell* 2009; **137**: 13-17 [PMID: 19345179 DOI: 10.1016/j.cell.2009.03.034]

111 **Mayshar Y**, Ben-David U, Lavon N, Biancotti JC, Yakir B, Clark AT, Plath K, Lowry WE, Benvenisty N. Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. *Cell Stem Cell* 2010; **7**: 521-531 [PMID: 20887957 DOI: 10.1016/j.stem.2010.07.017]

112 **Okano H**, Nakamura M, Yoshida K, Okada Y, Tsuji O, Nori S, Ikeda E, Yamanaka S, Miura K. Steps toward safe cell therapy using induced pluripotent stem cells. *Circ Res* 2013; **112**: 523-533 [PMID: 23371901 DOI: 10.1161/CIRCRESAHA.111.256149]

113 **Aguiar C**, Theoret C, Smith O, Segura M, Lemire P, Smith LC. Immune potential of allogeneic equine induced pluripotent stem cells. *Equine Vet J* 2015; **47**: 708-714 [PMID: 25196173 DOI: 10.1111/evj.12345]

114 **Olivera R**, Moro LN, Jordan R, Luzzani C, Miriuka S, Radrizzani M, Donadeu FX, Vichera G. In Vitro and In Vivo Development of Horse Cloned Embryos Generated with iPSCs, Mesenchymal Stromal Cells and Fetal or Adult Fibroblasts as Nuclear Donors. *PLoS One* 2016; **11**: e0164049 [PMID: 27732616 DOI: 10.1371/journal.pone.0164049]

115 **Bavin EP**, Smith O, Baird AE, Smith LC, Guest DJ. Equine Induced Pluripotent Stem Cells have a Reduced Tendon Differentiation Capacity Compared to Embryonic Stem Cells. *Front Vet Sci* 2015; **2**: 55 [PMID: 26664982 DOI: 10.3389/fvets.2015.00055]

116 **Aguiar C**, Therrien J, Lemire P, Segura M, Smith LC, Theoret CL. Differentiation of equine induced pluripotent stem cells into a keratinocyte lineage. *Equine Vet J* 2016; **48**: 338-345 [PMID: 25781637 DOI: 10.1111/evj.12438]

117 **Lepage SI**, Nagy K, Sung HK, Kandel RA, Nagy A, Koch TG. Generation, Characterization, and Multilineage Potency of Mesenchymal-Like Progenitors Derived from Equine Induced Pluripotent Stem Cells. *Stem Cells Dev* 2016; **25**: 80-89 [PMID: 26414480 DOI: 10.1089/scd.2014.0409]

118 **Baird A**, Lindsay T, Everett A, Iyemere V, Paterson YZ, McClellan A, Henson FMD, Guest DJ. Osteoblast differentiation of equine induced pluripotent stem cells. *Biol Open* 2018; **7**: pii: bio033514 [PMID: 29685993 DOI: 10.1242/bio.033514]

119 **Amilon KR**, Cortes-Araya Y, Moore B, Lee S, Lillico S, Breton A, Esteves CL, Donadeu FX. Generation of Functional Myocytes from Equine Induced Pluripotent Stem Cells. *Cell Reprogram* 2018; **20**: 275-281 [PMID: 30207795 DOI: 10.1089/cell.2018.0023]

120 **Payne E**, Bennett PC, McGreevy PD. Current perspectives on attachment and bonding in the dog-human dyad. *Psychol Res Behav Manag* 2015; **8**: 71-79 [PMID: 25750549 DOI: 10.2147/PRBM.S74972]

121 **Starkey MP**, Scase TJ, Mellersh CS, Murphy S. Dogs really are man's best friend--canine genomics has applications in veterinary and human medicine! *Brief Funct Genomic Proteomic* 2005; **4**: 112-128 [PMID: 16102268 DOI: 10.1093/bfgp/4.2.112]

122 **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]

123 **Lee AS**, Xu D, Plews JR, Nguyen PK, Nag D, Lyons JK, Han L, Hu S, Lan F, Liu J, Huang M, Narsinh KH, Long CT, de Almeida PE, Levi B, Kooreman N, Bangs C, Pacharinsak C, Ikeno F, Yeung AC, Gambhir SS, Robbins RC, Longaker MT, Wu JC. Preclinical derivation and imaging of autologously transplanted canine induced pluripotent stem cells. *J Biol Chem* 2011; **286**: 32697-32704 [PMID: 21719696 DOI: 10.1074/jbc.M111.235739]

124 **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]

125 **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]

126 **Chow L**, Johnson V, Regan D, Wheat W, Webb S, Koch P, Dow S. Safety and immune regulatory properties of canine induced pluripotent stem cell-derived mesenchymal stem cells. *Stem Cell Res* 2017; **25**: 221-232 [PMID: 29172152 DOI: 10.1016/j.scr.2017.11.010]

127 **Nishimura T**, Hatoya S, Kanegi R, Wijesekera DPH, Sanno K, Tanaka E, Sugiura K, Hiromitsu Tamada NK, Imai H, Inaba T. Feeder-independent canine induced pluripotent stem cells maintained under serum-free conditions. *Mol Reprod Dev* 2017; **84**: 329-339 [PMID: 28240438 DOI: 10.1002/mrd.22789]

128 **Gonçalves NJN**, Bressan FF, Roballo KCS, Meirelles FV, Xavier PLP, Fukumasu H, Williams C, Breen M, Koh S, Sper R, Piedrahita J, Ambrósio CE. Generation of LIF-independent induced pluripotent stem cells from canine fetal fibroblasts. *Theriogenology* 2017; **92**: 75-82 [PMID: 28237347 DOI: 10.1016/j.theriogenology.2017.01.013]

129 **Tsukamoto M**, Nishimura T, Yodoe K, Kanegi R, Tsujimoto Y, Alam ME, Kuramochi M, Kuwamura M, Ohtaka M, Nishimura K, Nakanishi M, Inaba T, Sugiura K, Hatoya S. Generation of Footprint-Free Canine Induced Pluripotent Stem Cells Using Auto-Erasable Sendai Virus Vector. *Stem Cells Dev* 2018; **27**: 1577-1586 [PMID: 30215317 DOI: 10.1089/scd.2018.0084]

130 **Baird A**, Barsby T, Guest DJ. Derivation of Canine Induced Pluripotent Stem Cells. *Reprod Domest Anim* 2015; **50**: 669-676 [PMID: 26074059 DOI: 10.1111/rda.12562]

131 **Herberts CA**, Kwa MS, Hermsen HP. Risk factors in the development of stem cell therapy. *J Transl Med* 2011; **9**: 29 [PMID: 21418664 DOI: 10.1186/1479-5876-9-29]

132 **Whitworth DJ**, Frith JE, Frith TJ, Ovchinnikov DA, Cooper-White JJ, Wolvetang EJ. Derivation of mesenchymal stromal cells from canine induced pluripotent stem cells by inhibition of the TGFβ/activin signaling pathway. *Stem Cells Dev* 2014; **23**: 3021-3033 [PMID: 25055193 DOI: 10.1089/scd.2013.0634]

133 **Phakdeedindan P**, Setthawong P, Tiptanavattana N, Rungarunlert S, Ingrungruanglert P, Israsena N, Techakumphu M, Tharasanit T. Rabbit induced pluripotent stem cells retain capability of in vitro cardiac differentiation. *Exp Anim* 2019; **68**: 35-47 [PMID: 30089733 DOI: 10.1538/expanim.18-0074]

134 **Mapara M**, Thomas BS, Bhat KM. Rabbit as an animal model for experimental research. *Dent Res J (Isfahan)* 2012; **9**: 111-118 [PMID: 22363373 DOI: 10.4103/1735-3327.92960]

135 **Honda A**, Hatori M, Hirose M, Honda C, Izu H, Inoue K, Hirasawa R, Matoba S, Togayachi S, Miyoshi H, Ogura A. Naive-like conversion overcomes the limited differentiation capacity of induced pluripotent stem cells. *J Biol Chem* 2013; **288**: 26157-26166 [PMID: 23880763 DOI: 10.1074/jbc.M113.502492]

136 **Osteil P**, Tapponnier Y, Markossian S, Godet M, Schmaltz-Panneau B, Jouneau 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]

137 **Tapponnier Y**, Afanassieff M, Aksoy I, Aubry M, Moulin A, Medjani L, Bouchereau W, Mayère C, Osteil P, Nurse-Francis J, Oikonomakos I, Joly T, Jouneau L, Archilla C, Schmaltz-Panneau B, Peynot N, Barasc H, Pinton A, Lecardonnel J, Gocza E, Beaujean N, Duranthon V, Savatier P. Reprogramming of rabbit induced pluripotent stem cells toward epiblast and chimeric competency using Krüppel-like factors. *Stem Cell Res* 2017; **24**: 106-117 [PMID: 28889080 DOI: 10.1016/j.scr.2017.09.001]

138 **Dai R**, Rossello R, Chen CC, Kessler J, Davison I, Hochgeschwender U, Jarvis ED. Maintenance and neuronal differentiation of chicken induced pluripotent stem-like cells. *Stem Cells Int* 2014; **2014**: 182737 [PMID: 25610469 DOI: 10.1155/2014/182737]

139 **Hawkridge AM**. The chicken model of spontaneous ovarian cancer. *Proteomics Clin Appl* 2014; **8**: 689-699 [PMID: 25130871 DOI: 10.1002/prca.201300135]

140 **Lu Y**, West FD, Jordan BJ, Mumaw JL, Jordan ET, Gallegos-Cardenas A, Beckstead RB, Stice SL. Avian-induced pluripotent stem cells derived using human reprogramming factors. *Stem Cells Dev* 2012; **21**: 394-403 [PMID: 21970437 DOI: 10.1089/scd.2011.0499]

141 **Rosselló RA**, Chen CC, Dai R, Howard JT, Hochgeschwender U, Jarvis ED. Mammalian genes induce partially reprogrammed pluripotent stem cells in non-mammalian vertebrate and invertebrate species. *Elife* 2013; **2**: e00036 [PMID: 24015354 DOI: 10.7554/eLife.00036]

142 **Yu P**, Lu Y, Jordan BJ, Liu Y, Yang JY, Hutcheson JM, Ethridge CL, Mumaw JL, Kinder HA, Beckstead RB, Stice SL, West FD. Nonviral minicircle generation of induced pluripotent stem cells compatible with production of chimeric chickens. *Cell Reprogram* 2014; **16**: 366-378 [PMID: 25084370 DOI: 10.1089/cell.2014.0028]

143 **Choi HW**, Kim JS, Choi S, Ju Hong Y, Byun SJ, Seo HG, Do JT. Mitochondrial Remodeling in Chicken Induced Pluripotent Stem-Like Cells. *Stem Cells Dev* 2016; **25**: 472-476 [PMID: 26795691 DOI: 10.1089/scd.2015.0299]

144 **Fuet A**, Pain B. Chicken Induced Pluripotent Stem Cells: Establishment and Characterization. *Methods Mol Biol* 2017; **1650**: 211-228 [PMID: 28809024 DOI: 10.1007/978-1-4939-7216-6_14]

145 **Katayama M**, Hirayama T, Tani T, Nishimori K, Onuma M, Fukuda T. Chick derived induced pluripotent stem cells by the poly-cistronic transposon with enhanced transcriptional activity. *J Cell Physiol* 2018; **233**: 990-1004 [PMID: 28387938 DOI: 10.1002/jcp.25947]

146 **Kim YM**, Park YH, Lim JM, Jung H, Han JY. Technical note: Induction of pluripotent stem cell-like cells from chicken feather follicle cells. *J Anim Sci* 2017; **95**: 3479-3486 [PMID: 28805906 DOI:

10.2527/jas.2017.1418]
147 **Shittu I**, Zhu Z, Lu Y, Hutcheson JM, Stice SL, West FD, Donadeu M, Dungu B, Fadly AM, Zavala G, Ferguson-Noel N, Afonso CL. Development, characterization and optimization of a new suspension chicken-induced pluripotent cell line for the production of Newcastle disease vaccine. *Biologicals* 2016; **44**: 24-32 [PMID: 26586283 DOI: 10.1016/j.biologicals.2015.09.002]
148 **Susta L**, He Y, Hutcheson JM, Lu Y, West FD, Stice SL, Yu P, Abdo Z, Afonso CL. Derivation of chicken induced pluripotent stem cells tolerant to Newcastle disease virus-induced lysis through multiple rounds of infection. *Virol J* 2016; **13**: 205 [PMID: 27919263 DOI: 10.1186/s12985-016-0659-3]
149 **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]
150 **Cox LA**, Comuzzie AG, Havill LM, Karere GM, Spradling KD, Mahaney MC, Nathanielsz PW, Nicolella DP, Shade RE, Voruganti S, VandeBerg JL. Baboons as a model to study genetics and epigenetics of human disease. *ILAR J* 2013; **54**: 106-121 [PMID: 24174436 DOI: 10.1093/ilar/ilt038]
151 **Mora-Bermúdez F**, Badsha F, Kanton S, Camp JG, Vernot B, Köhler K, Voigt B, Okita K, Maricic T, He Z, Lachmann R, Pääbo S, Treutlein B, Huttner WB. Differences and similarities between human and chimpanzee neural progenitors during cerebral cortex development. *Elife* 2016; **5**: pii: e18683 [PMID: 27669147 DOI: 10.7554/eLife.18683]
152 **Camacho P**, Fan H, Liu Z, He JQ. Large Mammalian Animal Models of Heart Disease. *J Cardiovasc Dev Dis* 2016; **3**: pii: E30 [PMID: 29367573 DOI: 10.3390/jcdd3040030]
153 **Zhang X**, Cao H, Bai S, Huo W, Ma Y. Differentiation and characterization of rhesus monkey atrial and ventricular cardiomyocytes from induced pluripotent stem cells. *Stem Cell Res* 2017; **20**: 21-29 [PMID: 28249229 DOI: 10.1016/j.scr.2017.02.002]
154 **Fang R**, Liu K, Zhao Y, Li H, Zhu D, Du Y, Xiang C, Li X, Liu H, Miao Z, Zhang X, Shi Y, Yang W, Xu J, Deng H. Generation of naive induced pluripotent stem cells from rhesus monkey fibroblasts. *Cell Stem Cell* 2014; **15**: 488-497 [PMID: 25280221 DOI: 10.1016/j.stem.2014.09.004]
155 **Chan AW**, Cheng PH, Neumann A, Yang JJ. Reprogramming Huntington monkey skin cells into pluripotent stem cells. *Cell Reprogram* 2010; **12**: 509-517 [PMID: 20936902 DOI: 10.1089/cell.2010.0019]
156 **Shimozawa N**, Ono R, Shimada M, Shibata H, Takahashi I, Inada H, Takada T, Nosaka T, Yasutomi Y. Cynomolgus monkey induced pluripotent stem cells established by using exogenous genes derived from the same monkey species. *Differentiation* 2013; **85**: 131-139 [PMID: 23792767 DOI: 10.1016/j.diff.2013.02.004]
157 **Thoma EC**, Heckel T, Keller D, Giroud N, Leonard B, Christensen K, Roth A, Bertinetti-Lapatki C, Graf M, Patsch C. Establishment of a translational endothelial cell model using directed differentiation of induced pluripotent stem cells from Cynomolgus monkey. *Sci Rep* 2016; **6**: 35830 [PMID: 27779219 DOI: 10.1038/srep35830]
158 **Ramaswamy K**, Yik WY, Wang XM, Oliphant EN, Lu W, Shibata D, Ryder OA, Hacia JG. Derivation of induced pluripotent stem cells from orangutan skin fibroblasts. *BMC Res Notes* 2015; **8**: 577 [PMID: 26475477 DOI: 10.1186/s13104-015-1567-0]
159 **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.e1-228.e2 [PMID: 22079579 DOI: 10.1016/j.theriogenology.2011.09.022]
160 **Verma R**, Liu J, Holland MK, Temple-Smith P, Williamson M, Verma PJ. Nanog is an essential factor for induction of pluripotency in somatic cells from endangered felids. *Biores Open Access* 2013; **2**: 72-76 [PMID: 23514873 DOI: 10.1089/biores.2012.0297]
161 **Weeratunga P**, Shahsavari A, Ovchinnikov DA, Wolvetang EJ, Whitworth DJ. Induced Pluripotent Stem Cells from a Marsupial, the Tasmanian Devil (Sarcophilus harrisii): Insight into the Evolution of Mammalian Pluripotency. *Stem Cells Dev* 2018; **27**: 112-122 [PMID: 29161957 DOI: 10.1089/scd.2017.0224]
162 **Katayama M**, Hirayama T, Horie K, Kiyono T, Donai K, Takeda S, Nishimori K, Fukuda T. Induced Pluripotent Stem Cells With Six Reprogramming Factors From Prairie Vole, Which Is an Animal Model for Social Behaviors. *Cell Transplant* 2016; **25**: 783-796 [PMID: 26777120 DOI: 10.3727/096368916X690502]
163 **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]
164 **Whitworth DJ**, Limnios IJ, Gauthier ME, Weeratunga P, Ovchinnikov DA, Baillie G, Grimmond SM, Graves JAM, Wolvetang EJ. Platypus Induced Pluripotent Stem Cells: The Unique Pluripotency Signature of a Monotreme. *Stem Cells Dev* 2019; **28**: 151-164 [PMID: 30417748 DOI: 10.1089/scd.2018.0179]
165 **Menzorov AG**, Matveeva NM, Markakis MN, Fishman VS, Christensen K, Khabarova AA, Pristyazhnyuk IE, Kizilova EA, Cirera S, Anistoroaei R, Serov OL. Comparison of American mink embryonic stem and induced pluripotent stem cell transcriptomes. *BMC Genomics* 2015; **16** Suppl 13: S6 [PMID: 26694224 DOI: 10.1186/1471-2164-16-S13-S6]

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REVIEW

Tonsil-derived stem cells as a new source of adult stem cells

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Abstract

Located near the oropharynx, the tonsils are the primary mucosal immune organ. Tonsil tissue is a promising alternative source for the high-yield isolation of adult stem cells, and recent studies have reported the identification and isolation of tonsil-derived stem cells (T-SCs) from waste surgical tissue following tonsillectomies in relatively young donors (*i.e.*, under 10 years old). As such, T-SCs offer several advantages, including superior proliferation and a shorter doubling time compared to bone marrow-derived mesenchymal stem cells (MSCs). T-SCs also exhibit multi-lineage differentiation, including mesodermal, endodermal (*e.g.*, hepatocytes and parathyroid-like cells), and even ectodermal cells (*e.g.*, Schwann cells). To this end, numbers of researchers have evaluated the practical use of T-SCs as an alternative source of autologous or allogenic MSCs. In this review, we summarize the details of T-SC isolation and identification and provide an overview of their application in cell therapy and regenerative medicine.

Key words: Stem cell; Tonsil-derived stem cell; Differentiation; Endoderm; Mesoderm; Ectoderm; Cell therapy

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Core tip: The use of adult stem cells is often limited by the lack of differentiation among stem cells isolated from certain germ layers. However, tonsil-derived stem cells (T-SCs) were able to differentiate into various tissue types from the three germ layers, which is the most advantageous feature of this new stem cell source. T-SCs can also be used as native cells in the treatment of various immune-related diseases. As a result, it can be concluded that T-SCs have great potential for clinical applications in cell therapy and regenerative medicine.

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INTRODUCTION

Recent achievements in the identification, isolation, *in vitro* culture, and differentiation of various adult stem cells are indicative of the unprecedented potential of these cells in treating various degenerative diseases[1]. Mesenchymal stem cells (MSCs) in particular, have been used clinically for more than 10 years. From animal studies to clinical trials, MSCs have demonstrated great promise in treating numerous diseases, particularly tissue injury and immune disorders[2]. To obtain the large volumes of cells required for testing and treatment, various tissue sources have been investigated for the isolation of MSCs, including bone marrow, adipose tissue, umbilical cord blood, amniotic fluid, the placenta, dental pulp, and urine[3]. However, the isolation yields of MSCs from different tissue sources vary greatly, and the differentiation potential, yield, and maximal lifespan of isolated MSCs decrease significantly with donor age. Therefore, it is important to locate new adult stem cell sources to overcome these limitations.

The human tonsils are located near the oropharynx (palatine tonsils) and nasopharynx (adenoid), which are part of the respiratory and digestive system. Tonsil tissue is one of the primary sensitization systems for the generation of B cells, and tonsil tissue is easily obtained from tonsillectomies, a minimally invasive surgery conducted most often on patients aged between 5 and 19. Tonsil-derived stem cells (T-SCs) were first introduced by Janjanin *et al*[4]. Due to the younger donors, the isolation yields of T-SCs are much higher than those from other tissue types. Therefore, T-SCs have received much attention as alternative allogeneic or autologous cell sources for clinical use. In this review, we highlight recent research on the isolation and development of T-SCs, which provides strong evidence of their superior characteristics. In addition to their high proliferation and expansion capacity, T-SCs can undergo differentiation into cells from all three germ layers (*i.e.*, ectoderm, mesoderm, and endoderm). This unique differentiation potential is described in detail. Finally, we provide an in-depth discussion of the use of T-SCs in cell therapy and regenerative medicine.

ISOLATING AND IDENTIFYING TONSIL-DERIVED MSCS

Isolating T-SCs consists of two major steps: Enzymatic disaggregation and density gradient centrifugation[5]. Briefly, small pieces of tonsillar tissues were exposed to enzymes, including collagenase type I and DNase for 30 min at 37 °C under stirring. This solution was then filtered through a wire mesh and 70-μm cell strainer to collect single-cell suspensions. The mononuclear cell (MNC) fraction was obtained using Ficoll-Paque (GE Healthcare, Little Chalfont, United Kingdom) density gradient centrifugation. The MNCs were plated at the density of 10^8 cells in a T-150 culture flask with Dulbecco's modified Eagle's medium-high glucose (DMEM-HG; Invitrogen) supplemented with fetal bovine serum and antibiotics. The primary culture (passage 0; P0) was cultivated until the adherent cells reached confluence and were passaged by trypsinization (Trypsin, Life Technologies GmbH, Vienna, Austria).

Immunophenotype characterization, which is based on the expression of cell surface markers, is the most common method for distinguishing different cell clusters. To date, extensive research has identified various cell surface markers that characterize the MSCs derived from different sources. In order to identify T-SCs as a cellular source for new adult MSCs, T-SC surface makers were investigated[5,6]. As with other MSCs, T-SCs expressed the standard positive markers for MSCs, CD73, CD90, CD105, CD29, CD44, CD166, CD58, and CD49e. Most of these markers represent cellular adhesion molecules which possibly render MSCs to act on other cell types *via* direct interaction. On the other hand, T-SCs were negative for the hematopoietic markers CD14, CD34, CD45, and CD133, the endothelial marker CD31, and co-stimulatory proteins such as the antigens CD40, CD80, and CD86. In addition, class II MHC antigens are entirely absent on T-SCs[5,6]. Because tonsil tissue is part of the mucosal immune system and contains large numbers of follicular dendritic cells (FDCs), additional research has been carried out to verify the lack of FDC markers CD11b, CD21, CD23, CD35, and CD54 in T-SCs[5,7] to confirm no-contamination with FDC. FDCs are known to originate from tonsillar stromata and proliferate on and adhere to plastic *in vitro*. Therefore, the lack of these markers is an important indicator that can be used to distinguish T-SCs from FDCs.

MAJOR ADVANTAGES OF T-SCS OVER BONE MARROW-DERIVED STEM CELLS

Although MSCs can be isolated from various tissue types, they were initially harvested from bone marrow (BM), which requires a highly invasive procedure[4]. Here, we highlight the significant benefits of using T-SCs in terms of isolation and clinical use compared with BM-MSCs.

Isolating BM-MSCs has several limitations, including donor morbidity, and they are challenging to harvest, thus requiring a high degree of skill. Bone marrow extraction takes approximately two hours under general anesthesia and requires the hospitalization and recovery of the donor. Therefore, it is always difficult to find a sufficient number of donors. In contrast, T-SCs are easily obtained from discarded tissue; more than 530000 tonsillectomies are performed annually in children younger than 15 years in the United States[8], meaning that tonsils are one of the most abundant tissue sources for stem cell isolation.

The age of the donor affects the isolation yield of MSCs, with the number of MSCs harvested from bone marrow decreasing with donor age. For example, infants have one colony forming units-fibroblast (CFU-f) per 10000 cells in bone marrow, but this falls to 1 per 400000 in donors in their 50 s[9]. In contrast, approximately 8-10 × 10^8 MSCs are isolated from one-third of one tonsil (2 cm x 1.5 cm x 1.5 cm) from donors under 10 years old[5].

When compared with BM-MSCs, T-SCs offer superior stem cell properties, such as high self-renewal and proliferation. For example, T-SCs show a doubling time of 37.1 ± 3.4 h for an initial population, compared to 58.2 ± 2.3 h for BM-MSCs[4]. Other research has also confirmed the more rapid proliferation of T-SCs compared with MSCs derived from adipose tissue[10].

The proliferation of BM-MSCs gradually decreases with passage number, whereas T-SCs retain their physiological properties for much longer. In general, most cells become more prominent, longer, less defined, and less proliferative during long-term *in vitro* culture as they experience senescence. T-SCs also exhibit the signs of senescence from passage 7, but the cells proliferate up to passage 15 with no change in the MSC markers. Tonsil tissue contains as many B cells and T cells as immune organs, and these cells affect the immune modulation of stem cells. Pro-inflammatory cytokines may also affect the positive differentiation and proliferation of T-SCs[4,11,12], and this has been supported by research on tissue obtained from tonsillectomies in response to chronic bacterial infections and chronic tonsillitis[13-15].

Bone marrow and adipose tissue originate from the mesoderm layer, whereas tonsil tissue has two origins: The epithelial cells derive from the second pharyngeal pouch in the endoderm layer, and lymphoid tissue comes from the mesoderm layer, which invades during fetal development. Research has confirmed that T-SCs can be easily differentiated into endodermal, ectodermal, and mesodermal cells (Figure 1).

THERAPEUTIC POTENTIAL OF T-SCS BASED ON THEIR DIFFERENTIATION PROPERTIES

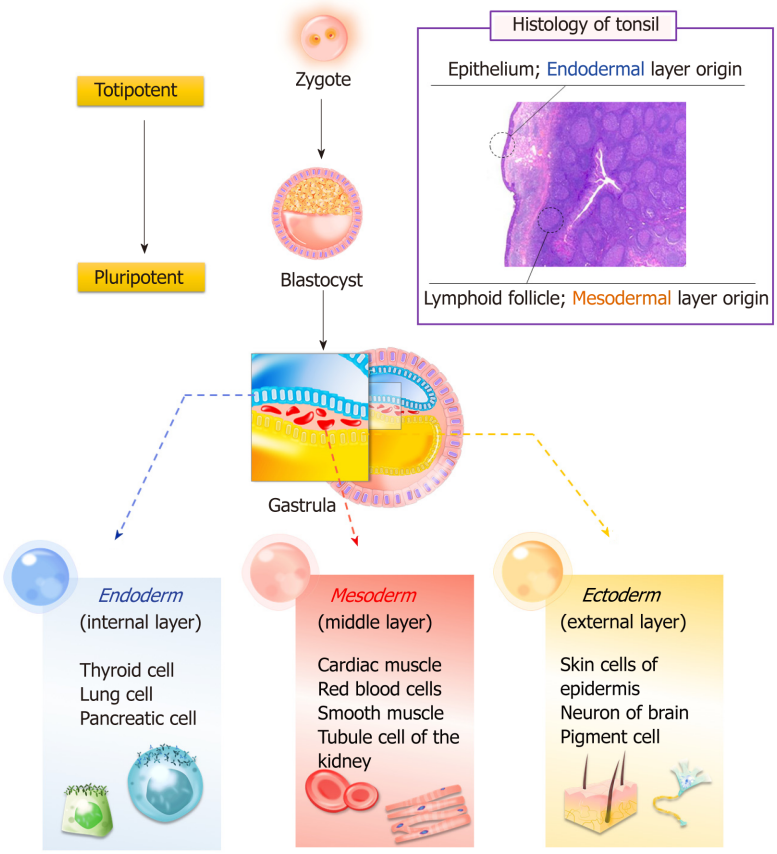

Figure 1 Possible reason enables diversity in tonsil-derived stem cells differentiation; constitutive features of tonsil. Zygote has most powerful differentiation potential (totipotent). It was able to differentiate to all human anatomies and to become human body. Embryonic stem cells (ESCs) are derived from the inner cell mass of the early embryo. ESCs also has great developmental potential and was able to differentiate to all cell lineages of an organism except for extraembryonic tissues (pluripotent). It is well known that ectodermal or endodermal differentiation is often difficult to achieve with mesenchymal stem cells isolated from bone marrow and adipose tissue (multipotent). Tonsil tissues consist of two different origin tissues; epithelial cells from endoderm origin and lymphoid tissues from mesoderm origin.

Cell therapy and tissue engineering have been investigated to regenerate lost or malfunctioning organs. These approaches utilize biomaterial scaffolds and MSCs to facilitate initial cell adhesion and retention while promoting cell growth for tissue regeneration[16,17]. In particular, the differentiation properties of MSCs are of great importance for tissue regeneration. It is generally known that isolated MSCs are often limited to germ-layer specific differentiation. As mentioned earlier, T-SCs offer multipotent differentiation potential that can be applied in regenerating various tissue types without concern for their germ layer origin.

ECTODERMAL DIFFERENTIATION OF T-SCS

Ectodermal differentiation is often difficult to achieve with MSCs isolated from bone marrow and adipose tissue. However, under the right conditions, T-SCs can be differentiated into non-mesenchymal lineages, including ectodermal differentiation into neurons, astrocytes, and Schwann-like cells to support nerve regeneration.

Neuronal differentiation of T-SCs

The neuronal differentiation of T-SCs was investigated in a three dimensional (3D) hybrid scaffold system by Patel *et al*[18]. This scaffold was fabricated by increasing the temperature of an aqueous solution of poly (ethylene glycol)-poly(L-alanine) to 37 °C, thus instigating the heat-induced sol-to-gel transition, in which T-SCs and growth factor-releasing microspheres were suspended. The gel exhibited a modulus of 800 Pa at 37 °C, similarly to that of brain tissue, and was robust enough to hold the microspheres and cells within the 3D cell culture. Neuronal growth factors were

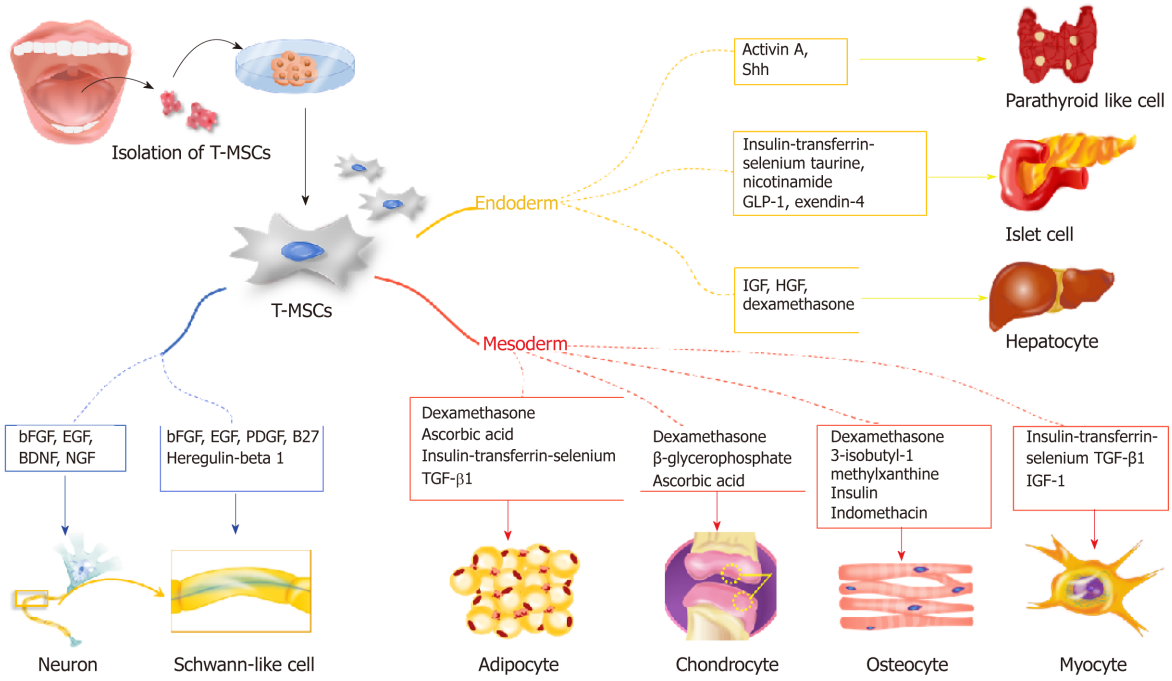

Figure 2 Differentiation potential of tonsil-derived stem cells. The multi-potency of the tonsil-derived stem cells (T-SCs) is confirmed in many studies. Under the proper conditions and signals (box), T-SCs can be differentiated into non-mesenchymal lineage, such as ectodermal differentiation (neurons, and Schwann-like cells) and endodermal cells (parathyroid like cells, islet cells and hepatocytes). T-MSCs: Tonsil- mesenchymal stem cells.

released over 12–18 d, and the encapsulated T-SCs gradually exhibited morphological changes from spherical to multipolar elongation. Significantly higher expression levels of neuronal biomarkers such as nuclear receptor-related protein, neuron-specific enolase, microtubule-associated protein-2, neurofilament-M, and glial fibrillary acidic protein were observed at both the mRNA and protein level in the hybrid system. This study clearly demonstrates the advantages of 3D hybrid scaffolds and highlights the importance of the sustained release of growth factors from hybrid systems to support the neuronal differentiation of T-SCs.

Schwann cell differentiation of T-SCs

Schwann cells are the glial cells of peripheral nerves that wrap around the axons to form myelin in the peripheral nervous system. Schwann cells promote nerve regeneration by secreting trophic support molecules and establishing a supportive growth matrix[19]. Jung *et al*[20] demonstrated that T-SCs could be differentiated into Schwann-like cells over several steps. Briefly, T-SCs were induced to form neurospheres under stimulation with EGF, bFGF, and B27 for 7 d. These neurospheres were then triturated and re-plated onto laminin-coated dishes with Schwann cell differentiation medium. After 10 d of culturing, the cells exhibited morphological changes, including the formation of elongated bipolar and tripolar spindle shapes. Schwann-like cells differentiated from T-SCs highly express the Schwann cell markers GFAP, NGFR, S100B, KROX20, and KROX24. Notably, Schwann cells differentiated from T-SCs were able to produce myelinate axons *in vitro* when co-cultured with mouse dorsal root ganglion neurons. In a mouse model with a sciatic nerve injury, a marked improvement in gait and increased nerve regeneration were observed with Schwann-cell treatment. Therefore, T-SCs can be a useful source for Schwann cell-based cell therapy to treat neuropathic diseases.

MESODERMAL DIFFERENTIATION OF T-SCS

Mesodermal differentiation is mainly achieved with adult MSCs from the mesodermal germ layer. Previously, a variety of cell sources from bone marrow and adipose tissue was utilized for mesodermal differentiation to treat bone, cartilage, and fat disorders. In this section, we highlight the potential use of T-SCs as an alternative cell source for mesodermal differentiation (Figure 2), and we provide a comparative study that illustrates the advantages of T-SCs.

Osteogenic differentiation of T-SCs

For osteogenic differentiation, Choi *et al*[21] cultured T-SCs in commercially available osteogenic media (α MEM supplemented with 10% FBS, 0.1 mmol/L dexamethasone, 10 μmol/L β glycerophosphate, and 50 μg/mL ascorbic acid) for three weeks. Alizarin Red S staining confirmed the successful deposition of extracellular calcium in culture. During osteogenic differentiation, mRNA expression of osteocalcin decreased 0.28-fold after cryopreservation, whereas ALP expression showed no difference. This profile remained stable even after passage 15 (P15). Interestingly, the osteogenic differentiation of T-SCs increased with the number of passages, with the peak osteogenic potential observed for passage 10 (P10), which exhibited a 1.4-fold increase over P3[6]. The expression of CCN1, a gene that is closely related to the osteogenic differentiation of MSCs, increased at P10. This finding is consistent with previous studies that have reported that CCN1 expression modulates the osteogenic potential of MSCs by regulating the Wnt3A pathway[6,22].

Various scaffolds have been employed to enhance skeletal regeneration to replace damaged bone. Because bone tissue is highly vascularized, integration with the host tissue followed by subsequent angiogenesis is critical for successful treatment. As an example, Park *et al*[23] encapsulated T-SCs within highly water-swollen hydrogel through the sol-gel transition of the thermoresponsive polymer poly(ethylene glycol)-poly(L-alanine-co-L-phenyl alanine) (PEG-PAF). The encapsulated T-SCs were cultured *in vitro* in the presence of an osteogenic-induction medium. The osteogenic differentiation of T-SCs was investigated by evaluating the expression of osteogenic genes, including Runx 2, ALP, and OCN. With support from the hydrogel, osteogenic gene expression was two times higher than that of conventional tissue cultures without soluble factor supplements.

Incorporating specific functional groups into scaffold substrates is also known to affect the osteogenic differentiation of MSCs[24,25]. T-SCs were encapsulated in poly (ethylene glycol)-poly(L-alanine) diblock copolymer (PEG-L-PA) thermogel that was modified with the phosphate functional groups of polystyrene microspheres to facilitate the osteogenic differentiation of encapsulated MSCs. The osteogenic differentiation of tonsil-MSCs (T-MSCs) was analyzed, and all osteogenic biomarker expressions were significantly higher for the modified thermogel than for the native thermogel. Immunofluorescence staining also confirmed high OCN expression, and Kye *et al*[24] also found that, compared with thermogel modified with carboxylate group microspheres, the phosphate functional group (-PO43-) more readily induced the osteogenic differentiation of T-SCs.

The osteogenic differentiation of T-SCs can be further improved by the over-expression of BMP-2 through genetic material transfer. In Jeong *et al*[26], BMP-2 minicircle DNA vectors were employed to form nano-sized polyplexes with the CBA-106 polymer. CBA-106 is a bioreducible cationic poly (amido amine) that facilitates the intracellular delivery of genetic material. The osteogenic differentiation of T-SCs was investigated by evaluating the gene expression of osteogenic markers such as osteocalcin, Runx2, and Col 1. *In vitro* calcium deposition was also confirmed using Alizarin Red S staining on day 7 and day 14 of the cell culture. In addition, *in vivo* bone regeneration was attempted using T-SCs transfected with the BMP-2 gene. The T-SCs were 3D cultured using PLLA/PLGA scaffolds for one week and then transplanted into the skulls of immunodeficient mice. It was shown that bone regeneration increased 1.96-fold compared to the control group five weeks after treatment.

Chondrogenic differentiation of T-SCs

To mimic a cartilage-like microenvironment, T-SCs were encapsulated in thermogel consisting of the PEG-PAF block copolymer by Park *et al*[23]. Under specific medium conditions (25 μL chondrogenic supplement/2.5 mL basal medium), the much higher expression of Col II and sulfated glycosaminoglycan in T-SCs was achieved using the thermogel than using a monolayer culture. Moreover, unique branching was observed among the encapsulated cells within the hydrogel. These changes in cellular morphology may influence the chondrogenic differentiation of T-SCs during 3D culturing. *In vivo* studies also confirmed that T-SCs successfully undergo chondrogenic differentiation with high expression levels of biomarkers such as Col II, AGG, and Col X.

Kye *et al*[24] also investigated the chondrogenic differentiation of T-SCs within PEG-L-PA thermogel. To enhance the cellular attachment in the 3D hydrogel micro-environment, various polystyrene microspheres with thiol (-SH), phosphate (-PO3¬), carboxylate (-COO) and amino (-NH2) functional groups were incorporated into the thermogel. Of the incorporated microparticles, the PS-S, PS-P, and PS-C microspheres exhibited significantly higher COL II expression. COL II mRNA expression was much higher in the PS-S thermogel than in the PS-N thermogel even though the PS-S and

the PS-N microspheres had similar sizes, clearly indicating that surface functional groups play an important role in stem cell differentiation.

Similarly, graphene oxide (GO) and reduced graphene oxide (rGO) were incorporated into PEG-L-PA thermogel by Park *et al*[27] to enhance surface functionality and thus provide good cellular adhesion. GO, or rGO (1 wt%) was suspended in a PEG- L-PA solution. T-SCs in GO/PEG-L-PA or rGO/PEG-L-PA were cultured in DMEM, and the spherical cellular morphology of the T-SCs was observed. When chondrogenic culture media enriched with TGF-β3 was utilized, T-SCs in hybrid systems aggregated extensively, and the expression levels of chondrogenic biomarkers such as SOX 9, COL II A1, COL II, and COL X increased. In particularly, COL II mRNA expression was 13 times higher in the GO/PEG-L-PA hybrid system than in the PEG-L-PA 3D system. Immunofluorescence analysis also revealed a significant increase in COL II expression and cell aggregation in the GO/PEG-L-PA hybrid system. In particular, the GO/PEG-L-PA 2D/3D hybrid system demonstrated the most significant increase in chondrogenic biomarker expression. These results indicate that the cooperative interaction among TGF-β3, COL II, and GO may be closely related to signaling cascades for chondrogenic differentiation.

Adipogenic differentiation of T-SCs

Ryu *et al*[5] investigated T-SCs as a source for adipogenic differentiation due to their multi-lineage differentiation potential and self-renewal capacity by culturing them using commercially available adipogenic media (Gibco StemPro™ Adipogenesis Differentiation Kit, Thermo Fisher Scientific, Waltham, MA, USA) for three weeks. The adipogenic potential of the T-SCs was evaluated for P3, P7, P10, and P15. Interestingly, unlike other types of T-SC differentiation, the adipogenesis of T-SCs decreased continuously with passage number, with P10 approximately two-thirds that of P3.

Adipogenic differentiation of T-SCs in thermogel was also investigated using PEG-L-PA (molecular weight of each block: 1000−1080 Da) by Kye *et al*[24]. The differentiation potential of T-SCs was investigated by incorporating polystyrene microspheres with different functional groups into the hydrogel. mRNA expression and immune histochemical assays indicated that T-SCs preferentially underwent adipogenesis in ammonium (−NH3+)- or thiol (−SH)-functionalized thermogels, whereas chondrogenesis occurred predominantly in phosphate (PO32−)- or carboxylate (−COO−)-functionalized thermogels. This study thus suggests that the surface functional groups of microspheres can control the preferential differentiation of stem cells into specific cell types in 3D cultures.

In addition to functionalized hydrogels, Patel *et al*[28] reported that a composite system of GO and polypeptide thermogel (GO/P), prepared using the temperature-sensitive sol-to-gel transition of GO-suspended poly(ethylene glycol)-poly(L-alanine) (PEG-PA), significantly enhanced the expression of adipogenic biomarkers, including PPAR-γ, CEBP-α, LPL, AP2, ELOVL3, and HSL when compared with a native hydrogel system. It appears that insulin, an adipogenic differentiation factor, can preferentially adhere to the surface of the GO incorporated into thermogels; insulin is then slowly released into the environment in a sustained manner during the cell culturing period. In contrast, more hydrophobic graphene may interfere with insulin, causing partial denaturation, reducing the adipogenic differentiation of T-SCs.

Myogenic differentiation of T-SCs

Various MSCs and progenitor cells have been evaluated their differentiation capacity into the myogenic cells for skeletal muscle regeneration. MSCs derived from BM, adipose, and umbilical cord tissue, and their use in cell therapy to augment the skeletal muscle injury response, have also been reported[29-31]. An alternative cellular source for MSCs, T-SCs have been shown to differentiate into myogenic cells *in vitro*, and transplanting the myoblasts and myocytes generated from T-SCs mediates the recovery of muscle function following injury *in vivo*[32]. For myogenic differentiation, T-SCs are treated in three sequential steps: Sphere formation on a petri dish in low-glucose DMEM, rosette-like spread formation on a collagen-coated dish, and two weeks of myogenic induction. In this final step, the cells express myogenic markers, including desmin, dystrophin, MHC, skeletal markers, α-Actinin, TNNI1, and myogenin. Furthermore, the intramuscular injection of T-MSC-derived myogenic cells into myectomized C57BL/6 mice enhances muscle function as demonstrated by gait assessment and the restoration of the skeletal muscle structure.

ENDODERMAL DIFFERENTIATION OF T-SCS

The endodermal differentiation of MSCs is important because many degenerative

diseases are related to organs that originate from the endoderm, including the liver, pancreas, and parathyroid. In this section, we highlight recent research that employs T-SCs to produce functional hepatocytes, pancreatic beta cells, and parathyroid cells (Figure 2).

Hepatocyte differentiation of T-SCs

Many clinical studies have indicated that BM-MSCs are safe and effective in the treatment of liver disease[33]. They can alleviate end-stage liver disease and improve symptoms and liver function[34,35]. However, some studies have indicated that BM-MSCs have the potential to aggravate fibrosis[36-38]. Thus, employing BM-MSCs as a therapy for liver fibrosis remains controversial.

As a novel cell source for treating liver disease, Park *et al*[39] demonstrated that T-SCs differentiate into hepatocyte-like cells and ameliorate live fibrosis *via* the activation of autophagy and the downregulation of TGF-β. A three-week culture in a differentiation medium containing IGF, HGF, dexamethasone, and oncostatin M led to the development of hepatocyte-like cells from T-SCs, as revealed by the expression of albumin and HNF-4α. In addition, transplanting T-SCs into a carbon tetrachloride (CCl4)-induced liver injury mouse model confirmed that T-SCs have a regenerative effect by migrating to the site of the liver injury and differentiating into hepatocyte-like cells. These results prove that T-SCs was able to differentiate into hepatocyte-like cells both *in vitro* and *in vivo*.

In addition to the direct differentiation of T-SCs into liver hepatocytes, T-SCs have also been investigated in terms of hepatogenic differentiation using PEG-L-PA thermogel[40,41]. The thermogel exhibited a physical modulus of 1000 Pa, which is similar to that of decellularized liver tissue. Three different 3D culture systems were compared in relation to the use of soluble factors such as hepatogenic growth factors. The spherical morphology and size of the encapsulated cells were maintained in the native 3D culture system during a culture period of 28 d, whereas the cells changed their morphology and aggregated significantly in 3D systems with growth factors. Hepatocyte-specific biomarker expression and metabolic functions were negligible in the native culture system. However, the expression levels of the hepatogenic genes of albumin and cytokeratin 18 and hepatocyte nuclear factor 4α were high in the two systems supplemented with growth factors. In addition, albumin and α-fetoprotein production were also significant[40]. PEG-L-PA thermogel thus provides a biocompatible microenvironment for the hepatogenic differentiation of T-SCs. In particular, the successful results of the growth factor encapsulated hydrogel system suggest that PEG-L-PA thermogel is a promising injectable tissue engineering system for liver tissue regeneration[41].

Pancreatic differentiation of T-SCs

Metabolic disturbances associated with diabetes lead to a number of complications ranging from cardiovascular and cerebrovascular disease to neuropathy, retinopathy, nephropathy, and the poor healing of wounds[42]. The only curative therapy available is pancreatic islet cell replacement, for which suitable donors are rare and which requires immunosuppressant therapy to reduce rejection. Recently, stem cell therapy has been proposed for the treatment of diabetes. Transplanting insulin-secreting cells produced from various stem cells, including embryonic and induced pluripotent stem cells and MSCs has shown therapeutic effects in diabetic animals[43]. In addition, differentiating various MSCs, including BM-MSCs and adipose MSCs, into insulin-producing cells has been suggested[44]. Kim *et al*[10] investigated the efficiency of differentiating T-SCs into insulin-producing cells by comparing two different methods and found that T-SCs differentiated more efficiently with insulin-transferrin-selenium (ITS) than with β-mercaptoethanol. The ITS method is composed of three steps: Two days of culturing in high-glucose α-MEM with 1% fatty acid-free bovine serum albumin (BSA) and 1 × ITS on a nonadherent dish; four days of culturing in high glucose α-MEM with 1% fatty acid-free BSA, ITS, 3 mM taurine, and 10 mM nicotinamide; and four days of culturing in high-glucose α-MEM with 1% fatty acid-free BSA, ITS, 3 mmol/L taurine, 10 mmol/L nicotinamide, 100 nmol/L glucagon-like peptide, and 10 Nm exendin-4. Notably, T-SCs exhibited a differentiation capability that was superior to that of adipose cell-derived MSCs. Further, implanting T-MSC-derived insulin-producing cells significantly alleviated streptozotocin-induced glucose intolerance in mice. These results suggest that T-SCs have the potential to be reprogrammed into pancreatic β-cells and applied to the clinical treatment of diabetes in the future.

Parathyroid differentiation of T-SCs

Hypoparathyroidism is a rare endocrine disorder, resulting in low serum calcium and increased serum phosphorus[45]. Hypoparathyroidism is the only hormonal

insufficiency state that does not have a hormone-replacement-therapy approved. Current managements include supplementation with oral calcium and active vitamin D, which cause various life-long adverse effects[45].

Stem cells have shown some promise in treating hypoparathyroidism in clinical applications. It has been reported that human embryonic stem cells (hESCs) and differentiated thymic stromal cells can be used in the *in vitro* regeneration of parathyroid-like cells[46-48]. However, the use of hESCs have critical ethical limitations, and it takes over 10 weeks for thymic stromal cells to differentiate and secrete PTH. Because of this, T-SCs have been considered as an alternative cell source for cell therapy. Park *et al*[49] demonstrated that T-SCs differentiate into parathyroid-like cells that release intact PTH using the modified Bingham protocol. Briefly, T-SCs at 90% confluence were cultured in a differentiation medium containing activin A and soluble sonic hedgehog for 7-21 d. Surprisingly, the T-SC-derived parathyroid-like cells differentially secreted PTH in response to extracellular calcium levels. Further, the therapeutic effects of T-SC-derived parathyroid-like cells embedded in Matrigel in rats that have undergone a parathyroidectomy suggest that embedding differentiated T-SCs in hydrogel scaffolds is a promising strategy for restoring parathyroid function.

ROLE OF T-SCS AS A NATIVE CELL THERAPY SOURCE

In addition to their multipotent differentiation potential, stem cells hold great promise for the treatment of numbers of diseases, especially those related to tissue damage involving immune reactions. The therapeutic effects of MSCs depend largely on their capacity to regulate inflammation and tissue homeostasis *via* an array of immunosuppressive factors, cytokines, growth factors, and differentiation factors[50]. Interestingly, depending on their type and intensity, inflammatory stimuli can lead MSCs to suppress the immune response in some cases or to enhance it in others. This plasticity of MSCs in immunomodulation leads them to act as suppressors or enhancers in response to the microenvironment[51]. In particular, the palatine tonsil is secondary lymphoid tissue that continuously encounters antigens and subsequently drives efficient immune response[52]. This tissue specificity may account for the intrinsic property of T-SCs in terms of immune regulatory plasticity.

IMMUNE MODULATION BY T-SCS

Previously, T-SCs have shown excellent immunomodulatory properties in targeting muscular fibrosis[53], skin inflammation[54], B-cell-mediated immune response[55], and autoimmune-mediated colitis[56]. In addition, T-SCs have been shown to improve the immune system by facilitating myelopoiesis in an allogeneic BMT mouse model[57]. These studies report that non-differentiated, non-stimulated native T-SCs constitutively secrete anti-inflammatory cytokines such as IL-1Ra, PD-L1, and EBI3 protein. Conditioned media from T-SCs that contain high levels of IL-1Ra efficiently regulate the mediation of the pro-fibrogenic process of myotubes by altering their IL-1β activity. Similarly, PD-L1 is a well-known immune-suppressive protein that targets numbers of immune and nonimmune cells. Notably, T-SCs express both soluble and membrane-bound forms of PD-L1 at higher levels compared with BM-MSCs and AT-MSCs. Indeed, T-MSC-derived PD-L1 has been demonstrated to attenuate Th17cell-mediated skin inflammation in psoriatic skin dermatitis in mice.

Recent studies have reported that IL-35 is a regulatory protein that acts on B cells[58] and that T-SCs constitutively produce EBI3, which is a critical component of IL-35[55]. Of note, T-SCs significantly ameliorate the estrogen-induced B-cell response both *in vitro* and *in vivo* in an IL-35-dependent manner.

Allogeneic hematopoietic stem cell transplantation is a routine treatment for intractable hematologic malignancies. The co-transplantation of BM-derived MSCs and donor HSCs promotes hematopoietic cell engraftment and prevents graft-versus-host disease with accelerated marrow stromal regeneration[59,60]. Research has also found enhanced myelocytic or megakaryocytic engraftment in the co-transplantation of MSCs and HSCs. Ryu *et al*[57] reported the supporting role of T-SCs in BM reconstitution and in supplementing hematopoiesis in a BMT mouse model. Considering that hematopoietic cells give rise to all of the mature blood cell types, including immune cells, normalizing hematopoiesis may eventually reverse immune deficiency induced by Bu/Cy preconditioning. Park *et al*[39] reported another study that demonstrated immune activation by T-SCs. In CCl4-induced liver fibrosis in mice, T-SCs migrated directly to injured tissue in the liver and promoted the restoration of liver function. Furthermore, T-SCs have been shown to promote the

activation of autophagy, which ultimately resolved fibrotic processes[39]. These results indicate that T-SCs can have a dual function on immunity by either activating or inhibiting the immune system.

FUTURE PERSPECTIVES OF T-SCS IN REGENERATIVE MEDICINE

The mass production of stem cells is vital for their widespread use, but, unfortunately, this process is expensive and time-consuming. Therefore, the regular use of stem cells as therapeutic agents lies in the distant future. However, T-SCs present new possibilities for the clinical application of stem cells. Obtaining T-SCs is more cost-effective than obtaining other types of stem cell; for example, tonsil tissue is readily obtained from tonsillectomies without the need for additional procedures. The yields and doubling times of T-SCs are also better than those for other stem cell types, and T-SCs from multiple donors can be used together[5]. The differential potential of T-SCs is also very cost-effective for clinical applications. For example, it takes only 14 d for tonsil tissue to differentiate and secrete PTH, whereas it takes over 10 weeks when thymic cells are employed[32]. T-SCs can also differentiate into various tissue types from all three germ layers. These features suggest that T-SCs can be a new cell source for regenerative medicine.

SHORTCOMINGS TO OVERCOME FOR CLINICAL USE

T-SCs have yet to be fully characterized. Tonsils are composed of various tissue types and cells (*e.g.*, connective tissue, endothelium, epithelium, and lymphocytes), which presents both advantages and disadvantages. As summarized above, T-SCs show promise for differentiation, and this is thought to be due to the various components of T-SCs and to the generally younger age of the donors. However, the histological diversity of T-SCs can be an obstacle to clinical applications because of the risk of tumorigenesis. Further, it possibly made variations of the T-SCs capacities, *e.g.*, differentiation into certain cell types. Therefore, the thorough characterization, including molecular mechanisms that facilitating differentiation of T-SCs should precede any clinical trials.

CONCLUSION

Tonsil tissue is a promising alternative source for the high-yield isolation of adult stem cells. Although T-SCs exhibit a cellular morphology and surface markers that are similar to those of bone marrow-derived MSCs, T-SCs possess superior stem cell properties that are very useful for various applications in regenerative medicine. Unlike other adult stem cell sources, T-SCs are typically isolated from young donors under age of 10. This is particularly beneficial in that tonsillectomies provide not only with a source of abundant tissue but also with good proliferation and differentiation potential of isolated T-SCs. In particular, isolated T-SCs exhibit multi-lineage differentiation, which is not often observed in other sources. As a result, it is clearly that T-SCs hold great promise for clinical applications in cell therapy and regenerative medicine.

REFERENCES

1 **Gimble J**, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* 2003; **5**: 362-369 [PMID: 14578098 DOI: 10.1080/14653240310003026]
2 **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]
3 **Malgieri A**, Kantzari E, Patrizi MP, Gambardella S. Bone marrow and umbilical cord blood human mesenchymal stem cells: state of the art. *Int J Clin Exp Med* 2010; **3**: 248-269 [PMID: 21072260]
4 **Janjanin S**, Djouad F, Shanti RM, Baksh D, Gollapudi K, Prgomet D, Rackwitz L, Joshi AS, Tuan RS. Human palatine tonsil: a new potential tissue source of multipotent mesenchymal progenitor cells. *Arthritis Res Ther* 2008; **10**: R83 [PMID: 18662393 DOI: 10.1186/ar2459]
5 **Ryu KH**, Cho KA, Park HS, Kim JY, Woo SY, Jo I, Choi YH, Park YM, Jung SC, Chung SM, Choi BO, Kim HS. Tonsil-derived mesenchymal stromal cells: evaluation of biologic, immunologic and genetic factors for successful banking. *Cytotherapy* 2012; **14**: 1193-1202 [PMID: 22900958 DOI: 10.3109/14653249.2012.706708]
6 **Yu Y**, Park YS, Kim HS, Kim HY, Jin YM, Jung SC, Ryu KH, Jo I. Characterization of long-term in vitro

culture-related alterations of human tonsil-derived mesenchymal stem cells: role for CCN1 in replicative senescence-associated increase in osteogenic differentiation. *J Anat* 2014; **225**: 510-518 [PMID: 25155898 DOI: 10.1111/joa.12229]
7 **Cho KA**, Kim JY, Kim HS, Ryu KH, Woo SY. Tonsil-derived mesenchymal progenitor cells acquire a follicular dendritic cell phenotype under cytokine stimulation. *Cytokine* 2012; **59**: 211-214 [PMID: 22578801 DOI: 10.1016/j.cyto.2012.04.016]
8 **Baugh RF**, Archer SM, Mitchell RB, Rosenfeld RM, Amin R, Burns JJ, Darrow DH, Giordano T, Litman RS, Li KK, Mannix ME, Schwartz RH, Setzen G, Wald ER, Wall E, Sandberg G, Patel MM; American Academy of Otolaryngology-Head and Neck Surgery Foundation. Clinical practice guideline: tonsillectomy in children. *Otolaryngol Head Neck Surg* 2011; **144**: S1-30 [PMID: 21493257 DOI: 10.1177/0194599810389949]
9 **Caplan AI**. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* 2007; **213**: 341-347 [PMID: 17620285 DOI: 10.1002/jcp.21200]
10 **Kim SY**, Kim YR, Park WJ, Kim HS, Jung SC, Woo SY, Jo I, Ryu KH, Park JW. Characterisation of insulin-producing cells differentiated from tonsil derived mesenchymal stem cells. *Differentiation* 2015; **90**: 27-39 [PMID: 26391447 DOI: 10.1016/j.diff.2015.08.001]
11 **Passàli D**, Damiani V, Passàli GC, Passàli FM, Boccazzi A, Bellussi L. Structural and immunological characteristics of chronically inflamed adenotonsillar tissue in childhood. *Clin Diagn Lab Immunol* 2004; **11**: 1154-1157 [PMID: 15539521 DOI: 10.1128/CDLI.11.6.1154-1157.2004]
12 **Tsukahara S**, Ikeda R, Goto S, Yoshida K, Mitsumori R, Sakamoto Y, Tajima A, Yokoyama T, Toh S, Furukawa K, Inoue I. Tumour necrosis factor alpha-stimulated gene-6 inhibits osteoblastic differentiation of human mesenchymal stem cells induced by osteogenic differentiation medium and BMP-2. *Biochem J* 2006; **398**: 595-603 [PMID: 16771708 DOI: 10.1042/BJ20060027]
13 **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]
14 **Wagner W**, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD. Replicative senescence of mesenchymal stem cells: a continuous and organized process. *PLoS One* 2008; **3**: e2213 [PMID: 18493317 DOI: 10.1371/journal.pone.0002213]
15 **Wagner W**, Bork S, Horn P, Krunic D, Walenda T, Diehlmann A, Benes V, Blake J, Huber FX, Eckstein V, Boukamp P, Ho AD. Aging and replicative senescence have related effects on human stem and progenitor cells. *PLoS One* 2009; **4**: e5846 [PMID: 19513108 DOI: 10.1371/journal.pone.0005846]
16 **Smart N**, Riley PR. The stem cell movement. *Circ Res* 2008; **102**: 1155-1168 [PMID: 18497316 DOI: 10.1161/CIRCRESAHA.108.175158]
17 **Ren G**, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008; **2**: 141-150 [PMID: 18371435 DOI: 10.1016/j.stem.2007.11.014]
18 **Patel M**, Moon HJ, Jung BK, Jeong B. Microsphere-Incorporated Hybrid Thermogel for Neuronal Differentiation of Tonsil Derived Mesenchymal Stem Cells. *Adv Healthc Mater* 2015; **4**: 1565-1574 [PMID: 26033880 DOI: 10.1002/adhm.201500224]
19 **Monk KR**, Feltri ML, Taveggia C. New insights on Schwann cell development. *Glia* 2015; **63**: 1376-1393 [PMID: 25921593 DOI: 10.1002/glia.22852]
20 **Jung N**, Park S, Choi Y, Park JW, Hong YB, Park HH, Yu Y, Kwak G, Kim HS, Ryu KH, Kim JK, Jo I, Choi BO, Jung SC. Tonsil-Derived Mesenchymal Stem Cells Differentiate into a Schwann Cell Phenotype and Promote Peripheral Nerve Regeneration. *Int J Mol Sci* 2016; 17 [PMID: 27834852 DOI: 10.3390/ijms17111867]
21 **Choi JS**, Lee BJ, Park HY, Song JS, Shin SC, Lee JC, Wang SG, Jung JS. Effects of donor age, long-term passage culture, and cryopreservation on tonsil-derived mesenchymal stem cells. *Cell Physiol Biochem* 2015; **36**: 85-99 [PMID: 25924984 DOI: 10.1159/000374055]
22 **Si W**, Kang Q, Luu HH, Park JK, Luo Q, Song WX, Jiang W, Luo X, Li X, Yin H, Montag AG, Haydon RC, He TC. CCN1/Cyr61 is regulated by the canonical Wnt signal and plays an important role in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells. *Mol Cell Biol* 2006; **26**: 2955-2964 [PMID: 16581771 DOI: 10.1128/MCB.26.8.2955-2964.2006]
23 **Park MH**, Yu Y, Moon HJ, Ko du Y, Kim HS, Lee H, Ryu KH, Jeong B. 3D culture of tonsil-derived mesenchymal stem cells in poly(ethylene glycol)-poly(L-alanine-co-L-phenyl alanine) thermogel. *Adv Healthc Mater* 2014; **3**: 1782-1791 [PMID: 24958187 DOI: 10.1002/adhm.201400140]
24 **Kye EJ**, Kim SJ, Park MH, Moon HJ, Ryu KH, Jeong B. Differentiation of tonsil-tissue-derived mesenchymal stem cells controlled by surface-functionalized microspheres in PEG-polypeptide thermogels. *Biomacromolecules* 2014; **15**: 2180-2187 [PMID: 24805903 DOI: 10.1021/bm500342r]
25 **Moon HJ**, Patel M, Chung H, Jeong B. Nanocomposite versus Mesocomposite for Osteogenic Differentiation of Tonsil-Derived Mesenchymal Stem Cells. *Adv Healthc Mater* 2016; **5**: 353-363 [PMID: 26634888 DOI: 10.1002/adhm.201500558]
26 **Jeong H**, Lee ES, Jung G, Park J, Jeong B, Ryu KH, Hwang NS, Lee H. Bioreducible-Cationic Poly(amido amine)s for Enhanced Gene Delivery and Osteogenic Differentiation of Tonsil-Derived Mesenchymal Stem Cells. *J Biomed Nanotechnol* 2016; **12**: 1023-1034 [PMID: 27305823]
27 **Park J**, Kim, IY, Patel M, Moon HJ, Hwang SJ, Jeong B. 2D and 3D hybrid systems for enhancement of chondrogenic differentiation of tonsil-derived mesenchymal stem cells. Adv Funct Mater. 2015; 2573-2582 [DOI: 10.1002/adfm.201500299]
28 **Patel M**, Moon HJ, Ko du Y, Jeong B. Composite System of Graphene Oxide and Polypeptide Thermogel As an Injectable 3D Scaffold for Adipogenic Differentiation of Tonsil-Derived Mesenchymal Stem Cells. *ACS Appl Mater Interfaces* 2016; **8**: 5160-5169 [PMID: 26844684 DOI: 10.1021/acsami.5b12324]
29 **De Bari C**, Dell'Accio F, Vandenabeele F, Vermeesch JR, Raymackers JM, Luyten FP. Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *J Cell Biol* 2003; **160**: 909-918 [PMID: 12629053 DOI: 10.1083/jcb.200212064]
30 **Ferrari G**, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* 1998; **279**: 1528-1530 [PMID: 9488650]
31 **Hosoyama T**, McGivern JV, Van Dyke JM, Ebert AD, Suzuki M. Derivation of myogenic progenitors directly from human pluripotent stem cells using a sphere-based culture. *Stem Cells Transl Med* 2014; **3**: 564-574 [PMID: 24657962 DOI: 10.5966/sctm.2013-0143]
32 **Park S**, Choi Y, Jung N, Yu Y, Ryu KH, Kim HS, Jo I, Choi BO, Jung SC. Myogenic differentiation

potential of human tonsil-derived mesenchymal stem cells and their potential for use to promote skeletal muscle regeneration. *Int J Mol Med* 2016; **37**: 1209-1220 [PMID: 27035161 DOI: 10.3892/ijmm.2016.2536]

33 **Suk KT**, Yoon JH, Kim MY, Kim CW, Kim JK, Park H, Hwang SG, Kim DJ, Lee BS, Lee SH, Kim HS, Jang JY, Lee CH, Kim BS, Jang YO, Cho MY, Jung ES, Kim YM, Bae SH, Baik SK. Transplantation with autologous bone marrow-derived mesenchymal stem cells for alcoholic cirrhosis: Phase 2 trial. *Hepatology* 2016; **64**: 2185-2197 [PMID: 27339398 DOI: 10.1002/hep.28693]

34 **Liang J**, Zhang H, Zhao C, Wang D, Ma X, Zhao S, Wang S, Niu L, Sun L. Effects of allogeneic mesenchymal stem cell transplantation in the treatment of liver cirrhosis caused by autoimmune diseases. *Int J Rheum Dis* 2017; **20**: 1219-1226 [PMID: 28217916 DOI: 10.1111/1756-185X.13015]

35 **Margini C**, Vukotic R, Brodosi L, Bernardi M, Andreone P. Bone marrow derived stem cells for the treatment of end-stage liver disease. *World J Gastroenterol* 2014; **20**: 9098-9105 [PMID: 25083082 DOI: 10.3748/wjg.v20.i27.9098]

36 **Baertschiger RM**, Serre-Beinier V, Morel P, Bosco D, Peyrou M, Clément S, Sgroi A, Kaelin A, Buhler LH, Gonelle-Gispert C. Fibrogenic potential of human multipotent mesenchymal stromal cells in injured liver. *PLoS One* 2009; **4**: e6657 [PMID: 19684854 DOI: 10.1371/journal.pone.0006657]

37 **Li C**, Kong Y, Wang H, Wang S, Yu H, Liu X, Yang L, Jiang X, Li L, Li L. Homing of bone marrow mesenchymal stem cells mediated by sphingosine 1-phosphate contributes to liver fibrosis. *J Hepatol* 2009; **50**: 1174-1183 [PMID: 19398237 DOI: 10.1016/j.jhep.2009.01.028]

38 **Yang L**, Chang N, Liu X, Han Z, Zhu T, Li C, Yang L, Li L. Bone marrow-derived mesenchymal stem cells differentiate to hepatic myofibroblasts by transforming growth factor-β1 via sphingosine kinase/sphingosine 1-phosphate (S1P)/S1P receptor axis. *Am J Pathol* 2012; **181**: 85-97 [PMID: 22609227 DOI: 10.1016/j.ajpath.2012.03.014]

39 **Park M**, Kim YH, Woo SY, Lee HJ, Yu Y, Kim HS, Park YS, Jo I, Park JW, Jung SC, Lee H, Jeong B, Ryu KH. Tonsil-derived mesenchymal stem cells ameliorate CCl4-induced liver fibrosis in mice via autophagy activation. *Sci Rep* 2015; **5**: 8616 [PMID: 25722117 DOI: 10.1038/srep08616]

40 **Kim SJ**, Park MH, Moon HJ, Park JH, Ko du Y, Jeong B. Polypeptide thermogels as a three dimensional culture scaffold for hepatogenic differentiation of human tonsil-derived mesenchymal stem cells. *ACS Appl Mater Interfaces* 2014; **6**: 17034-17043 [PMID: 25192309 DOI: 10.1021/am504652y]

41 **Hong JH**, Lee HJ, Jeong B. Injectable Polypeptide Thermogel as a Tissue Engineering System for Hepatogenic Differentiation of Tonsil-Derived Mesenchymal Stem Cells. *ACS Appl Mater Interfaces* 2017; **9**: 11568-11576 [PMID: 28290667 DOI: 10.1021/acsami.7b02488]

42 **Khamaisi M**, Balanson SE. Stem Cells for Diabetes Complications: A Future Potential Cure. *Rambam Maimonides Med J* 2017; 8 [PMID: 28178432 DOI: 10.5041/RMMJ.10283]

43 **Lilly MA**, Davis MF, Fabie JE, Terhune EB, Gallicano GI. Current stem cell based therapies in diabetes. *Am J Stem Cells* 2016; **5**: 87-98 [PMID: 27853630]

44 **Gabr MM**, Zakaria MM, Refaie AF, Abdel-Rahman EA, Reda AM, Ali SS, Khater SM, Ashamallah SA, Ismail AM, Ismail HEA, El-Badri N, Ghoneim MA. From Human Mesenchymal Stem Cells to Insulin-Producing Cells: Comparison between Bone Marrow- and Adipose Tissue-Derived Cells. *Biomed Res Int* 2017; **2017**: 3854232 [PMID: 28584815 DOI: 10.1155/2017/3854232]

45 **Abate EG**, Clarke BL. Review of Hypoparathyroidism. *Front Endocrinol (Lausanne)* 2017; **7**: 172 [PMID: 28138323 DOI: 10.3389/fendo.2016.00172]

46 **Bingham EL**, Cheng SP, Woods Ignatoski KM, Doherty GM. Differentiation of human embryonic stem cells to a parathyroid-like phenotype. *Stem Cells Dev* 2009; **18**: 1071-1080 [PMID: 19025488 DOI: 10.1089/scd.2008.0337]

47 **Sulzbacher S**, Schroeder IS, Truong TT, Wobus AM. Activin A-induced differentiation of embryonic stem cells into endoderm and pancreatic progenitors-the influence of differentiation factors and culture conditions. *Stem Cell Rev* 2009; **5**: 159-173 [PMID: 19263252 DOI: 10.1007/s12015-009-9061-5]

48 **Woods Ignatoski KM**, Bingham EL, Frome LK, Doherty GM. Directed trans-differentiation of thymus cells into parathyroid-like cells without genetic manipulation. *Tissue Eng Part C Methods* 2011; **17**: 1051-1059 [PMID: 21797755 DOI: 10.1089/ten.tec.2011.0170]

49 **Park YS**, Kim HS, Jin YM, Yu Y, Kim HY, Park HS, Jung SC, Han KH, Park YJ, Ryu KH, Jo I. Differentiated tonsil-derived mesenchymal stem cells embedded in Matrigel restore parathyroid cell functions in rats with parathyroidectomy. *Biomaterials* 2015; **65**: 140-152 [PMID: 26156233 DOI: 10.1016/j.biomaterials.2015.06.044]

50 **Kyurkchiev D**, Bochev I, Ivanova-Todorova E, Mourdjeva M, Oreshkova T, Belemezova K, Kyurkchiev S. Secretion of immunoregulatory cytokines by mesenchymal stem cells. *World J Stem Cells* 2014; **6**: 552-570 [PMID: 25426252 DOI: 10.4252/wjsc.v6.i5.552]

51 **Wang Y**, Chen X, Cao W, Shi Y. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol* 2014; **15**: 1009-1016 [PMID: 25329189 DOI: 10.1038/ni.3002]

52 **Sada-Ovalle I**, Talayero A, Chavéz-Galán L, Barrera L, Castorena-Maldonado A, Soda-Merhy A, Torre-Bouscoulet L. Functionality of CD4+ and CD8+ T cells from tonsillar tissue. *Clin Exp Immunol* 2012; **168**: 200-206 [PMID: 22471281 DOI: 10.1111/j.1365-2249.2012.04573.x]

53 **Cho KA**, Park M, Kim YH, Woo SY, Ryu KH. Conditioned media from human palatine tonsil mesenchymal stem cells regulates the interaction between myotubes and fibroblasts by IL-1Ra activity. *J Cell Mol Med* 2017; **21**: 130-141 [PMID: 27619557 DOI: 10.1111/jcmm.12947]

54 **Kim JY**, Park M, Kim YH, Ryu KH, Lee KH, Cho KA, Woo SY. Tonsil-derived mesenchymal stem cells (T-MSCs) prevent Th17-mediated autoimmune response via regulation of the programmed death-1/programmed death ligand-1 (PD-1/PD-L1) pathway. *J Tissue Eng Regen Med* 2018; **12**: e1022-e1033 [PMID: 28107610 DOI: 10.1002/term.2423]

55 **Cho KA**, Lee JK, Kim YH, Park M, Woo SY, Ryu KH. Mesenchymal stem cells ameliorate B-cell-mediated immune responses and increase IL-10-expressing regulatory B cells in an EBI3-dependent manner. *Cell Mol Immunol* 2017 [PMID: 28042143 DOI: 10.1038/cmi.2016.59]

56 **Yu Y**, Song EM, Lee KE, Joo YH, Kim SE, Moon CM, Kim HY, Jung SA, Jo I. Therapeutic potential of tonsil-derived mesenchymal stem cells in dextran sulfate sodium-induced experimental murine colitis. *PLoS One* 2017; **12**: e0183141 [PMID: 28854223 DOI: 10.1371/journal.pone.0183141]

57 **Ryu JH**, Park M, Kim BK, Kim YH, Woo SY, Ryu KH. Human tonsilderived mesenchymal stromal cells enhanced myelopoiesis in a mouse model of allogeneic bone marrow transplantation. *Mol Med Rep* 2016; **14**: 3045-3051 [PMID: 27511380 DOI: 10.3892/mmr.2016.5604]

58 **Wang RX**, Yu CR, Dambuza IM, Mahdi RM, Dolinska MB, Sergeev YV, Wingfield PT, Kim SH,

Egwuagu CE. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. *Nat Med* 2014; **20**: 633-641 [PMID: 24743305 DOI: 10.1038/nm.3554]

59 **Battiwalla M**, Barrett AJ. Bone marrow mesenchymal stromal cells to treat complications following allogeneic stem cell transplantation. *Tissue Eng Part B Rev* 2014; **20**: 211-217 [PMID: 24410434 DOI: 10.1089/ten.TEB.2013.0566]

60 **Battiwalla M**, Hematti P. Mesenchymal stem cells in hematopoietic stem cell transplantation. *Cytotherapy* 2009; **11**: 503-515 [PMID: 19728189 DOI: 10.1080/14653240903193806]

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REVIEW

Linking stemness with colorectal cancer initiation, progression, and therapy

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Abstract

The discovery of cancer stem cells caused a paradigm shift in the concepts of origin and development of colorectal cancer. Several unresolved questions remain in this field though. Are colorectal cancer stem cells the cause or an effect of the disease? How do cancer stem cells assist in colorectal tumor dissemination to distant organs? What are the molecular or environmental factors affecting the roles of these cells in colorectal cancer? Through this review, we investigate the key findings until now and attempt to elucidate the origins, physical properties, microenvironmental niches, as well as the molecular signaling network that support the existence, self-renewal, plasticity, quiescence, and the overall maintenance of cancer stem cells in colorectal cancer. Increasing data show that the cancer stem cells play a crucial role not only in the establishment of the primary colorectal tumor but also in the distant spread of the disease. Hence, we will also look at the mechanisms adopted by cancer stem cells to influence the development of metastasis and evade therapeutic targeting and its role in the overall disease prognosis. Finally, we will illustrate the importance of understanding the biology of these cells to develop improved clinical strategies to tackle colorectal cancer.

Key words: Cancer stem cell; Colorectal cancer; Tumor microenvironment; Metastasis; Extracellular matrix; Tumor heterogeneity; Resistance; Stemness; Quiescence; Recurrence

Core tip: With the advancement of technology, the importance of deciphering the roles of stem cells in normal and malignant intestinal biology has grown tremendously. Aided by several molecular and environmental factors, evidence suggests that colorectal cancer stem cells exploit the intestinal cellular framework causing the development and spread of the disease, simultaneously promoting a poor prognosis through drug resistance and recurrence-based events. Only by a better understanding of the biology of these cells can

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there be an improvement in the strategies associated with clinical monitoring and therapeutic targeting required for disease management.

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INTRODUCTION

Colorectal cancer (CRC) is a heterogeneous disease. Approximately three decades ago, the genetic roadmap for the origin and development of CRC was identified[1]. Since then, massive technological progress has allowed the identification of numerous genetic and epigenetic components of the disease, further improving our understanding of the heterogeneity associated with CRC. We now know that not only does CRC exhibit a highly complex inter-tumor heterogeneity across multiple cases, warranting the need for personalized medicine[2], but it also displays a component of intra-tumor heterogeneity (morphological, genotypic, and phenotypic differences within the same tumor)[3]. The overall picture was further complicated by the discovery of cancer stem cells (CSCs) that led to an altered component of intra-tumor heterogeneity (*i.e*. heterogeneity between clonal populations), owing to the highly dynamic nature of CSCs. Major components affecting this behavior of CSCs include tumor genetics, epigenetic signals, and most importantly, the surrounding tumor microenvironment[4]. Notably, these factors have brought a landmark change in our understanding of the landscape of CRC development and progression. Considering these developments, here we review the current understanding as well as the evolving concepts of CSCs in the context of origin, development, and outcome of CRC. At the outset, we wish to clarify that the understanding of several aspects of CSCs, particularly within the field of CRC, is still at its infancy. Regardless, we aim to provide critical shreds of evidence from clinically relevant discoveries that would attempt to bridge, if not all, certain knowledge gaps existent within this field.

THE STEM CELL NICHE: PERSPECTIVES OF THE ADULT INTESTINE

An insight into the biology of intestinal stem cells (ISCs) will fuel our existing knowledge of the regulatory mechanisms of development and function of colorectal CSCs, owing to the similarities in several signaling pathways within normal and cancerous stem cells[5,6]. Structurally, the intestinal epithelium is organized into several finger-like villi protrusions extending into the gut lumen that is surrounded at the base by multiple glandular invaginations, the crypts of Lieberkühn, that extend into the extracellular matrix. The villus architecture comprises of non-dividing differentiated polyclonal cells with divergent functions of nutrient absorption-enterocytes, protective mucus barrier secretion - goblet cells, and gastrointestinal hormone secretion-enteroendocrine cells; all of which, including the post-mitotic Paneth cells that reside at the bottom of the crypt, are generated from the undifferentiated, rapidly proliferating multipotent stem cells residing as monoclonal compartments within the crypts. Unequivocally, the exorbitant rate of proliferation exhibited by the ISCs within the crypts is responsible for providing a high rate of self-renewal to the intestinal epithelium; essential to protect it by the persistent fusillade from physical, chemical, and/or biological insult[7].

Two functionally unique ISC populations are characterized within human and mice small intestine, the quiescent DNA label-retaining ISC (LRCs) identified at the +4 crypt position (characterized by the high expression of the polycomb complex protein Bmi1[8], homeodomain-containing protein Hopx[9], Tert[10], and Lrig1[11,12] markers), and the leucine-rich repeat–containing G protein-coupled receptor 5 ($Lgr5^+$) expressing crypt base columnar cells (CBCs)[13]; both of which exhibit a self-renewal ability (self-renewal) as well as the potency to differentiate into cells of the intestinal epithelium (multi-potency), certifying them as true stem cells[7,14]. The identification of these ISC markers has been hugely possible through lineage tracing studies employing mouse models. Evidence from clonal analysis and knock-in experiments suggests that the

Lgr5$^+$ CBC cells represent the actively cycling stem cell niche that gives rise to daughter cells that are transferred into a transit amplifying (TA) compartment; subsequently dividing and moving towards the crypt-villus axis as differentiated cells, promoting intestinal homeostasis[13]. Contrastingly, the Lgr5$^-$ ISCs located on the +4 position of crypts are under 2% proliferative, and consequently serve as a reserve ISC pool that can assist the LRCs, directly or by regenerating the Lgr5$^+$ cell population, in case of injury from chemotherapy or radiation to the functional stem cell population[8,15]. Indeed, through carefully derived genetic models, it has been shown that selective killing of Lgr5 cells can be restored by Lgr5$^-$/Bmi1$^+$ cells located anywhere in the crypt[16].

Muñoz *et al*[17] identified, however, that the Lgr5$^+$ cells can also express the +4 markers; suggesting a high degree of plasticity between the two ISC populations. Furthermore, secretory precursors [including the Paneth cells, enteroendocrine cells, goblet cells, and Tuft cells (IL-25 secreting chemosensory cells that increase in numbers as a type-2 immune response)] derived from Lgr5$^+$ cells expressing the Notch ligand DLL1, have also been shown to generate short-lived clones composed of differentiated secretory cells of intestinal epithelium, upon radiation-induced damage[18]. Yet another regenerative mechanism was identified by Barriga *et al*[19], who observed through single-cell transcriptomics, the existence of a subpopulation of Lgr5$^+$ cells expressing an RNA-binding protein Mex3a that divides at a relatively slower rate, making it more resistant to radiation or chemotherapy-induced damage, and can help regenerate all intestinal lineages. To summarize, the small intestinal crypt functions as a well-defined network of interdependent cellular niches that serve as multiple layers of backups to ensure the smooth and continual functioning of the stem cell machinery to maintain intestinal homeostasis.

Within the ISC research community, while the small intestine has received a major focus, the colon stem cells lack significant characterization[5]. As compared to the small intestine, the colon shows large differences with the overall anatomy of the intestinal epithelium as well as the cellular architecture. The large intestine is devoid of any finger-like villi protrusions, or crypt based Bmi1$^+$ cells, and Paneth cells[5]. The colonic stem cell niche has been characterized primarily with cells showing a high expression of Lgr5 as well as ephrin type-B receptor 2, olfactomedin-4, and achaete-scute complex homolog-2 markers; which are capable of self-renewal and giving rise to the cells of the intestinal lineage[13,20-22]. Studies also indicate, however, the presence of slow-cycling colonic Lrig1$^+$ cells that attempt to replenish the Lgr5 cell population upon injury[11]. Reports also show the presence of doublecortin-like kinase 1 cells within the colonic crypts that have been found to be actively proliferating in the presence of growth factors and give rise to intestinal lineage cells, forming enteroids[23,24]. Furthermore, unlike small intestine where the Paneth cells serve as the primary source of wingless/integrated (Wnt) signaling molecules that guide the renewal of the epithelium, recent work by Degirmenci *et al*[25] showed the existence of a group of subepithelial mesenchymal cells expressing zinc finger protein Gli1 that act as a critical source for Wnt secretion that directs colonic stem cell renewal. Moreover, the crypt structural elements, specifically the colonocytes, have also been found to yield a protective function to the proliferating cells within the crypt from potent metabolites produced by the intestinal microbiota[26]. Notwithstanding these crucial bits of research, a lot must be done to understand better the colonic crypt and the associated stem cell niche.

ROLE OF INTESTINAL STEMNESS IN THE ORIGIN OF CRC

The original model for colorectal carcinogenesis and progression, the "Vogelgram", was laid down rather elaborately by Fearon and Vogelstein[1]. It provided a schematic in which loss of the adenomatous polyposis coli (*APC*) tumor suppressor gene would result in an adenoma and subsequently mutations in *KRAS*, *TP53*, phosphoinositide 3-kinase (*PI3K)*, and other genes would cause the development of a metastatic disease. A principal feature of CRC identified through this model was the monoclonal origin of the disease (*i.e.* CRC originates from the clonal expansion of one hyperproliferating cell). Importantly, the involvement of the crypt and the corresponding ISCs residing within them as CRC initiators were debated upon, since the analysis of several spontaneous adenomas found dysplastic cells with mutations in *APC* only on the luminal surface of the colon, while the underneath crypt and the ISCs were normal[27]. This finding caused the development of the 'top-down' model of tumor initiation that begins at the top of the crypt, in the intra-cryptal zones between crypt orifices, and then spreads laterally and downward, displacing the normal epithelium of crypts[27]. Though this was true for patients with familial adenomatous polyposis (FAP),

immunohistochemical studies of early sporadic colorectal adenomas have shown proliferative adenomatous epithelium with nuclear beta-catenin within the entire crypt; pointing at the role of crypt based stem cells as progenitors of CRC[28]. In parallel, the Wright lab also examined the mucosa from FAP patients post-surgery and found that both sporadic and FAP adenomas originate as a uni-cryptal adenoma, with dysplastic lesions in a single but entire crypt, and grows 'bottom-up' by a division of the crypt at the base, termed as crypt fission. Although the lateral and downward spreading model was not completely excluded, several studies pointed out that crypt fission is the primary mode of adenoma progression in FAP[29,30] as well as sporadic adenomas[31]. Indeed, an alternative explanation for the top-down model was suggested by Shih *et al*[27] wherein the stem cells at the base of a single crypt develop the neoplasm, which subsequently transforms and migrates up the crypt and become a part of the superficial mucosae, which then spreads laterally and downward into adjacent crypts.

It has been shown in the past that the severity of intestinal cancer depends largely on the initiation than the progression, indicating the importance associated with the early events of CRC development[32,33]. Importantly, the identification of ISC specific expression markers has allowed for functional techniques that can be used to determine if they can function as progenitors of colorectal carcinogenesis. In 2007, O'Brien *et al*[34] characterized the first tumor stem cell marker, CD133, and pointed towards a CSC model of tumor initiation driven by $CD133^+$ cells in CRC. A plethora of studies has indicated that specific deletion of *APC* in ISCs expressing Lgr5, LRIG1, or CD133 markers can induce rapid adenoma generation[11,35-37]. Moreover, activation of the β-catenin pathway within these cells as well as $BMI1^+$ cells resulted in a similar outcome, indicating that ISCs are the primary cells of origin of CRC[8]. Mutations within key signaling pathways, including Wnt, Notch, and Hedgehog pathways, can dislodge the wild-type ISCs from the control of regulatory signals, allowing them to develop precursor lesions[38,39]. Most of these approaches, however, caused the generation of intestinal adenomas in mice that commonly occur in the small intestine and do not generally progress to carcinoma[40,41]. In contrast, human intestinal malignancies mostly occur in the colon. Additionally, the development of human CRC is also strongly dependent on environmental factors such as inflammatory conditions[42], which are lacking in genetic mouse models[5].

Owing to multiple differences between carcinogenesis in genetically modified mice and human patients, several studies have looked at the dynamics of stem cells in response to key genetic mutations and its influence on the development of CRC to improve our grasp on the actual mechanisms of CRC origin. Mutations within the intestine were assumed to arise due to several factors, including DNA replication errors and environmental factors such as carcinogen exposure, inflammation, *etc*. Since the TA cells represent the most actively proliferating population within the crypt, they are more prone to mutations; although given the short life span of these cells and the mild phenotype of the mutation, mutated TA cells offer a lower risk of serving as tumor initiators[43]. Indeed, it was shown that the wild-type ISC division follows the neutral drift principle to replace randomly any of the other crypt ISC populations[44,45]. Although oncogenic mutations aim towards preventing this, Vermeulen *et al*[46] and Snippert *et al*[47] suggest that the mutated cells can also be stochastically replaced by wild-type ISCs. What this means is that the likelihood of an inactivating mutation in a key tumor suppressor, like *APC*, to get fixed is less than 50%, making the mutated cell highly susceptible of getting lost in the continuous process of replacement[43]. Such a low probability makes CRC an extremely slow disease, postulated to take over a decade for cellular mutations to accumulate that could drive the initiation and progression of the malignancy[48]. Importantly, the presence of accompanying conditions such as intestinal inflammation tends to allow the mutated cells to prevail, pointing at the importance of environmental factors in conjunction with genetic factors in playing a critical role in CRC initiation[46]. Not surprisingly, while a competition exists between the normal and the mutated ISCs during the tumor initiation process, disease progression is associated with a rivalry between the CSCs, with stronger clones characterized by a larger number of accumulated mutations and resistance to environmental factors such as therapy[5].

In addition to a stem cell-based CRC origin model, a few studies have indicated a role of differentiated cells in serving as the cell of origin for the disease. Like the ISC to colorectal CSC model, most of these studies also indicate that genetic events combined with environmental factors can favor the development of CRC. A loss of *APC* in the tuft cells accompanied by microenvironmental disturbances was found to induce colonic tumors[49]. Moreover, transgenic mice models have shown that intestinal epithelial cells can also dedifferentiate into tumor-initiating stem cells under the influence of enhanced Wnt and the inflammatory nuclear factor-kappa B (NFκB) signaling pathway[50]. Alternatively, accumulation of mutations such as $KRAS^{G12D}$ that

activates inflammatory signaling, accompanied with a loss of *APC* that results in the activation of Wnt pathway, yielded similar tumor initiation from the differentiated cells[50]. Taken together, the studies indicate that while CRC can have a stem cell (primary) or a non-stem cell (secondary) origin, the contributing factors include accumulation of mutations as well as environmental factors to confer a functional advantage for the development and progression of the tumor.

INFLUENTIAL PATHWAYS REGULATING THE COLORECTAL CSCs

There exists a molecular network surrounding the complex development of CSCs associated with CRC, and they are only recently emerging. Deciphering this network will not only improve our understanding of the role of stem cells in the origin and pathogenesis of CRC, rather will provide better therapeutic avenues to deal with the malignancy. Although several signaling pathways have been implicated, notable ones that have been found to play crucial roles in the growth and functional maintenance of CSCs include the Wnt, Notch and Hedgehog, and the bone morphogenetic protein (BMP) pathways.

The canonical Wnt signaling pathway has been identified as a hallmark in the regulation of stem cells–from maintenance, proliferation, differentiation to apoptosis[51]. Under normal signaling, binding of the Wnt ligand to the transmembrane receptors tends to stabilize and allows the nuclear translocation of β-catenin causing transcriptional activation of important targets including *c-Myc*, *Axin2*, *Lgr5*, and *ASCL2* that govern stem cell fate, proliferation, as well as maintenance[52-56]. Specifically, within the intestine, active Wnt signaling is essential to maintain the stem cell niche within the crypt and promote gut homeostasis[57]. Intuitively, abnormal Wnt signaling has been implicated in several cancers, including CRC[58]. Inactivating mutations in *APC* and consequently a hyperactive Wnt signaling or activating mutations in β-catenin have been found in most of the CRC cases and has been identified as one of the initiating steps in tumor development[59]. In line with the ISC as the CRC cell of origin theory, Vermeulen *et al*[60] reported that $CD133^+$ CRC cells growing as tumor spheres in culture contain a subpopulation of cells with constitutively high Wnt signaling. However, only a subset of these cells with the highest Wnt signaling was observed to show nuclear localization of β-catenin and behaved as CSCs. Denoted as the "β-catenin paradox"[61], the existence of intra-tumoral heterogeneity of Wnt signaling indicated that the pathogenesis of CSCs in CRC required contribution from other factors in addition to the loss of *APC*, such as *KRAS* mutations[62], PI3K[63], Notch[64] and Hedgehog signaling[65]. Moreover, mutations in essential Wnt pathway components, including the R-spondin/Lgr5/RNF43 module have been identified in almost $1/5^{th}$ of CRC cases, which commonly co-occur with *APC* inactivation/deletion[66].

Importantly, $Lgr5^+$ cells have been found to propagate CSCs within colon adenoma, and subsequently, Lgr5 has been identified as an important CSC marker[67]. More recently, studies have pointed out that while $Lgr5^-$ cells can revert into an $Lgr5^+$ cell phenotype, allowing the development, maintenance, and metastasis of the growing tumor[68]; inhibition of Lgr5 strongly suppressed the growth of patient-derived tumor organoids[69]. These findings suggest that $Lgr5^+$ CSCs are detrimental for the growth and propagation of CRC. Furthermore, Myant *et al*[70] show that following *APC* loss, the small GTPase RAC1 helps in the propagation of $Lgr5^+$ CSCs in colon cancer by activating reactive oxygen species production, which activates NFκB signaling that promotes Wnt signaling. Co-activation of NFκB signaling and Wnt signaling has also been shown to promote colorectal tumorigenesis by causing dedifferentiation of intestinal cells into stem cells[50].

Cross-talk has also been observed between the Wnt signaling pathway and critical members of the Notch pathway. Like Wnt, Notch signaling is predominantly higher within the stem cell populations of the crypt and gradually decreases in the differentiated compartment, suggesting that Notch also contributes to ISC maintenance. An early study by the Clevers group[71] on Apc^{Min} mice carrying a heterozygous mutation for *APC* that causes multiple intestinal neoplasia, identified a collaboration between active Notch signaling and Wnt pathway that is indispensable to maintain the proliferative adenoma cells. Moreover, suppression of Notch signals by deletion or by inhibition with a γ-secretase inhibitor resulted in an increase in the levels Math1, a basic helix-loop-helix transcriptional activator of cell differentiation in the intestine[72], consequently causing the arrest of cell proliferation within the crypt and the conversion of the crypt cells into differentiated secretory goblet cells[71]. It has been indicated, however, that goblet cells are commonly absent in CRC and show

downregulated expression of Hath1, the human orthologue of Math1, suggesting an active Notch signaling in most CRC cases[73].

In contrast to the Wnt and the Notch pathways, the Hedgehog and BMP pathways are primarily active within the differentiated cells of the crypt. Although Hedgehog genes are commonly upregulated in CRC[74,75], numerous studies indicate that Gli-dependent canonical Hedgehog pathway antagonizes Wnt signaling promoting tumor cell differentiation[65,76,77]. This makes it difficult to treat CRC with drugs targeting members of the Hedgehog signaling since this strategy seemed to promote Wnt-based proliferation of CSCs[78,79]. However, recently, Regan *et al*[80] clarified that while the Gli-dependent Hedgehog signaling downregulates Wnt signaling, the non-canonical PTCH1-dependent Hedgehog signaling promotes Wnt signaling to allow the maintenance of CSCs in CRC. This breakthrough lets physicians target the two pathways (Hedgehog and Wnt) independently, allowing improved management of the disease.

Like Hedgehog, the transforming growth factor (TGF)-β/BMP pathway has been found to have diverse associations with the Wnt signaling network: From inhibition - by promoting cell differentiation and apoptosis[81], and to collaboration - by causing CRC tumorigenesis[82]. More recently, it was demonstrated that BMP signaling inhibits Lgr5 stem cell signature through a Wnt signaling independent mechanism by SMAD1/SMAD4 recruitment of histone deacetylase that blocks transcription of key factors essential to maintain the stemness of CSCs[83]. Indeed, germline mutations within the BMP receptor type I or its downstream effector SMAD4 have been shown to have a high risk of CRC[84,85]. Additionally, Whissell *et al*[86] reported a key role for the zinc-finger transcription factor GATA6, which was found to help maintain the $Lgr5^+$ CSCs in adenoma, simultaneously suppressing BMP signaling by blocking the binding of β-catenin/TCF4 transcriptional complex to a regulatory region of the BMP4 locus within the differentiated tumor cells. *In vivo* knockdown of GATA6 was found to upregulate BMP signaling, suppressing CRC development.

Put together, these pathways offer a telescopic view of the multiple mechanisms of regulation of stem cells in the origin and development of CRC. Only by improving our understanding of these mechanisms of CSC regulation can we advance the therapeutic strategies required to deal with the progress of the disease.

ROLE OF STEMNESS IN CRC METASTASIS

Since stem cells have been implicated as the primary cell of origin of CRC, it is safe to assume that CSCs originating from ISCs would play a crucial role in the maintenance as well as the spread of the disease to distant sites. In 2010, our laboratory published the earliest account of a subpopulation of $CD26^+$ CSCs from a primary CRC tumor responsible for the development of distant metastasis[87]. An important observation was the discovery of the ability of $CD26^+$ CSCs isolated from the CRC tumor of a patient with liver metastasis to cause the formation of liver metastasis in mice, regardless of their CD133 or CD44 expression status. High expression of CD26 was also found to be associated with advanced tumor staging and poor overall survival of the patients[88]. While CSCs were considered as the key factors responsible for branching and spreading of the primary tumor, it was interesting to note that only small sub-populations of CSCs could initiate metastasis. Indeed, Brabletz *et al*[89] had deduced the existence of two subgroups of colorectal CSCs: The stationary CSCs that remain active within the primary tumor yet cannot disseminate to newer sites and, The mobile CSCs that are derivatives of stationary CSCs, but can form metastatic colonies. Importantly, for a CSC to be considered within the second group, the CSCs must have undergone epithelial-mesenchymal transition (EMT) to disseminate and form metastases while retaining its self-renewal capacity, heterogeneity acquired from the asymmetric division, as well as plasticity to adapt to the newer environment[89,90]. Moreover, a higher number of mobile CSCs at the tumor-host interface associated with the EMT phenotype has been found to correlate with an overall poor disease prognosis[91,92].

While distinct populations of CSCs initiated tumor progression, it was also essential to identify whether the same group of cells colonized target organs at random or through a tight-knit molecular pathway. Since the colorectal CSCs commonly enter the mesenteric circulation, metastasis is more often observed in the liver, followed by the lungs[93]. An elegant study by Gao and colleagues characterized CRC-specific migrating CSCs responsible for organ-specific metastasis[94]. The authors identified a specific group of CSCs expressing CD110, the thrombopoietin receptor that caused liver metastasis; considering that liver is the primary site for thrombopoietin production and hence serves as a chemotactic signal for the $CD110^+$

CSCs. Furthermore, CSCs expressing CUB-domain-containing protein 1 could alone colonize the lungs by homing to the lung endothelial cells[94]. Thus, metastatic colonization not just depends on the existence of specific markers on the initiator cells but requires a specific complement of the target biological organ with prometastatic functions.

Although CSCs were hypothesized as the primary force for CRC progression and metastasis, it was not clear how this trait was orchestrated. What are the genetic or epigenetic or environmental mechanisms forcing the conversion of CSCs from tumor instigators to propagators? A crucial step towards malignancy is the induction of the EMT pathway–A fundamental process of embryonic development as well as cancer metastasis, characterized by the loss of the epithelial morphology and the accompanying markers simultaneously acquiring the mesenchymal phenotype. The process is primarily driven by the activation of a cohort of transcription factors, including snail, zinc-finger E-box binding factor, twist, and several others[95]. Accumulation of key genetic mutations and epigenetic changes in combination with an invasive environmental signal triggers the formation of migratory CSCs that show a high expression of genes critical for EMT as well as for maintaining the CSC phenotype, such as Slug, β-catenin, N-cadherin, as well as Lgr5, CD133, and CD44[90]. Importantly, Brabletz *et al*[89] suggested that microenvironmental alterations have a greater say over genetic factors in inducing EMT, since a reduction of these signals at the target site reverses the EMT pathway, allowing organ colonization. Indeed, the presence of pro-CSC microenvironmental cytokines, including, stromal cell-derived factor 1, osteopontin, and hepatocyte growth factor, promote the activation of PI3K and nuclear accumulation of β-catenin, causing a concomitant increase in the migratory metastatic CSC pool[96,97]. In fact, the pro-CSC cytokines are known to induce CSC plasticity by causing the dedifferentiation of tumor cells into a CSC phenotype that may subsequently adopt the metastatic CSC feature[60,97].

Another study identified the influence of tumor-associated macrophages secreted milk-fat globule-epidermal growth factor-VIII in conferring the CSC with a self-renewal and chemoresistance ability, by activating the Stat3 and Sonic Hedgehog pathways in CSC populations[98]. Contrarily, increase in the levels of the BMPs, a tumor suppressive cytokine, promotes CSC differentiation, inhibits the Wnt pathway by upregulating the phosphatase and tensin homolog and suppressing PI3K[99], and limits the expression of CD44v6; a diagnostic marker of metastatic CSCs[97]. Interestingly, the BMPs belong to the TGF-β superfamily and tend to inhibit tumor progression, while Todaro *et al*[97] suggest that TGF-β may contribute to the metastatic activity of CD44v6+ cells. Indeed, TGF-β serves as a key microenvironmental factor that plays dual roles, serving as a tumor suppressor during the early transformation phase and subsequently playing a pivotal role as an oncogene during the progression phase; a switch catalyzed by the accumulation of key mutations, such as p53[100] and SMAD4[101].

An early study by Calon *et al*[102] showed that the migratory CSCs ready for colonization can instruct the stroma of the host organ, by inducing an increase in the levels of TGF-β either *via* active secretion or, indirectly by the recruitment of macrophages, cancer-associated fibroblasts or platelets that produce TGF-β. Moreover, by activating the Smad proteins, specifically Smad2, Smad3, and Smad1/5/8, TGFβ1 has been shown to induce both EMT as well as stemness in CRC cells, leading to liver metastasis[103]. Additionally, a functional loss of Smad 4, a critical member of the TGF-β/Smad signaling, has been found to correlate with an increase in EMT signaling characterized by the loss of E-cadherin, leading to distant metastasis and overall poor patient prognosis[104-106]. Oncogenic TGF-β has been found to cooperate with a hyperactive Raf/mitogen-activated protein kinase pathway to cause an EMT phenotype[107]. Moreover, a collaboration between the loss of the epithelial E-cadherin protein and an increase in Wnt/β-catenin signaling, with an increased TGF-β release, allows cells to maintain the mesenchymal phenotype in the EMT process[108]. Following dissemination of the CSCs to the target organs, the new microenvironment is generally hostile towards the incoming tumor cells, which may affect the stemness as well as the survival of the cancer cells. CSCs in CRC have been reported to suffer apoptosis almost immediately after reaching the liver[102]. Survival environmental signals in the form of cytokines and growth factors are hence generated by infiltrating cells from the primary tumor, along with the activation of stemness promoting Wnt as well as Notch pathways, to promote the creation of pre-metastatic niches within the target organs and improve the endurance of CRC based CSCs as well as allow colonization. A model of the role of stem cells in the normal colon, CRC development, as well as metastasis is shown in Figure 1.

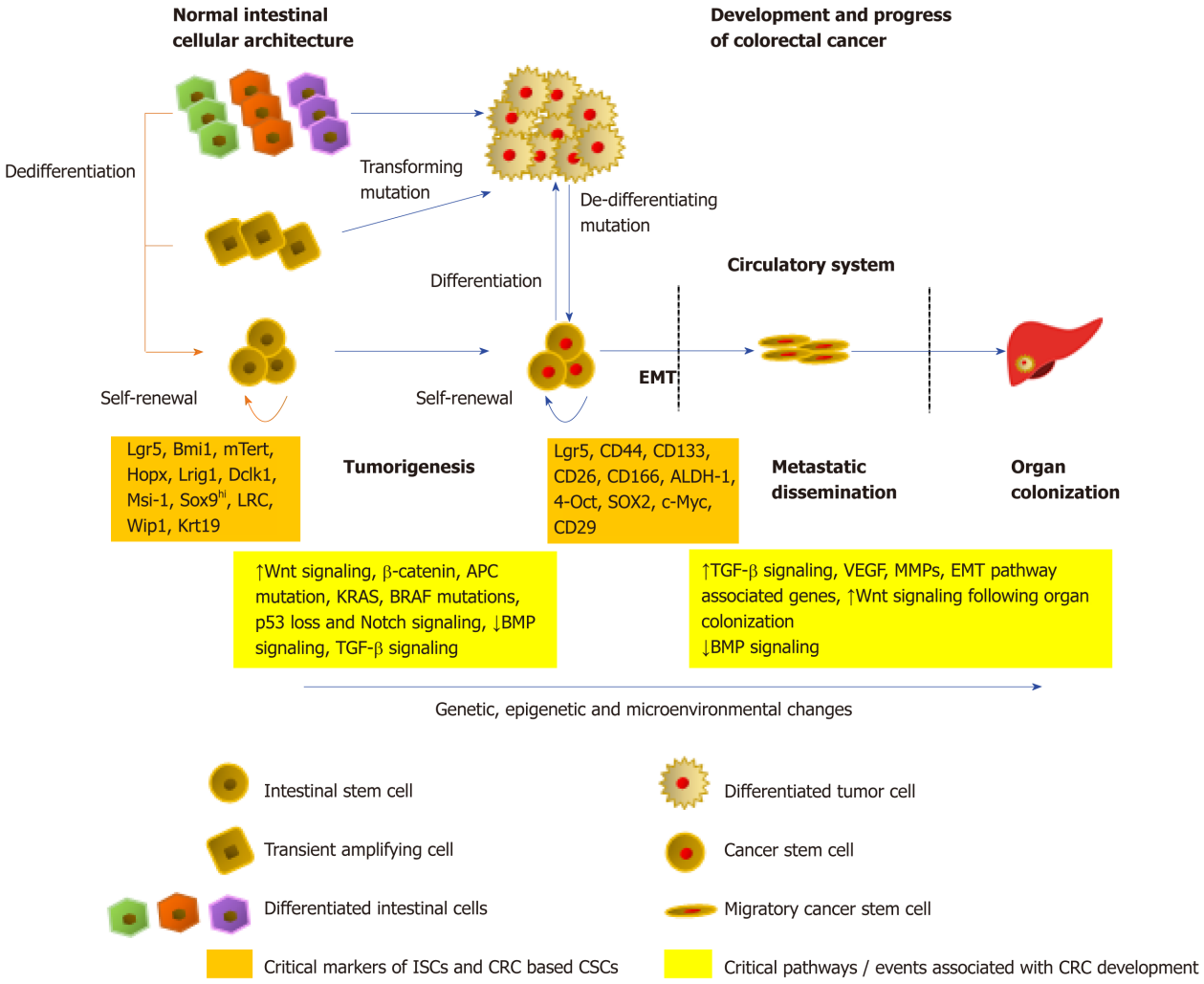

Figure 1 Role of stem cells in normal colon and CRC. Within the normal colon, ISCs constantly divide to give rise to the differentiated cells within the epithelium that have a multitude of functions. In colorectal cancer, the ISCs can develop into CSCs that lead to tumor formation. Moreover, the transient amplifying cells as well as the differentiated intestinal cells can mutate into mature tumor cells that can subsequently dedifferentiate to form CSCs. Owing to genetic, epigenetic or environmental signals, the stationary CSCs can transform into migratory CSCs that pass *via* circulation to target organs and colonize. Critical pathways and genetic events have been shown for the entire process of CRC tumorigenesis and progression. Moreover, key biomarkers have been shown for the ISCs and the CRC based CSCs, although there is some overlap in this panel. CRC: Colorectal cancer; CSC: Cancer stem cells; ISC: Intestinal stem cell; EMT: Epithelial mesenchymal transition; Wnt: Wingless/integrated; APC: Adenomatous polyposis coli; KRAS: Kirsten rat sarcoma viral oncogene homolog; BMP: Bone morphogenetic protein; TGF-β: Transforming growth factor-β; VEGF: Vascular endothelial growth factor; MMP: Matrix metalloproteinase.

THE RELEVANCE OF STEMNESS IN CRC PROGNOSIS AND THERAPY

Several factors affect the outcome in CRC—cancer spread (metastases), chemo-resistance, and recurrence; all of these being mutually exclusive events. Moreover, numerous studies have pointed out a stronger involvement of CSCs, accompanied with our current inefficiency in understanding the biology of these cells in the malignancy, in allowing the factors to become dominant, leading to poor disease prognosis. Traditional therapy tends to debulk the tumor off the mature, differentiated cells, while the CSCs stay quiescent, and hence become resistant to drug or radio-therapy, allowing for improved opportunities to promote recurrence. An early report in this direction identified that the quiescence of CSCs can be attributed to an increased expression of ATP-binding cassette drug transporters, active DNA-repair machinery, as well as an innate resistance towards apoptotic cell death[109]. Furthermore, variants of key CSC associated markers including Lgr5, CD44, and aldehyde dehydrogenase 1A1 have been found to be associated with a shorter time to tumor recurrence in high-risk stage II and stage III CRC patients treated with fluoropyrimidine-based therapy; suggesting the association of the variants with improved survival and chemoresistance abilities of the CSCs[110]. It is important to realize that while traditional adjuvant fluoropyrimidine and/or platinum-based

therapy has been found to be effective in CRC, exposure to these chemical agents may enrich a pool of CSCs responsible for resistance and recurrence. As proof of principle, the treatment of patients with unresectable CRC with mFOLFOX6 therapy was found to increase the levels of several CSC markers in distant metastases[111]. A previous study in this direction made use of cyclophosphamide or Irinotecan-based chemotherapy to treat xenogeneic CRC tumors and subsequently found an enrichment of a group of drug-resistant CSC populations with elevated levels of ALDH1 that could regenerate tumors[112].

While more aggressive CSCs tend to emerge in tumor development, it is the feature of plasticity that allows CSCs to pioneer resistance to therapy as well as recurrence. A study by Kobayashi *et al*[113] identified the interconversion of CSCs from a proliferative $Lgr5^+$ state to a quiescent, drug-resistant $Lgr5^-$ state in the presence of an anticancer drug. Following reseeding and drug removal, the $Lgr5^-$ cells transitioned back to the $Lgr5^+$ state, while maintaining the *in vivo* tumor-initiating properties all the time in both states. It is hence essential to identify and target key molecular factors that are common to multiple states of CSCs to achieve a better therapeutic cleanup in CRC. Indeed, by gene profiling studies the authors demonstrate that an epidermal growth factor receptor ligand, epiregulin, is expressed by both the $Lgr5^+$ and $Lgr5^-$ states that could be targeted using an anti-epiregulin antibody[113]. Several reports have also indicated that the resistance and recurrence abilities of CSCs are strongly influenced by the tumor microenvironment as well as key signaling pathways and epigenetic modifications. Numerous cytokines and chemokines secreted by cancer-associated fibroblasts, particularly the MET receptor ligand hepatocyte growth factor, were found to promote CSC proliferation while making them resistant to apoptosis in response to epidermal growth factor receptor therapy[114]. Overactivation of Wnt signaling, a critical signaling network in the growth and development of stem cells, has been observed in 5-fluorouracil resistant CRC, while downregulation of Wnt transcription factor T cell factor 4 increases the sensitivity of the tumor to radiation therapy[115].

Recently, the role of microRNAs has also been identified as potent modulators of stem cell signaling within CRC. Notable ones include miR-15a and miR-16-1, which are frequently deleted in CRC cell lines as well as clinical specimens, are found to be associated with a greater number of B cells positive for immunoglobulin A (IgA^+ B cells) and shorter survival periods[116]. At the molecular level, deletion/inhibition of miR-15a/miR-16-1 results in the upregulation of AP4, a c-Myc target[117], through a double negative feedback loop, resulting in distant metastases and poor survival[118]. MiR-15a has also been found to impact several other key genes implicated in the origin, maintenance, as well as chemoresistance of CSCs in CRC, including YAP1, doublecortin-like kinase 1, BMI1, and BCL2[119]. Similarly, the expression of miR-16-1 is negatively correlated with cyclooxygenase-2 level[120] which is also a downstream effector of the Wnt signaling pathway and thus has an active role in regulating the stem cell biology in CRC. Altogether, the miR-15a/miR-16-1 complex serves as a valuable therapeutic target to specifically tackle pathways associated with CSC maintenance in CRC.

Successful CRC targeting requires the inhibition of key pathways and environmental signals that function to promote the self-renewal ability, apoptotic resistance, stemness, as well as prolonged survival of the CSCs. Several potential CSC targeting drugs have been identified in the past several years, a few of which are under trial as well. Studies by Todaro *et al*[121,122] demonstrated a mechanism of apoptosis evasion by $CD133^+$ CSCs by expressing IL-4, which could be neutralized by the treatment of the cells with an anti-IL4 antibody, IL-4DM. Moreover, silencing of the Aurora-A kinase, a critical regulator of mitosis, has been found to affect the colorectal CSCs by inhibiting proliferation, promoting the apoptotic potential, and sensitizing the cells to chemotherapy[123]. The role of mitochondrial targeting molecules as potential therapeutic agents has also been identified. A remarkable study by Colak *et al*[124] identified a role of BCLXL in protecting colon CSCs from chemotherapy, determined by decreased mitochondrial priming. By making use of BH3 mimetics, the authors successfully inhibit the BCL2 family members, sensitizing the CSCs to chemotherapy. Additionally, several molecules targeting critical members of Notch signaling[125,126] as well as the Wnt pathway[127] have been identified.

Although there are many more therapeutic targets as well as potential drugs under pre-clinical/clinical trials, understanding the clinical phenotype of the patient is critical to the usage of these drugs. Recent studies focusing on the development of CSC-targeting drugs advise upon the combined use of these drugs with the conventional adjuvant therapy to maximize the potential (Figure 2). The efficiency of CSC-targeting drugs is particularly higher on circulating CSCs due to the absence of a safe microenvironment as well as the presence of a toxic adjuvant therapy[5]. In advanced CRC, debulking of the tumor would not directly correspond to a similar

loss of volume of the associated CSCs. In addition, in aggressive tumors, combination therapies increase the stress on tumor microenvironment, which has been known to contribute to an increase in the CSC pool. The situation is made further complex by the ability of CSCs and differentiated cells to interconvert. Strategies for monitoring the efficacy of CSC-targeting are still at infancy. Though several CSC markers have been identified in CRC, most of them are also expressed by ISCs. The success of CSC-targeting drugs hence strongly depends on the improvement of CSC monitoring techniques. Additionally, studying of patient-derived models of CRC is essential to increase our knowledge of the roles of CSCs and help piece the missing gaps within this field.

CONCLUSION

The landscape of CRC has progressed from a simple hierarchical model to a complex setup interspersed with multiple roles of dynamic CSCs that are modulated constantly by genetic, epigenetic, and specifically, microenvironmental factors. Although the discovery of CSCs in CRC was made roughly a decade ago, our understanding of the biology of these cells is still quite limited[34]. With the progress of technology, our existing knowledge of the complex roles and the dynamic nature of colonic stem cells, as well as CSCs, is undergoing constant evolution. However, better techniques for detection and isolation as well as the usage of patient-derived CRC models is essential to further our understanding of CSCs in CRC.

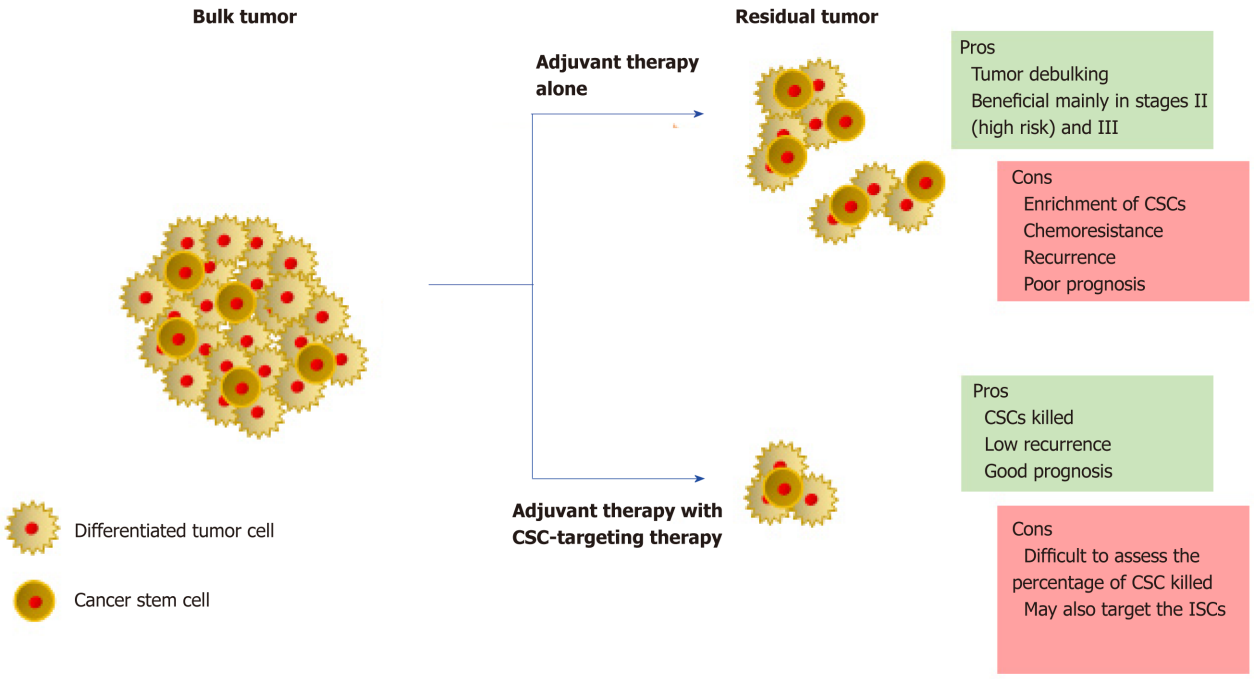

Figure 2 Therapeutic strategies in colorectal cancer. Targeting the tumor bulk in colorectal cancer with adjuvant therapy leads to tumor regression, although CSCs may escape cell death. Such a chemoresistance event leads to tumor recurrence and overall poor prognosis. In contrast, treatment with a combination of adjuvant therapy and CSC-targeting therapy would also eradicate the CSC population, along with the tumor bulk. In the long term, reduction in CSC volume would kill the tumor. CSC: Cancer stem cells; ISC: Intestinal stem cells.

REFERENCES

1 **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767 [PMID: 2188735 DOI: 10.1016/0092-8674(90)90186-I]
2 **De Sousa E Melo F**, Vermeulen L, Fessler E, Medema JP. Cancer heterogeneity--a multifaceted view. *EMBO Rep* 2013; **14**: 686-695 [PMID: 23846313 DOI: 10.1038/embor.2013.92]
3 **Wersto RP**, Liblit RL, Deitch D, Koss LG. Variability in DNA measurements in multiple tumor samples of human colonic carcinoma. *Cancer* 1991; **67**: 106-115 [PMID: 1985705 DOI: 10.1002/1097-0142(19910101)67:1<106::AID-CNCR2820670120>3.0.CO;2-I]
4 **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]
5 **Zeuner A**, Todaro M, Stassi G, De Maria R. Colorectal cancer stem cells: From the crypt to the clinic. *Cell Stem Cell* 2014; **15**: 692-705 [PMID: 25479747 DOI: 10.1016/j.stem.2014.11.012]
6 **Beck B**, Blanpain C. Unravelling cancer stem cell potential. *Nat Rev Cancer* 2013; **13**: 727-738 [PMID: 24060864 DOI: 10.1038/nrc3597]
7 **Barker N**, van Oudenaarden A, Clevers H. Identifying the stem cell of the intestinal crypt: Strategies and pitfalls. *Cell Stem Cell* 2012; **11**: 452-460 [PMID: 23040474 DOI: 10.1016/j.stem.2012.09.009]
8 **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]
9 **Takeda N**, Jain R, LeBoeuf MR, Wang Q, Lu MM, Epstein JA. Interconversion between intestinal stem cell populations in distinct niches. *Science* 2011; **334**: 1420-1424 [PMID: 22075725 DOI: 10.1126/science.1213214]
10 **Montgomery RK**, Carlone DL, Richmond CA, Farilla L, Kranendonk ME, Henderson DE, Baffour-Awuah NY, Ambruzs DM, Fogli LK, Algra S, Breault DT. Mouse telomerase reverse transcriptase (mTert) expression marks slowly cycling intestinal stem cells. *Proc Natl Acad Sci U S A* 2011; **108**: 179-184 [PMID: 21173232 DOI: 10.1073/pnas.1013004108]
11 **Powell AE**, Wang Y, Li Y, Poulin EJ, Means AL, Washington MK, Higginbotham JN, Juchheim A, Prasad N, Levy SE, Guo Y, Shyr Y, Aronow BJ, Haigis KM, Franklin JL, Coffey RJ. The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. *Cell* 2012; **149**: 146-158 [PMID: 22464327 DOI: 10.1016/j.cell.2012.02.042]
12 **Wong VW**, Stange DE, Page ME, Buczacki S, Wabik A, Itami S, van de Wetering M, Poulsom R, Wright NA, Trotter MW, Watt FM, Winton DJ, Clevers H, Jensen KB. Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. *Nat Cell Biol* 2012; **14**: 401-408 [PMID: 22388892 DOI: 10.1038/ncb2464]
13 **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 DOI: 10.1038/nature06196]
14 **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]
15 **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 U S A* 2012; **109**: 466-471 [PMID: 22190486 DOI:

10.1073/pnas.1118857109]
16 **Tian H**, Biehs B, Warming S, Leong KG, Rangell L, Klein OD, de Sauvage FJ. A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* 2011; **478**: 255-259 [PMID: 21927002 DOI: 10.1038/nature10408]
17 **Muñoz J**, Stange DE, Schepers AG, van de Wetering M, Koo BK, Itzkovitz S, Volckmann R, Kung KS, Koster J, Radulescu S, Myant K, Versteeg R, Sansom OJ, van Es JH, Barker N, van Oudenaarden A, Mohammed S, Heck AJ, Clevers H. The Lgr5 intestinal stem cell signature: Robust expression of proposed quiescent '+4' cell markers. *EMBO J* 2012; **31**: 3079-3091 [PMID: 22692129 DOI: 10.1038/emboj.2012.166]
18 **van Es JH**, Sato T, van de Wetering M, Lyubimova A, Yee Nee AN, Gregorieff A, Sasaki N, Zeinstra L, van den Born M, Korving J, Martens ACM, Barker N, van Oudenaarden A, Clevers H. Dll1+ secretory progenitor cells revert to stem cells upon crypt damage. *Nat Cell Biol* 2012; **14**: 1099-1104 [PMID: 23000963 DOI: 10.1038/ncb2581]
19 **Barriga FM**, Montagni E, Mana M, Mendez-Lago M, Hernando-Momblona X, Sevillano M, Guillaumet-Adkins A, Rodriguez-Esteban G, Buczacki SJA, Gut M, Heyn H, Winton DJ, Yilmaz OH, Attolini CS, Gut I, Batlle E. Mex3a Marks a Slowly Dividing Subpopulation of Lgr5+ Intestinal Stem Cells. *Cell Stem Cell* 2017; **20**: 801-816.e7 [PMID: 28285904 DOI: 10.1016/j.stem.2017.02.007]
20 **Jung P**, Sato T, Merlos-Suárez A, Barriga FM, Iglesias M, Rossell D, Auer H, Gallardo M, Blasco MA, Sancho E, Clevers H, Batlle E. Isolation and in vitro expansion of human colonic stem cells. *Nat Med* 2011; **17**: 1225-1227 [PMID: 21892181 DOI: 10.1038/nm.2470]
21 **van der Flier LG**, van Gijn ME, Hatzis P, Kujala P, Haegebarth A, Stange DE, Begthel H, van den Born M, Guryev V, Oving I, van Es JH, Barker N, Peters PJ, van de Wetering M, Clevers H. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell* 2009; **136**: 903-912 [PMID: 19269367 DOI: 10.1016/j.cell.2009.01.031]
22 **van der Flier LG**, Haegebarth A, Stange DE, van de Wetering M, Clevers H. OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology* 2009; **137**: 15-17 [PMID: 19450592 DOI: 10.1053/j.gastro.2009.05.035]
23 **Chandrakesan P**, May R, Qu D, Weygant N, Taylor VE, Li JD, Ali N, Sureban SM, Qante M, Wang TC, Bronze MS, Houchen CW. Dclk1+ small intestinal epithelial tuft cells display the hallmarks of quiescence and self-renewal. *Oncotarget* 2015; **6**: 30876-30886 [PMID: 26362399 DOI: 10.18632/oncotarget.5129]
24 **Sarkar S**, Swiercz R, Kantara C, Hajjar KA, Singh P. Annexin A2 mediates up-regulation of NF-κB, β-catenin, and stem cell in response to progastrin in mice and HEK-293 cells. *Gastroenterology* 2011; **140**: 583-595.e4 [PMID: 20826156 DOI: 10.1053/j.gastro.2010.08.054]
25 **Degirmenci B**, Valenta T, Dimitrieva S, Hausmann G, Basler K. GLI1-expressing mesenchymal cells form the essential Wnt-secreting niche for colon stem cells. *Nature* 2018; **558**: 449-453 [PMID: 29875413 DOI: 10.1038/s41586-018-0190-3]
26 **Kaiko GE**, Ryu SH, Koues OI, Collins PL, Solnica-Krezel L, Pearce EJ, Pearce EL, Oltz EM, Stappenbeck TS. The Colonic Crypt Protects Stem Cells from Microbiota-Derived Metabolites. *Cell* 2016; **165**: 1708-1720 [PMID: 27264604 DOI: 10.1016/j.cell.2016.05.018]
27 **Shih IM**, Wang TL, Traverso G, Romans K, Hamilton SR, Ben-Sasson S, Kinzler KW, Vogelstein B. Top-down morphogenesis of colorectal tumors. *Proc Natl Acad Sci U S A* 2001; **98**: 2640-2645 [PMID: 11226292 DOI: 10.1073/pnas.051629398]
28 **Preston SL**, Wong WM, Chan AO, Poulsom R, Jeffery R, Goodlad RA, Mandir N, Elia G, Novelli M, Bodmer WF, Tomlinson IP, Wright NA. Bottom-up histogenesis of colorectal adenomas: Origin in the monocryptal adenoma and initial expansion by crypt fission. *Cancer Res* 2003; **63**: 3819-3825 [PMID: 12839979 DOI: 10.1097/00130404-200307000-00015]
29 **Wasan HS**, Park HS, Liu KC, Mandir NK, Winnett A, Sasieni P, Bodmer WF, Goodlad RA, Wright NA. APC in the regulation of intestinal crypt fission. *J Pathol* 1998; **185**: 246-255 [PMID: 9771477 DOI: 10.1002/(SICI)1096-9896(199807)185:3<246::AID-PATH90>3.0.CO;2-8]
30 **Chang WW**, Whitener CJ. Histogenesis of tubular adenomas in hereditary colonic adenomatous polyposis. *Arch Pathol Lab Med* 1989; **113**: 1042-1049 [PMID: 2774856 DOI: 10.1016/0264-410X(89)90186-2]
31 **Wong WM**, Mandir N, Goodlad RA, Wong BC, Garcia SB, Lam SK, Wright NA. Histogenesis of human colorectal adenomas and hyperplastic polyps: The role of cell proliferation and crypt fission. *Gut* 2002; **50**: 212-217 [PMID: 11788562 DOI: 10.1136/gut.50.2.212]
32 **Crabtree MD**, Tomlinson IP, Talbot IC, Phillips RK. Variability in the severity of colonic disease in familial adenomatous polyposis results from differences in tumour initiation rather than progression and depends relatively little on patient age. *Gut* 2001; **49**: 540-543 [PMID: 11559652 DOI: 10.1136/gut.49.4.540]
33 **Crabtree MD**, Tomlinson IP, Hodgson SV, Neale K, Phillips RK, Houlston RS. Explaining variation in familial adenomatous polyposis: Relationship between genotype and phenotype and evidence for modifier genes. *Gut* 2002; **51**: 420-423 [PMID: 12171967 DOI: 10.1136/gut.51.3.420]
34 **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]
35 **Barker N**, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M, Danenberg E, Clarke AR, Sansom OJ, Clevers H. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* 2009; **457**: 608-611 [PMID: 19092804 DOI: 10.1038/nature07602]
36 **Powell AE**, Vlacich G, Zhao ZY, McKinley ET, Washington MK, Manning HC, Coffey RJ. Inducible loss of one Apc allele in Lrig1-expressing progenitor cells results in multiple distal colonic tumors with features of familial adenomatous polyposis. *Am J Physiol Gastrointest Liver Physiol* 2014; **307**: G16-G23 [PMID: 24833705 DOI: 10.1152/ajpgi.00358.2013]
37 **Zhu L**, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, Poppleton H, Zakharenko S, Ellison DW, Gilbertson RJ. Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. *Nature* 2009; **457**: 603-607 [PMID: 19092805 DOI: 10.1038/nature07589]
38 **Pardal R**, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer* 2003; **3**: 895-902 [PMID: 14737120 DOI: 10.1038/nrc1232]
39 **Jordan CT**. Cancer stem cell biology: From leukemia to solid tumors. *Curr Opin Cell Biol* 2004; **16**: 708-712 [PMID: 15530785 DOI: 10.1016/j.ceb.2004.09.002]
40 **Su LK**, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* 1992; **256**: 668-

670 [PMID: 1350108 DOI: 10.1126/science.1350108]
41 **Taketo MM**, Edelmann W. Mouse models of colon cancer. *Gastroenterology* 2009; **136**: 780-798 [PMID: 19263594 DOI: 10.1053/j.gastro.2008.12.049]
42 **Itzkowitz SH**, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: The role of inflammation. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G7-17 [PMID: 15194558 DOI: 10.1152/ajpgi.00079.2004]
43 **Huels DJ**, Sansom OJ. Stem vs non-stem cell origin of colorectal cancer. *Br J Cancer* 2015; **113**: 1-5 [PMID: 26110974 DOI: 10.1038/bjc.2015.214]
44 **Lopez-Garcia C**, Klein AM, Simons BD, Winton DJ. Intestinal stem cell replacement follows a pattern of neutral drift. *Science* 2010; **330**: 822-825 [PMID: 20929733 DOI: 10.1126/science.1196236]
45 **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]
46 **Vermeulen L**, Snippert HJ. Stem cell dynamics in homeostasis and cancer of the intestine. *Nat Rev Cancer* 2014; **14**: 468-480 [PMID: 24920463 DOI: 10.1038/nrc3744]
47 **Snippert HJ**, Schepers AG, van Es JH, Simons BD, Clevers H. Biased competition between Lgr5 intestinal stem cells driven by oncogenic mutation induces clonal expansion. *EMBO Rep* 2014; **15**: 62-69 [PMID: 24355609 DOI: 10.1002/embr.201337799]
48 **Beerenwinkel N**, Antal T, Dingli D, Traulsen A, Kinzler KW, Velculescu VE, Vogelstein B, Nowak MA. Genetic progression and the waiting time to cancer. *PLoS Comput Biol* 2007; **3**: e225 [PMID: 17997597 DOI: 10.1371/journal.pcbi.0030225]
49 **Westphalen CB**, Asfaha S, Hayakawa Y, Takemoto Y, Lukin DJ, Nuber AH, Brandtner A, Setlik W, Remotti H, Muley A, Chen X, May R, Houchen CW, Fox JG, Gershon MD, Quante M, Wang TC. Long-lived intestinal tuft cells serve as colon cancer-initiating cells. *J Clin Invest* 2014; **124**: 1283-1295 [PMID: 24487592 DOI: 10.1172/JCI73434]
50 **Schwitalla S**, Fingerle AA, Cammareri P, Nebelsiek T, Göktuna SI, Ziegler PK, Canli O, Heijmans J, Huels DJ, Moreaux G, Rupec RA, Gerhard M, Schmid R, Barker N, Clevers H, Lang R, Neumann J, Kirchner T, Taketo MM, van den Brink GR, Sansom OJ, Arkan MC, Greten FR. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* 2013; **152**: 25-38 [PMID: 23273993 DOI: 10.1016/j.cell.2012.12.012]
51 **Clevers H**. Wnt/beta-catenin signaling in development and disease. *Cell* 2006; **127**: 469-480 [PMID: 17081971 DOI: 10.1016/j.cell.2006.10.018]
52 **He TC**, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. *Science* 1998; **281**: 1509-1512 [PMID: 9727977 DOI: 10.1126/science.281.5382.1509]
53 **Tan SH**, Barker N. Stemming Colorectal Cancer Growth and Metastasis: HOXA5 Forces Cancer Stem Cells to Differentiate. *Cancer Cell* 2015; **28**: 683-685 [PMID: 26678334 DOI: 10.1016/j.ccell.2015.11.004]
54 **Jubb AM**, Chalasani S, Frantz GD, Smits R, Grabsch HI, Kavi V, Maughan NJ, Hillan KJ, Quirke P, Koeppen H. Achaete-scute like 2 (ascl2) is a target of Wnt signalling and is upregulated in intestinal neoplasia. *Oncogene* 2006; **25**: 3445-3457 [PMID: 16568095 DOI: 10.1038/sj.onc.1209382]
55 **Lustig B**, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Clevers H, Schlag PM, Birchmeier W, Behrens J. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Mol Cell Biol* 2002; **22**: 1184-1193 [PMID: 11809809 DOI: 10.1128/MCB.22.4.1184-1193.2002]
56 **de Sousa EM**, Vermeulen L, Richel D, Medema JP. Targeting Wnt signaling in colon cancer stem cells. *Clin Cancer Res* 2011; **17**: 647-653 [PMID: 21159886 DOI: 10.1158/1078-0432.CCR-10-1204]
57 **Gregorieff A**, Pinto D, Begthel H, Destrée O, Kielman M, Clevers H. Expression pattern of Wnt signaling components in the adult intestine. *Gastroenterology* 2005; **129**: 626-638 [PMID: 16083717 DOI: 10.1016/j.gastro.2005.06.007]
58 **Kinzler KW**, Nilbert MC, Su LK, Vogelstein B, Bryan TM, Levy DB, Smith KJ, Preisinger AC, Hedge P, McKechnie D. Identification of FAP locus genes from chromosome 5q21. *Science* 1991; **253**: 661-665 [PMID: 1651562 DOI: 10.1126/science.1651562]
59 **Kinzler KW**, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159-170 [PMID: 8861899 DOI: 10.1016/S0092-8674(00)81333-1]
60 **Vermeulen L**, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman 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]
61 **Fodde R**, Brabletz T. Wnt/beta-catenin signaling in cancer stemness and malignant behavior. *Curr Opin Cell Biol* 2007; **19**: 150-158 [PMID: 17306971 DOI: 10.1016/j.ceb.2007.02.007]
62 **Janssen KP**, Alberici P, Fsihi H, Gaspar C, Breukel C, Franken P, Rosty C, Abal M, El Marjou F, Smits R, Louvard D, Fodde R, Robine S. APC and oncogenic KRAS are synergistic in enhancing Wnt signaling in intestinal tumor formation and progression. *Gastroenterology* 2006; **131**: 1096-1109 [PMID: 17030180 DOI: 10.1053/j.gastro.2006.08.011]
63 **Vivanco I**, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002; **2**: 489-501 [PMID: 12094235 DOI: 10.1038/nrc839]
64 **Peignon G**, Durand A, Cacheux W, Ayrault O, Terris B, Laurent-Puig P, Shroyer NF, Van Seuningen I, Honjo T, Perret C, Romagnolo B. Complex interplay between β-catenin signalling and Notch effectors in intestinal tumorigenesis. *Gut* 2011; **60**: 166-176 [PMID: 21205878 DOI: 10.1136/gut.2009.204719]
65 **van den Brink GR**, Bleuming SA, Hardwick JC, Schepman BL, Offerhaus GJ, Keller JJ, Nielsen C, Gaffield W, van Deventer SJ, Roberts DJ, Peppelenbosch MP. Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. *Nat Genet* 2004; **36**: 277-282 [PMID: 14770182 DOI: 10.1038/ng1304]
66 **Giannakis M**, Hodis E, Jasmine Mu X, Yamauchi M, Rosenbluh J, Cibulskis K, Saksena G, Lawrence MS, Qian ZR, Nishihara R, Van Allen EM, Hahn WC, Gabriel SB, Lander ES, Getz G, Ogino S, Fuchs CS, Garraway LA. RNF43 is frequently mutated in colorectal and endometrial cancers. *Nat Genet* 2014; **46**: 1264-1266 [PMID: 25344691 DOI: 10.1038/ng.3127]
67 **Schepers AG**, Snippert HJ, Stange DE, van den Born M, van Es JH, van de Wetering M, Clevers H. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science* 2012; **337**: 730-735

[PMID: 22855427 DOI: 10.1126/science.1224676]
68 **de Sousa e Melo F**, Kurtova AV, Harnoss JM, Kljavin N, Hoeck JD, Hung J, Anderson JE, Storm EE, Modrusan Z, Koeppen H, Dijkgraaf GJ, Piskol R, de Sauvage FJ. A distinct role for Lgr5⁺ stem cells in primary and metastatic colon cancer. *Nature* 2017; **543**: 676-680 [PMID: 28358093 DOI: 10.1038/nature21713]
69 **Shimokawa M**, Ohta Y, Nishikori S, Matano M, Takano A, Fujii M, Date S, Sugimoto S, Kanai T, Sato T. Visualization and targeting of LGR5⁺ human colon cancer stem cells. *Nature* 2017; **545**: 187-192 [PMID: 28355176 DOI: 10.1038/nature22081]
70 **Myant KB**, Cammareri P, McGhee EJ, Ridgway RA, Huels DJ, Cordero JB, Schwitalla S, Kalna G, Ogg EL, Athineos D, Timpson P, Vidal M, Murray GI, Greten FR, Anderson KI, Sansom OJ. ROS production and NF-κB activation triggered by RAC1 facilitate WNT-driven intestinal stem cell proliferation and colorectal cancer initiation. *Cell Stem Cell* 2013; **12**: 761-773 [PMID: 23665120 DOI: 10.1016/j.stem.2013.04.006]
71 **van Es JH**, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* 2005; **435**: 959-963 [PMID: 15959515 DOI: 10.1038/nature03659]
72 **Yang Q**, Bermingham NA, Finegold MJ, Zoghbi HY. Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science* 2001; **294**: 2155-2158 [PMID: 11739954 DOI: 10.1126/science.1065718]
73 **Leow CC**, Romero MS, Ross S, Polakis P, Gao WQ. Hath1, down-regulated in colon adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells. *Cancer Res* 2004; **64**: 6050-6057 [PMID: 15342386 DOI: 10.1158/0008-5472.CAN-04-0290]
74 **Yoshikawa K**, Shimada M, Miyamoto H, Higashijima J, Miyatani T, Nishioka M, Kurita N, Iwata T, Uehara H. Sonic hedgehog relates to colorectal carcinogenesis. *J Gastroenterol* 2009; **44**: 1113-1117 [PMID: 19662327 DOI: 10.1007/s00535-009-0110-2]
75 **Oniscu A**, James RM, Morris RG, Bader S, Malcomson RD, Harrison DJ. Expression of Sonic hedgehog pathway genes is altered in colonic neoplasia. *J Pathol* 2004; **203**: 909-917 [PMID: 15258993 DOI: 10.1002/path.1591]
76 **Akiyoshi T**, Nakamura M, Koga K, Nakashima H, Yao T, Tsuneyoshi M, Tanaka M, Katano M. Gli1, downregulated in colorectal cancers, inhibits proliferation of colon cancer cells involving Wnt signalling activation. *Gut* 2006; **55**: 991-999 [PMID: 16299030 DOI: 10.1136/gut.2005.080333]
77 **van Dop WA**, Uhmann A, Wijgerde M, Sleddens-Linkels E, Heijmans J, Offerhaus GJ, van den Bergh Weerman MA, Boeckxstaens GE, Hommes DW, Hardwick JC, Hahn H, van den Brink GR. Depletion of the colonic epithelial precursor cell compartment upon conditional activation of the hedgehog pathway. *Gastroenterology* 2009; **136**: 2195-2203.e1-7 [PMID: 19272384 DOI: 10.1053/j.gastro.2009.02.068]
78 **Berlin J**, Bendell JC, Hart LL, Firdaus I, Gore I, Hermann RC, Mulcahy MF, Zalupski MM, Mackey HM, Yauch RL, Graham RA, Bray GL, Low JA. A randomized phase II trial of vismodegib versus placebo with FOLFOX or FOLFIRI and bevacizumab in patients with previously untreated metastatic colorectal cancer. *Clin Cancer Res* 2013; **19**: 258-267 [PMID: 23082002 DOI: 10.1158/1078-0432.CCR-12-1800]
79 **Catenacci DV**, Junttila MR, Karrison T, Bahary N, Horiba MN, Nattam SR, Marsh R, Wallace J, Kozloff M, Rajdev L, Cohen D, Wade J, Sleckman B, Lenz HJ, Stiff P, Kumar P, Xu P, Henderson L, Takebe N, Salgia R, Wang X, Stadler WM, de Sauvage FJ, Kindler HL. Randomized Phase Ib/II Study of Gemcitabine Plus Placebo or Vismodegib, a Hedgehog Pathway Inhibitor, in Patients With Metastatic Pancreatic Cancer. *J Clin Oncol* 2015; **33**: 4284-4292 [PMID: 26527777 DOI: 10.1200/JCO.2015.62.8719]
80 **Regan JL**, Schumacher D, Staudte S, Steffen A, Haybaeck J, Keilholz U, Schweiger C, Golob-Schwarzl N, Mumberg D, Henderson D, Lehrach H, Regenbrecht CRA, Schäfer R, Lange M. Non-Canonical Hedgehog Signaling Is a Positive Regulator of the WNT Pathway and Is Required for the Survival of Colon Cancer Stem Cells. *Cell Rep* 2017; **21**: 2813-2828 [PMID: 29212028 DOI: 10.1016/j.celrep.2017.11.025]
81 **He XC**, Zhang J, Tong WG, Tawfik O, Ross J, Scoville DH, Tian Q, Zeng X, He X, Wiedemann LM, Mishina Y, Li L. BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat Genet* 2004; **36**: 1117-1121 [PMID: 15378062 DOI: 10.1038/ng1430]
82 **Takaku K**, Oshima M, Miyoshi H, Matsui M, Seldin MF, Taketo MM. Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell* 1998; **92**: 645-656 [PMID: 9506519 DOI: 10.1016/S0092-8674(00)81132-0]
83 **Qi Z**, Li Y, Zhao B, Xu C, Liu Y, Li H, Zhang B, Wang X, Yang X, Xie W, Li B, Han JJ, Chen YG. BMP restricts stemness of intestinal Lgr5⁺ stem cells by directly suppressing their signature genes. *Nat Commun* 2017; **8**: 13824 [PMID: 28059064 DOI: 10.1038/ncomms13824]
84 **Howe JR**, Bair JL, Sayed MG, Anderson ME, Mitros FA, Petersen GM, Velculescu VE, Traverso G, Vogelstein B. Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. *Nat Genet* 2001; **28**: 184-187 [PMID: 11381269 DOI: 10.1038/88919]
85 **Howe JR**, Roth S, Ringold JC, Summers RW, Järvinen HJ, Sistonen P, Tomlinson IP, Houlston RS, Bevan S, Mitros FA, Stone EM, Aaltonen LA. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* 1998; **280**: 1086-1088 [PMID: 9582123 DOI: 10.1126/science.280.5366.1086]
86 **Whissell G**, Montagni E, Martinelli P, Hernando-Momblona X, Sevillano M, Jung P, Cortina C, Calon A, Abuli A, Castells A, Castellvi-Bel S, Nacht AS, Sancho E, Stephan-Otto Attolini C, Vicent GP, Real FX, Batlle E. The transcription factor GATA6 enables self-renewal of colon adenoma stem cells by repressing BMP gene expression. *Nat Cell Biol* 2014; **16**: 695-707 [PMID: 24952462 DOI: 10.1038/ncb2992]
87 **Pang R**, Law WL, Chu AC, Poon JT, Lam CS, Chow AK, Ng L, Cheung LW, Lan XR, Lan HY, Tan VP, Yau TC, Poon RT, Wong BC. A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. *Cell Stem Cell* 2010; **6**: 603-615 [PMID: 20569697 DOI: 10.1016/j.stem.2010.04.001]
88 **Lam CS**, Cheung AH, Wong SK, Wan TM, Ng L, Chow AK, Cheng NS, Pak RC, Li HS, Man JH, Yau TC, Lo OS, Poon JT, Pang RW, Law WL. Prognostic significance of CD26 in patients with colorectal cancer. *PLoS One* 2014; **9**: e98582 [PMID: 24870408 DOI: 10.1371/journal.pone.0098582]
89 **Brabletz T**, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: Migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer* 2005; **5**: 744-749 [PMID: 16148886 DOI: 10.1038/nrc1694]
90 **Zhou Y**, Xia L, Wang H, Oyang L, Su M, Liu Q, Lin J, Tan S, Tian Y, Liao Q, Cao D. Cancer stem cells

in progression of colorectal cancer. *Oncotarget* 2017; **9**: 33403-33415 [PMID: 30279970 DOI: 10.18632/oncotarget.23607]
91 **Ueno H**, Murphy J, Jass JR, Mochizuki H, Talbot IC. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology* 2002; **40**: 127-132 [PMID: 11952856 DOI: 10.1046/j.1365-2559.2002.01324.x]
92 **Ueno H**, Price AB, Wilkinson KH, Jass JR, Mochizuki H, Talbot IC. A new prognostic staging system for rectal cancer. *Ann Surg* 2004; **240**: 832-839 [PMID: 15492565 DOI: 10.1097/01.sla.0000143243.81014.f2]
93 **Oskarsson T**, Batlle E, Massagué J. Metastatic stem cells: Sources, niches, and vital pathways. *Cell Stem Cell* 2014; **14**: 306-321 [PMID: 24607405 DOI: 10.1016/j.stem.2014.02.002]
94 **Gao W**, Chen L, Ma Z, Du Z, Zhao Z, Hu Z, Li Q. Isolation and phenotypic characterization of colorectal cancer stem cells with organ-specific metastatic potential. *Gastroenterology* 2013; **145**: 636-46.e5 [PMID: 23747337 DOI: 10.1053/j.gastro.2013.05.049]
95 **Peinado H**, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: An alliance against the epithelial phenotype? *Nat Rev Cancer* 2007; **7**: 415-428 [PMID: 17508028 DOI: 10.1038/nrc2131]
96 **Ormanns S**, Neumann J, Horst D, Kirchner T, Jung A. WNT signaling and distant metastasis in colon cancer through transcriptional activity of nuclear β-Catenin depend on active PI3K signaling. *Oncotarget* 2014; **5**: 2999-3011 [PMID: 24930890 DOI: 10.18632/oncotarget.1626]
97 **Todaro M**, Gaggianesi M, Catalano V, Benfante A, Iovino F, Biffoni M, Apuzzo T, Sperduti I, Volpe S, Cocorullo G, Gulotta G, Dieli F, De Maria R, Stassi G. CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. *Cell Stem Cell* 2014; **14**: 342-356 [PMID: 24607406 DOI: 10.1016/j.stem.2014.01.009]
98 **Jinushi M**, Chiba S, Yoshiyama H, Masutomi K, Kinoshita I, Dosaka-Akita H, Yagita H, Takaoka A, Tahara H. Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells. *Proc Natl Acad Sci U S A* 2011; **108**: 12425-12430 [PMID: 21746895 DOI: 10.1073/pnas.1106645108]
99 **Lombardo Y**, Scopelliti A, Cammareri P, Todaro M, Iovino F, Ricci-Vitiani L, Gulotta G, Dieli F, de Maria R, Stassi G. Bone morphogenetic protein 4 induces differentiation of colorectal cancer stem cells and increases their response to chemotherapy in mice. *Gastroenterology* 2011; **140**: 297-309 [PMID: 20951698 DOI: 10.1053/j.gastro.2010.10.005]
100 **Adorno M**, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B, Solari A, Bobisse S, Rondina MB, Guzzardo V, Parenti AR, Rosato A, Bicciato S, Balmain A, Piccolo S. A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. *Cell* 2009; **137**: 87-98 [PMID: 19345189 DOI: 10.1016/j.cell.2009.01.039]
101 **Zhang B**, Halder SK, Kashikar ND, Cho YJ, Datta A, Gorden DL, Datta PK. Antimetastatic role of Smad4 signaling in colorectal cancer. *Gastroenterology* 2010; **138**: 969-80.e1-3 [PMID: 19909744 DOI: 10.1053/j.gastro.2009.11.004]
102 **Calon A**, Espinet E, Palomo-Ponce S, Tauriello DV, Iglesias M, Céspedes MV, Sevillano M, Nadal C, Jung P, Zhang XH, Byrom D, Riera A, Rossell D, Mangues R, Massagué J, Sancho E, Batlle E. Dependency of colorectal cancer on a TGF-β-driven program in stromal cells for metastasis initiation. *Cancer Cell* 2012; **22**: 571-584 [PMID: 23153532 DOI: 10.1016/j.ccr.2012.08.013]
103 **Zubeldia IG**, Bleau AM, Redrado M, Serrano D, Agliano A, Gil-Puig C, Vidal-Vanaclocha F, Lecanda J, Calvo A. Epithelial to mesenchymal transition and cancer stem cell phenotypes leading to liver metastasis are abrogated by the novel TGFβ1-targeting peptides P17 and P144. *Exp Cell Res* 2013; **319**: 12-22 [PMID: 23153552 DOI: 10.1016/j.yexcr.2012.11.004]
104 **Reinacher-Schick A**, Baldus SE, Romdhana B, Landsberg S, Zapatka M, Mönig SP, Hölscher AH, Dienes HP, Schmiegel W, Schwarte-Waldhoff I. Loss of Smad4 correlates with loss of the invasion suppressor E-cadherin in advanced colorectal carcinomas. *J Pathol* 2004; **202**: 412-420 [PMID: 15095268 DOI: 10.1002/path.1516]
105 **Miyaki M**, Iijima T, Konishi M, Sakai K, Ishii A, Yasuno M, Hishima T, Koike M, Shitara N, Iwama T, Utsunomiya J, Kuroki T, Mori T. Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. *Oncogene* 1999; **18**: 3098-3103 [PMID: 10340381 DOI: 10.1038/sj.onc.1202642]
106 **Alazzouzi H**, Alhopuro P, Salovaara R, Sammalkorpi H, Järvinen H, Mecklin JP, Hemminki A, Schwartz S, Aaltonen LA, Arango D. SMAD4 as a prognostic marker in colorectal cancer. *Clin Cancer Res* 2005; **11**: 2606-2611 [PMID: 15814640 DOI: 10.1158/1078-0432.CCR-04-1458]
107 **Janda E**, Lehmann K, Killisch I, Jechlinger M, Herzig M, Downward J, Beug H, Grünert S. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: Dissection of Ras signaling pathways. *J Cell Biol* 2002; **156**: 299-313 [PMID: 11790801 DOI: 10.1083/jcb.200109037]
108 **Eger A**, Stockinger A, Park J, Langkopf E, Mikula M, Gotzmann J, Mikulits W, Beug H, Foisner R. beta-Catenin and TGFbeta signalling cooperate to maintain a mesenchymal phenotype after FosER-induced epithelial to mesenchymal transition. *Oncogene* 2004; **23**: 2672-2680 [PMID: 14755243 DOI: 10.1038/sj.onc.1207416]
109 **Dean M**, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer* 2005; **5**: 275-284 [PMID: 15803154 DOI: 10.1038/nrc1590]
110 **Gerger A**, Zhang W, Yang D, Bohanes P, Ning Y, Winder T, LaBonte MJ, Wilson PM, Benhaim L, Paez D, El-Khoueiry R, El-Khoueiry A, Kahn M, Lenz HJ. Common cancer stem cell gene variants predict colon cancer recurrence. *Clin Cancer Res* 2011; **17**: 6934-6943 [PMID: 21918173 DOI: 10.1158/1078-0432.CCR-11-1180]
111 **Tsuji S**, Midorikawa Y, Takahashi T, Yagi K, Takayama T, Yoshida K, Sugiyama Y, Aburatani H. Potential responders to FOLFOX therapy for colorectal cancer by Random Forests analysis. *Br J Cancer* 2012; **106**: 126-132 [PMID: 22095227 DOI: 10.1038/bjc.2011.505]
112 **Dylla SJ**, Beviglia L, Park IK, Chartier C, Raval J, Ngan L, Pickell K, Aguilar J, Lazetic S, Smith-Berdan S, Clarke MF, Hoey T, Lewicki J, Gurney AL. Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS One* 2008; **3**: e2428 [PMID: 18560594 DOI: 10.1371/journal.pone.0002428]
113 **Kobayashi S**, Yamada-Okabe H, Suzuki M, Natori O, Kato A, Matsubara K, Jau Chen Y, Yamazaki M, Funahashi S, Yoshida K, Hashimoto E, Watanabe Y, Mutoh H, Ashihara M, Kato C, Watanabe T, Yoshikubo T, Tamaoki N, Ochiya T, Kuroda M, Levine AJ, Yamazaki T. LGR5-positive colon cancer stem cells interconvert with drug-resistant LGR5-negative cells and are capable of tumor reconstitution. *Stem Cells* 2012; **30**: 2631-2644 [PMID: 23081779 DOI: 10.1002/stem.1257]
114 **Luraghi P**, Reato G, Cipriano E, Sassi F, Orzan F, Bigatto V, De Bacco F, Menietti E, Han M, Rideout WM, Perera T, Bertotti A, Trusolino L, Comoglio PM, Boccaccio C. MET signaling in colon cancer stem-

like cells blunts the therapeutic response to EGFR inhibitors. *Cancer Res* 2014; **74**: 1857-1869 [PMID: 24448239 DOI: 10.1158/0008-5472.CAN-13-2340-T]
115 **Kendziorra E**, Ahlborn K, Spitzner M, Rave-Fränk M, Emons G, Gaedcke J, Kramer F, Wolff HA, Becker H, Beissbarth T, Ebner R, Ghadimi BM, Pukrop T, Ried T, Grade M. Silencing of the Wnt transcription factor TCF4 sensitizes colorectal cancer cells to (chemo-) radiotherapy. *Carcinogenesis* 2011; **32**: 1824-1831 [PMID: 21983179 DOI: 10.1093/carcin/bgr222]
116 **Liu R**, Lu Z, Gu J, Liu J, Huang E, Liu X, Wang L, Yang J, Deng Y, Qian J, Luo F, Wang Z, Zhang H, Jiang X, Zhang D, Qian J, Liu G, Zhu H, Qian Y, Liu Z, Chu Y. MicroRNAs 15A and 16-1 Activate Signaling Pathways That Mediate Chemotaxis of Immune Regulatory B cells to Colorectal Tumors. *Gastroenterology* 2018; **154**: 637-651.e7 [PMID: 29031499 DOI: 10.1053/j.gastro.2017.09.045]
117 **Jung P**, Menssen A, Mayr D, Hermeking H. AP4 encodes a c-MYC-inducible repressor of p21. *Proc Natl Acad Sci U S A* 2008; **105**: 15046-15051 [PMID: 18818310 DOI: 10.1073/pnas.0801773105]
118 **Shi L**, Jackstadt R, Siemens H, Li H, Kirchner T, Hermeking H. p53-induced miR-15a/16-1 and AP4 form a double-negative feedback loop to regulate epithelial-mesenchymal transition and metastasis in colorectal cancer. *Cancer Res* 2014; **74**: 532-542 [PMID: 24285725 DOI: 10.1158/0008-5472.CAN-13-2203]
119 **Fesler A**, Liu H, Ju J. Modified miR-15a has therapeutic potential for improving treatment of advanced stage colorectal cancer through inhibition of BCL2, BMI1, YAP1 and DCLK1. *Oncotarget* 2017; **9**: 2367-2383 [PMID: 29416778 DOI: 10.18632/oncotarget.23414]
120 **Young LE**, Moore AE, Sokol L, Meisner-Kober N, Dixon DA. The mRNA stability factor HuR inhibits microRNA-16 targeting of COX-2. *Mol Cancer Res* 2012; **10**: 167-180 [PMID: 22049153 DOI: 10.1158/1541-7786.MCR-11-0337]
121 **Todaro M**, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russo A, Gulotta G, Medema JP, Stassi G. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 2007; **1**: 389-402 [PMID: 18371377 DOI: 10.1016/j.stem.2007.08.001]
122 **Todaro M**, Perez Alea M, Scopelliti A, Medema JP, Stassi G. IL-4-mediated drug resistance in colon cancer stem cells. *Cell Cycle* 2008; **7**: 309-313 [PMID: 18235245 DOI: 10.4161/cc.7.3.5389]
123 **Cammareri P**, Scopelliti A, Todaro M, Eterno V, Francescangeli F, Moyer MP, Agrusa A, Dieli F, Zeuner A, Stassi G. Aurora-a is essential for the tumorigenic capacity and chemoresistance of colorectal cancer stem cells. *Cancer Res* 2010; **70**: 4655-4665 [PMID: 20460511 DOI: 10.1158/0008-5472.CAN-09-3953]
124 **Colak S**, Zimberlin CD, Fessler E, Hogdal L, Prasetyanti PR, Grandela CM, Letai A, Medema JP. Decreased mitochondrial priming determines chemoresistance of colon cancer stem cells. *Cell Death Differ* 2014; **21**: 1170-1177 [PMID: 24682005 DOI: 10.1038/cdd.2014.37]
125 **Hoey T**, Yen WC, Axelrod F, Basi J, Donigian L, Dylla S, Fitch-Bruhns M, Lazetic S, Park IK, Sato A, Satyal S, Wang X, Clarke MF, Lewicki J, Gurney A. DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. *Cell Stem Cell* 2009; **5**: 168-177 [PMID: 19664991 DOI: 10.1016/j.stem.2009.05.019]
126 **Funahashi Y**, Hernandez SL, Das I, Ahn A, Huang J, Vorontchikhina M, Sharma A, Kanamaru E, Borisenko V, Desilva DM, Suzuki A, Wang X, Shawber CJ, Kandel JJ, Yamashiro DJ, Kitajewski J. A notch1 ectodomain construct inhibits endothelial notch signaling, tumor growth, and angiogenesis. *Cancer Res* 2008; **68**: 4727-4735 [PMID: 18559519 DOI: 10.1158/0008-5472.CAN-07-6499]
127 **Krishnamurthy N**, Kurzrock R. Targeting the Wnt/beta-catenin pathway in cancer: Update on effectors and inhibitors. *Cancer Treat Rev* 2018; **62**: 50-60 [PMID: 29169144 DOI: 10.1016/j.ctrv.2017.11.002]

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REVIEW

Derivation and applications of human hepatocyte-like cells

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Abstract

Human hepatocyte-like cells (HLCs) derived from human pluripotent stem cells (hPSCs) promise a valuable source of cells with human genetic background, physiologically relevant liver functions, and unlimited supply. With over 10 years' efforts in this field, great achievements have been made. HLCs have been successfully derived and applied in disease modeling, toxicity testing and drug discovery. Large cohorts of induced pluripotent stem cells-derived HLCs have been recently applied in studying population genetics and functional outputs of common genetic variants *in vitro*. This has offered a new paradigm for genome-wide association studies and possibly *in vitro* pharmacogenomics in the nearly future. However, HLCs have not yet been successfully applied in bioartificial liver devices and have only displayed limited success in cell transplantation. HLCs still have an immature hepatocyte phenotype and exist as a population with great heterogeneity, and HLCs derived from different hPSC lines display variable differentiation efficiency. Therefore, continuous improvement to the quality of HLCs, deeper investigation of relevant biological processes, and proper adaptation of recent advances in cell culture platforms, genome editing technology, and bioengineering systems are required before HLCs can fulfill the needs in basic and translational research. In this review, we summarize the discoveries, achievements, and challenges in the derivation and applications of HLCs.

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Core tip: Hepatocyte-like cells (HLCs) derived from human pluripotent stem cells (hPSCs) have a great application prospect as an unlimited supply of human hepatocytes in disease modeling, toxicity testing and drug discovery. In this review, we summarize the derivation of HLCs from hPSCs, and the limitations and optimization of current differentiation protocols. We also discuss progress in the application of HLCs, and reveal the exciting future of HLCs for use in the study of rare diseases, population genetics, and *in vitro* pharmacogenomics.

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INTRODUCTION

The liver represents one of the most pivotal organs of the human body in regulating glucose homeostasis, lipid metabolism, detoxification and many other physiological processes. As liver diseases, including fatty liver diseases, hepatic carcinoma, and viral hepatitis, continue to increase in prevalence, there is an urgent need for development of effective treatments, and sufficiently cell or tissue sources for transplantation. Primary human hepatocytes and liver donors offer immediate resources for studying liver diseases and transplantation. However, both primary cells and available donor transplants are in persistent shortage. Although different culture systems have been identified recently that enable long-term culture and expansion of both rodent and human primary hepatocytes[1-4], the capacity of expansion is still limited and has donor-dependent variability. As stem cells are known to have potent self-renewal ability as well as the capacity to differentiate into different somatic cell types, they have been proposed as an ideal alternative cell source for large or even unlimited supplies of hepatocytes and even liver tissues. Human hepatocytes can be derived from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells and hepatic progenitor cells[5]. As the cells derived from stem cells often have incomplete function and exhibit characteristics of fetal liver cells, they are generally defined as hepatocyte-like cells (HLCs). The discovery made by Gurdon and Yamanaka that mature cells from individual patients can be reprogrammed to iPSCs, opened up the possibility that these cells can be applied to disease modeling and organ transplantation. Furthermore, intense efforts have been made in recent years in generating better HLCs and liver organoids from PSCs, and in applications of these cells in various fields. Therefore, in this review, we focus on HLCs derived from human pluripotent stem cells (hPSCs) and discuss recent progress in the derivation and applications of HLCs in biomedical research.

DERIVATION OF HUMAN HLCs

hPSCs include human ESCs, mostly derived from the inner cell mass of the fertilized eggs, and iPSCs reprogramed from terminally differentiated somatic cells. hPSCs promise an unlimited supply of human somatic cells, due to their theoretical capacity for self-renewal and differentiation into any kind of somatic cell types in human body. To date, many protocols have been established to generate human hepatocytes derived from hPSCs. Most induction methods are based on the understanding of the embryonic development processes of the liver, and aimed to imitate in Petri dishes the endoderm development, endoderm hepatic specification and hepatic maturation stages. The directed differentiation protocols either rely on the use of embryoid body (EB) formation[6,7] or start with monolayer culture, with the latter more frequently adapted currently in laboratories. EB formation means to mimic the blastocyst and

epiblast architecture; however, it can be easily disturbed by suboptimal culture conditions and sources of reagents, for example, different batches of fetal bovine serum can affect to a large degree the quality of generated EBs. Most protocols currently in use apply similar strategies with contributions from individual laboratories by improving inducers of differentiation and optimizing their combinations (Table 1). These protocols can be largely specified to three consecutive steps: endoderm differentiation, hepatic induction, and liver maturation.

Endoderm formation

Transforming growth factor (TGF) β family member Nodal is vital in endoderm formation, based on studies in developmental biology in models including frogs, zebrafish, and mice[8-10]. Although Nodal is an attractive candidate for inducing hPSCs to differentiate into definitive endoderm (DE), it is difficult to get highly active protein. Activin is another TGFβ family member, which mimics Nodal activity in triggering similar intracellular signaling events[11], thus is often used as a substitution of Nodal *in vitro*[12]. In 2005, D'Amour *et al*[12] demonstrated efficient endoderm induction from monolayers of hPSCs by applying activin A, which was subsequently reproduced by many other groups. The monolayer culture here seems important to the endoderm differentiation in that cells can be exposed evenly to the endodermal inducer, activin A, and can better synchronize development of the endodermal cell fate[13]. Levels of Nodal signaling comprise key elements in cell fate determination, with high level promotes endoderm differentiation, whereas low level initiates mesoderm specification[14-17]. Therefore, high concentrations of activin A are now widely utilized for endoderm induction in hPSC culture[18-22]. Besides, activation of fibroblast growth factor (FGF), bone morphogenetic protein (BMP) and Wnt signaling pathways also promote endoderm development[7,19,23]. Phosphatidylinositol 3-kinase (PI3K) inhibitors, such as LY 294002 and AKT1-II, also promote activin-A-induced endoderm development[17]. Several studies have shown that low doses of serum are necessary for activin A to induce an efficient endoderm program[12,17,24].

Hepatic specification

In early embryo development, FGF signals and BMP signals initiate the liver gene program and simultaneously block that for pancreas development[25]. Consistent with the *in vivo* discoveries, the signaling molecules FGF and BMP have also been demonstrated to be important in generating hepatic cells from DE cells *in vitro*. The combination of FGFs and BMPs are thus widely used to induce hepatic endoderm programs[18,21,23]. Dimethylsulfoxide (DMSO) can assist in promoting hPSC differentiation and specific generation of hepatic progenitors, and is usually used in hepatic differentiation[19,22,26,27].

Liver maturation

As for further liver maturation, hepatic progenitors are mostly treated by hepatocyte growth factor (HGF), oncostatin M (OSM), and glucocorticoid dexamethasone (Dex). HGF binds to its tyrosine kinase receptor c-Met, promoting hepatoblast proliferation, increasing cell migration and improving cell survival[28,29]. OSM produced by hematopoietic cells is an interleukin(IL)-6 family cytokine, which induces hepatic maturation by the phosphorylation of signal transducer and activator of transcription[28,30]. The glucocorticoid dexamethasone has also been implicated in the maturation of the hepatocytes[31,32]. After the maturation stage, obtained HLCs display many hepatocyte features, such as albumin expression and secretion, urea secretion, low-density lipoprotein (LDL) uptake, indocyanine green (ICG) uptake, and glycogen storage (Table 1). However, those cells express fetal liver markers, such as α-fetoprotein (AFP), and have lower activities of CYP450 enzymes when compared to primary liver tissue. With comparison of a set of human adult and fetal liver markers, it is roughly estimated that the HLCs have the characteristics of fetal hepatocytes at < 20 wk gestation[33].

Protocol optimization

Different strategies have been adopted with the aim to promote maturation and to reduce the large heterogeneity of HLCs. One strategy is to use 3D culture, mimicking liver development in the body, thus promoting further maturation. Indeed, it has been shown that cells demonstrate more matured phenotypes in 3D than other culture systems. For example, it has been demonstrated that cAMP signaling within the 3D hepatoblast aggregates can promote further maturation of HLCs that display comparable metabolic enzyme levels to those of primary human hepatocytes[34] . The other main strategy is to optimize the current protocols through screening for molecules that can improve differentiation, and to understand better the molecular mechanisms underlying liver development. Towards this aim, by screening 4000

Table 1 Summary of hepatocyte-like cells differentiation protocols

Ref.	EB / monolayer	Protocol features		*In vitro* functional assays	*In vivo* assay
		Endoderm induction	Hepatic specification and maturation		
Cai *et al*[18], 2007	Monolayer	Activin A, ITS	FGF4, BMP2, HGF, OSM, Dex	ALB, Glycogen, ICG, LDL, CYP450	Yes
Hay *et al*[19], 2008	Monolayer	Activin A, Wnt3a	Serum, DMSO, Insulin, HGF, OSM	Urea, Gluconeogenesis, AFP	Yes
Agarwal *et al*[24], 2008	Monolayer	Activin A, low serum	FGF4, HGF, OSM, Dex	ALB, Glycogen, ICG	Yes
Basma *et al*[7], 2009	EB/monolayer	Activin A, bFGF	FGF, DMSO, Dex	ALB, Urea, AAT, CYP450	Yes
Song *et al*[20], 2009	Monolayer	Activin A	FGF4, BMP2, HGF, KGF, OSM, Dex	ALB, Urea, Glycogen, CYP450	No
Si-Tayeb *et al*[21], 2010	Monolayer	Activin A	BMP4 FGF2, HGF, OSM	Glycogen, LDL, oil red O storage, ICG, Urea	Yes
Sullivan *et al*[22], 2010	Monolayer	Activin A, Wnt3a	β-ME, DMSO, Insulin, HGF, OSM	CYP450, Fibrinogen, Fibronectin, Transthyretin, AFP	No
Touboul *et al*[23], 2010	Monolayer	Activin A, FGF2, BMP4, LY294002	FGF10, RA, SB431542, FGF4, HGF, EGF	Glycogen, CYP450, ICG, LDL	Yes
Borowiak *et al*[35], 2009	Monolayer	Activin A, Wnt3a, HGF	OSM, Dex, ITS	CYP450, Urea, LDL, Glycogen	Yes
Ogawa *et al*[34], 2013	EB/monolayer	BMP4, Activin A, Wnt3a	FGF10, bFGF, BMP4, HGF, OSM, Dex, cAMP	ICG, Glycogen, ALB, CYP450	No
Siller *et al*[37], 2015	Monolayer	CHIR99021	DMSO, dihexa, Dex	ALB, Glycogen, ICG, CYP450	No

EB: Embryoid bodies; KGF: Keratinocyte growth factor; β-ME: 2-mercaptoethanol; RA: Retinoic acid; EGF: Epidermal growth factor; ITS: Insulin, transferrin, selenium; dihexa: Hepatocyte growth factor receptor agonist N-hexanoic-Tyr, Ile-(6) aminohexanoic amide; ALB: Albumin secretion; AFP: Alpha-fetoprotein secretion; AAT: Alpha-1-antitrypsin secretion; LDL: Low-density lipoprotein uptake; glycogen: Glycogen storage; ICG: Indocyanine green uptake; Urea: Urea secretion and production; CYP450: CYP450 activity.

compounds, the Melton group identified IDE1 and IDE2, which can efficiently promote differentiation of mouse and human ESCs into DE cells[35]. Other groups have also identified other small molecules, and demonstrated their effects in improving hPSC differentiation toward endoderm[36]. In 2015, the Siller *et al*[37] group developed a new method for HLC differentiation with a combination of small molecules without the inclusion of growth factors in a defined minimum medium. Shan *et al*[38] developed a high-throughput chemical screening platform and identified two different classes of small molecules, which are able to induce functional proliferation of human primary hepatocytes *in vitro* and improve HLC maturation. By utilizing an established hepatic lineage hPSC reporter line, our laboratory performed genetic and chemical screenings, and identified several modulators involved in hepatic differentiation, and CI-994 compound (histone deacetylase 3 inhibitor) that can promote HLC differentiation at a late stage[39].

APPLICATIONS OF HLCs

Disease models

Human PSCs offer a unique *in vitro* cellular model system for disease modeling. Induced PSCs derived from patients or hPSCs engineered with specific disease-causing mutations using genome editing technologies allow researchers to study the consequences of genetic mutations with a human- and patient-specific genetic background; whereas the differentiation processes *in vitro* often recapitulate aspects of normal development, thus providing the opportunity to investigate the developmental and degenerative processes of certain human diseases. Furthermore, as hPSCs possess great capacity in self-renewal, they can offer large-scale cellular materials with identical genetic background for disease modeling and for possible compound screenings to develop potential treatments.

Studying rare genetic variants

For modeling liver diseases with rare mutations in Mendelian diseases, patient-

specific iPSCs carrying certain genetic mutations are often derived and differentiated to HLCs. Many disease models of inborn liver metabolic disorders, such as α1-antitrypsin deficiency, familial hypercholesterolemia, glycogen storage disease type 1a, and Wilson's disease, have been generated[40-42]. Upon differentiation to HLCs, these cells with genetic mutations displayed certain disease phenotypes that are reflected in patients, highlighting potential utility of these models for studying diseases or screening for therapeutic interventions. In situations in which patients are not available, disease mutations of interest can be engineered using genome editing technologies into wild-type hPSCs to create mutant hPSCs for disease study[43,44]. Drug screening with these disease models can highlight novel discoveries for disease treatment. In a study by the Duncan and Rader groups[45], HLCs derived from familial hypercholesterolemia iPSCs were applied to drug screening to identify potential LDL–cholesterol (LDL-C)-lowering drugs, which has successfully revealed cardiac glycosides as a candidate treatment for hypercholesterolemia. Other than studying diseases harboring genetic mutations, hPSC-derived HLCs are also powerful in providing cellular models for studying the lifecycle of hepatitis viruses. hPSC-derived HLCs have be used in hepatitis C virus (HCV) infection and screening for anti-HCV drugs[46], as well as modeling hepatitis B virus infection[47].

Studying common genetic variants

As a remarkable improvement in the recent iPSC disease modeling fields, large, diverse population cohorts of iPSCs have been generated and differentiated in parallel to HLCs as well as other cell types, offering valuable tissue substitutes for studies to reveal the relationship between genotype and phenotype; for example, expression quantitative trait locus (eQTL) analysis[48,49]. Two independent cohorts of iPSCs have been generated from healthy donors (68 iPSC lines from 34 donors in one study and 91 iPSC lines from 91 donors in the other study) and used for subsequent hepatic differentiation and genetic analysis. Studies either successfully confirmed eQTLs previously characterized *in vivo*[49], or identified a number of loci controlling hepatic gene expression with these *in vitro* HLCs[48]. In one study, the cohort of iPSC-derived HLCs were also subjected to metabolite abundance quantitative trait locus (mQTL) analysis, leading to the discovery of a strong association between a lipid-dysregulating phenotype and the minor allele at the 1p13 locus[49]. For the first time, these two studies demonstrated the capacity for iPSCs-derived cells to reproduce *in vivo* phenotypes driven by common genetic variants, and uncovered a potentially unlimited supply of human cells that allow to discover cell-type-specific QTL phenotypes (eQTL, mQTL and potentially others) that would be inaccessible using *in vivo* tissues. Together with several other studies that have performed genome-wide QTL analyses and identified a number of loci that contribute to interline heterogeneity using hundreds of undifferentiated iPSC lines[50-52], these studies have offered a new paradigm for human research, with iPSC-driven disease modeling being applied to study population genetics *in vitro*.

In vitro pharmacogenomics

Aside from drug discovery with iPSC-derived disease models with small cohorts, large cohorts of iPSCs and iPSC-derived cells have been proposed to perform trials-in-dish, to assist in translating the discoveries of genome-wide association studies (GWASs) into improved treatment regimens and drug discovery; that is, to apply genotype analysis to patient stratification and design of individual treatment plans[53]. In possible scenarios, iPSC-derived cells may provide an important link between drug development and Phase I trials, where iPSC-derived hepatocytes, cardiomyocytes or neurons can be used for preliminary safety screens with candidate drugs that might induce hepatotoxicity, cardiotoxicity, neurotoxicity or other off-target effects. Furthermore, between Phase I and Phase II trials, drug target cells derived from large cohorts of iPSCs can serve as the surrogate human population and be used in testing for drug efficacy; results from which can be applied to classify patients into responder and non-responder groups, thus increasing the relevance and successfully rate of further Phase 2 and 3 trials. Altogether, small or large cohorts of iPSCs and iPSC-derived function cell types are revolutionizing the field of drug discovery.

Making liver organoids

The liver is a highly specialized organ consisting of mostly hepatocytes, but also several other cell types, such as Kupffer cells, endothelia cells, bile duct cells, and hepatic stellate cells. These cells all contribute to the highly organized architecture and functions of liver tissue. Compared to HLCs in 2D culture, liver tissue organoids constitute more than one cell type, can resemble part of the architecture of liver tissue, and possess some functions that may not exist in HLCs. Liver organoids can either be derived from adult stem cells[54,55] or hPSCs[56-59]. Other than HLCs, development of

protocols to obtain other cell types derived from hPSCs that constitute the liver tissue are important. To date, protocols of directed differentiation to obtain cholangiocytes[56,57], endothelia cells[60] and hepatic stellate cells[61] have been established, which may further aid the generation of functional liver tissue organoids. Other reviews discuss the generation and application of tissue organoids, which can assist in better understanding the opportunities as well as challenges in this field[62,63].

Bioartificial livers

Artificial liver support systems have been developed to provide an alternative to orthotopic liver transplantation (OLT). Artificial livers use nonbiological components to perform hepatic detoxification, removing toxins and drugs that accumulate in the blood during liver failure[64]. However, artificial livers do not have the capacity to adequately replicate the physiological liver function. The incorporation of live cells harboring liver functions into these artificial liver systems, which establishes the bioartificial livers (BALs) systems, offers a solution to overcome these limitations[64]. BAL support systems are extracorporeal bioreactors in which whole livers or liver cells are cultured in a 3D manner within a network of hollow fibers for blood plasma perfusion. BAL systems provide both biotransformation and hepatic synthetic functions[65]. To date, different sources of liver cells have been tested in BAL devices, for example, human primary hepatocytes, immortalized human hepatoma cell lines, porcine hepatocytes[66], as well as induced human hepatocytes transdifferentiated from human fibroblasts (hiHeps)[67]. While human hepatocytes are the preferred cells, obtaining sufficient human hepatocytes faces the same difficulty of organ shortage. Porcine hepatocytes are close to human hepatocytes, but have potential risk of xenozoonosis and immunological response. Hepatoma cells can provide large amounts of materials, but suffer from incompetent metabolism and ammonia clearance[68]. HiHeps representing a new invaluable cell source for BAL devices, and have been successful in pigs[67] as well as in primary tests in patients. While we have not seen reports of HLCs being applied in BAL devices, we envisage that HLCs will be a potential cell source for the treatment of liver failure in BAL support systems in the future. The advantages of HLCs are obvious: human or patient-specific genetic background, normal karyotype, potentially unlimited supply, and better liver functions. However, to obtain a large amount of functional and homogeneous hepatocytes from hPSCs still depends on continuous improvement to the differentiation protocols and development of optimal large-scale culture systems.

In vivo transplantation

OLT remains the most effective treatment for end-stage liver diseases. However, liver donor shortage and life-long need for immunosuppression are the main limitations to liver transplantation. A potential alternative to liver transplantation is hepatocyte transplantation[69-71]. However, cell transplantation is also limited by the availability of effective cell sources, generation of alternative hepatocytes is thus an urgent problem. The ideal cell source should at least meet the following requirements: (1) Available in large quantity. Similar to hepatocytes needed in BAL devices, a large number of cells ($> 10^9$) may be needed for transplantation to every adult patient; (2) High efficiency of *in vivo* homing and repopulation. Transplanted cells can home and adapt to the microenvironment in recipient and successfully repopulate the liver; (3) Low immunogenicity. Cells have no or low immunogenic responses, which can be suppressed by low doses of immunosuppressant; (4) No tumorigenic risk. Transplanted cells should have normal karyotype and be free of potential tumorigenic modulations, such as modifications in oncogenic or tumor suppressor genes. To date, several mouse models have been adopted in testing the transplantation efficiency of human hepatocytes, which in general can be divided into two categories[72]. One is a mouse model with a genetic disorder that causes depletion of the host hepatocytes, such as mice expressing urinary plasminogen activator (uPA) driven by the albumin or Mup promoter[73,74], and immunodeficient FRG [*Fah*(-/-) *Rag2*(-/-) *Il2rg* (-/-)] mice[75]; another is a mouse model with drug- or surgery-induced liver damage, including mice receiving treatment with retrorsine[7], CCl_4[24,76], diethylnitrosamine[77] or partial hepatectomy[7,78] (Table 2). Transplantation using primary human hepatocytes has been successful in mouse models, for example, with the FRG mouse model, the ratio of human hepatocytes in a mouse liver can be up to 90%[75]. However, there are no definitive conclusions so far regarding whether the maturity of transplanted liver cells affects the efficiency of transplantation when HLCs are used. Cells in endoderm, hepatoblasts, and mature hepatocyte stages along the HLC differentiation process all have possibilities as donor cells in cell transplantation[7,24,73,76,77] (Table 2). The microenvironment in recipient liver is thought to supply necessary signals to promote further maturation of transplanted cells, although direct evidence and the underlying mechanism are lacking. However, the overall HLC transplantation efficiency is lower

compared to that of human primary hepatocytes[75] (Table 2). Furthermore, transplantation with HLCs may suffer tumorigenic risks due to remnant undifferentiated hPSCs, and the immunogenicity has not been addressed so far, as most studies were performed with immunocompromised animals.

To improve the transplantation efficiency, several ectopic sites have been investigated, including spleen, peritoneal cavity, kidney, lung, pancreas and fat pads. Bioengineering approaches have also been applied in cell transplantation. For example, Song *et al*[20] transplanted hPSC-derived HLCs in immunocompetent mice via 3D cell coaggregates with stromal cells and encapsulation. This study demonstrated an improved approach for the engraftment of hPSC-derived HLCs[79]. In a different study, Nagamoto *et al*[78]. used a cell sheet engineering technology by attaching HLC sheets onto the surface of mouse liver with acute liver failure, which showed improved hepatocyte engraftment and animal survival in contrast, genetic modification to HLCs represents another approach to improve transplantation efficiency. For example, Nagamoto *et al*[74] demonstrated higher transplantation efficiency using HLCs transduced with an adenovirus vector expressing FNK (Ad-FNK), by inhibiting apoptosis in the process of integration into liver. However, there is still a long way to go before HLCs can be used in clinical liver transplantation. Strenuous efforts are needed to understand the complex processes of cell transplantation, for example, the donor–host interactions, to improve the quality of HLCs and optimize the transplantation strategy. Plus, the potential tumorigenic risk of transplanted HLCs had to be carefully considered. Specifically, tumor cells can arise from cells with residual expression of factors in iPSC reprogramming process (*e.g.*, the myc expression), undifferentiated iPSCs remaining in the culture, and cells with mutations or karyotype abnormalities caught in the rather long *in vitro* culture and differentiation processes. Several approaches can be adopted to reduce the tumorigenic risk: (1) Use integrating-free viruses or small molecules for iPSC reprogramming[80,81]; (2) Improve the *in vitro* culture conditions and enhance the differentiation efficiency of hPSC-derived HLCs[82]; (3) Remove undifferentiated iPSCs, *e.g.* through treatments with small molecules or antibodies that can specifically target iPSCs[83,84]; or enrich HLCs using HLC specific surface markers before transplantation[85]; (4) Monitor the genome integrity of cells at the iPSC stage and the HLC stage, through karyotype analysis and whole-genome sequencing; (5) Engineer a self-killing circuit in cells that would allow the trigger of cell death *in vivo* to remove tumorigenic cells, if necessary, to further assure safety[86]. Nonetheless, hPSC-derived HLCs provide a potential valuable cell source to OLT for liver diseases that is worth pursuing.

CONCLUSION

The generation of iPSCs has revolutionized the whole field of cell biology. It is truly inspiring to imagine that we can grow any person's pluripotent cells indefinitely in a dish and turn them into any cell type. With this capability of iPSCs, the approach to the study of human biology has been profoundly changed. HLCs were among the first batch of adult cell types that have been derived from iPSCs, and have been tested ever since for disease modeling, toxicity screening, and drug discovery, and as donor cells for transplantation (Figure 1). Complexities and difficulties in the derivation and applications of these HLCs seem beyond our initial expectations. More than 10 years have passed, but HLCs derived from hPSCs remain a largely heterogeneous population with incompetent liver cell function and low transplantation efficiency. Protocols to grow HLCs from hPSCs need to be substantially and continuously improved and standardized on the basis of deeper understanding of liver development. Despite the gap between the reality and ideal conditions, efforts have paid off well and the field has made tremendous achievements in recent years, such as generation of functional liver organoids, successful modeling of certain liver diseases, identification of candidate treatments, and application of large cohorts of HLCs for human genetic studies, to name a few (Figure 1). With advances in cell culture systems including 3D culture platforms[87], coculturing conditions[88], tissue-on-a-chip approaches[89], and invention of new technologies including genome editing tools and bioengineering systems, HLCs obtained from hPSCs will eventually be able to fulfill the needs in biomedical research and clinical translation.

Table 2 Summary of transplantation studies using hepatocyte-like cells

Ref.	Animal model	Route	Proliferative stimulus	Type and number of cells	Donor / recipient (% engrafted)	Donor / recipient (% repopulated)	Time post-transplantation
Agarwal *et al*[24], 2008	NOD-SCID mice	Portal vein	CCl_4-injured	10^6 hES-DEs	< 1%	NA	28 d
Basma *et al*[7], 2009	NOD-SCID mice	Spleen	Retrorsine and partial hepatectomy	1 × 10^6 hES-HLCs	NA	NA	21 d
Liu *et al*[77], 2011	NSG mice	Tail vein	dimethylnitrosamine -injured	0.1 - 2 × 10^6 hiPSC- multistage hepatic cells	2%–17%	8%–15%	56 d
Asgari *et al*[76], 2013	Normal mouse	Tail vein	CCl_4-injured	1 × 10^6 hiPSC-HLCs	2 ± 0.7%	NA	35 d
Carpentier *et al*[73], 2014	MUP-uPA/ SCID/Bg mice	Spleen	NA	4 × 10^6 hiPSC-HLCs	1%-7%	< 1 to up to 20%	100 d
Song *et al*[20], 2015	Immunocompetent mice	Intraperitoneal cavity	NA	4.4×10^5 hiPSC-HLCs in capsules	NA	NA	24 d
Nagamoto *et al*[74], 2015	uPA/SCID mice	Spleen	NA	1 × 10^6 Ad-FNK-transduced hiPSC-HLCs	NA	NA	28 d
Nagamoto *et al*[78], 2016	Mice	hiPS-HLC sheet transplantation	2/3 partial hepatectomy and CCl_4-injured	8 × 10^5 hiPSC-HLCs	NA	NA	14 d

DE: Definitive endoderm; HLCs: Hepatocyte-like cells; hPSCs: Human pluripotent stem cells.

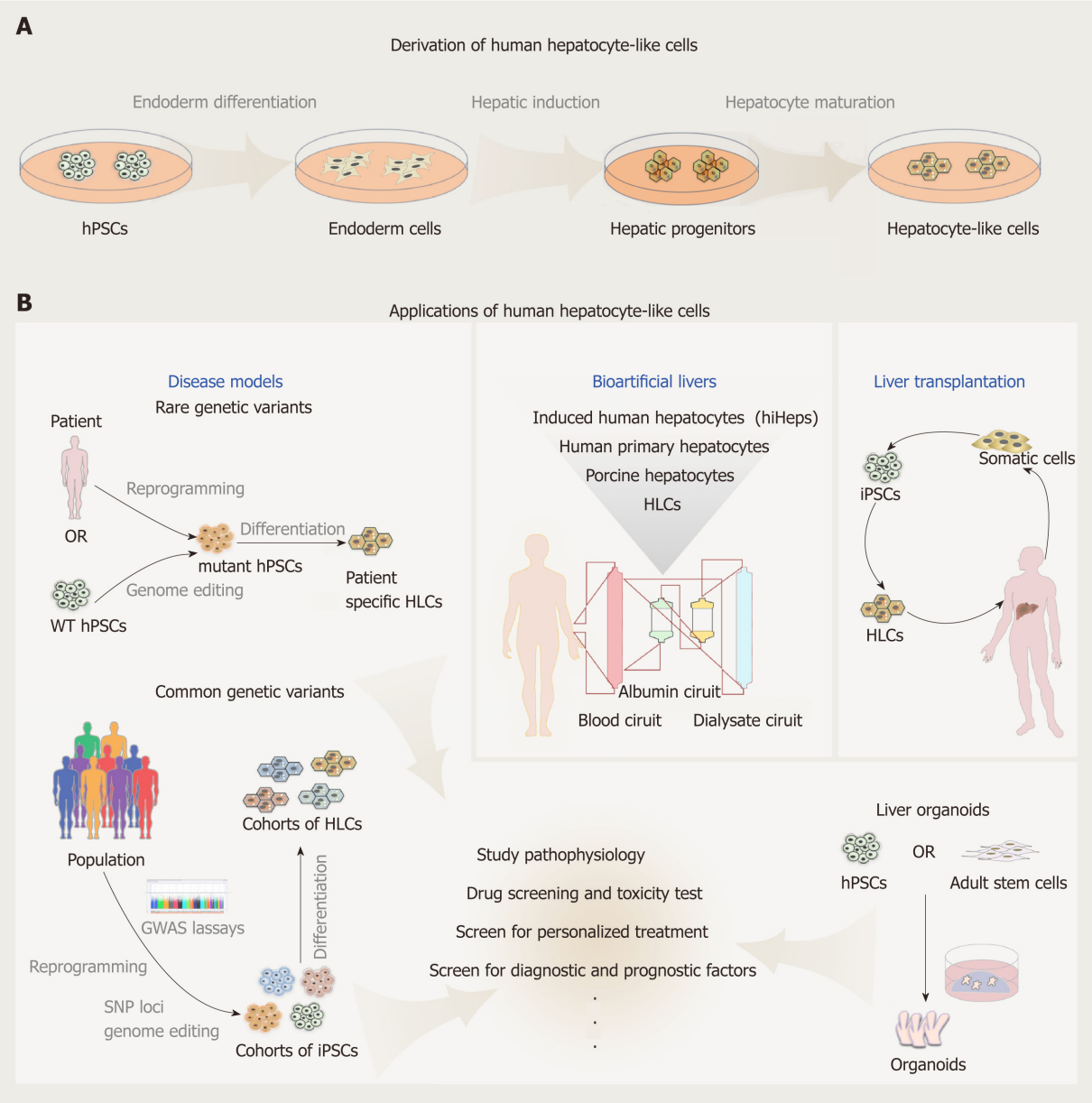

Figure 1 Derivation and applications of human hepatocyte-like cells. A: Directed differentiation process of human pluripotent stem cells (hPSCs)-derived hepatocyte-like cells (HLCs) *in vitro* includes endoderm development, endoderm hepatic specification, and hepatic maturation stages; B: Applications of human HLCs. HPSC-derived HLCs can be used to generate disease models to study rare or common genetic variants. These cellular models can be applied in pathophysiological research, drug screening, and toxicity testing. Cohorts of HLCs provide *in vitro* cell models for genome-wide association studies and potentially pharmacogenomics in dishes. HLCs also offer a potential cell source for bioartificial livers or liver transplantation. HLCs: Hepatocyte-like cells; hPSCs: Human pluripotent stem cells.

REFERENCES

1 **Zhang K**, Zhang L, Liu W, Ma X, Cen J, Sun Z, Wang C, Feng S, Zhang Z, Yue L, Sun L, Zhu Z, Chen X, Feng A, Wu J, Jiang Z, Li P, Cheng X, Gao D, Peng L, Hui L. In Vitro Expansion of Primary Human Hepatocytes with Efficient Liver Repopulation Capacity. *Cell Stem Cell* 2018; **23**: 806-819.e4 [PMID: 30416071 DOI: 10.1016/j.stem.2018.10.018]

2 **Hu H**, Gehart H, Artegiani B, LÖpez-Iglesias C, Dekkers F, Basak O, van Es J, Chuva de Sousa Lopes SM, Begthel H, Korving J, van den Born M, Zou C, Quirk C, Chiriboga L, Rice CM, Ma S, Rios A, Peters PJ, de Jong YP, Clevers H. Long-Term Expansion of Functional Mouse and Human Hepatocytes as 3D Organoids. *Cell* 2018; **175**: 1591-1606.e19 [PMID: 30500538 DOI: 10.1016/j.cell.2018.11.013]

3 **Fu GB**, Huang WJ, Zeng M, Zhou X, Wu HP, Liu CC, Wu H, Weng J, Zhang HD, Cai YC, Ashton C, Ding M, Tang D, Zhang BH, Gao Y, Yu WF, Zhai B, He ZY, Wang HY, Yan HX. Expansion and differentiation of human hepatocyte-derived liver progenitor-like cells and their use for the study of hepatotropic pathogens. *Cell Res* 2019; **29**: 8-22 [PMID: 30361550 DOI: 10.1038/s41422-018-0103-x]

4 **Peng WC**, Logan CY, Fish M, Anbarchian T, Aguisanda F, Álvarez-Varela A, Wu P, Jin Y, Zhu J, Li B, Grompe M, Wang B, Nusse R. Inflammatory Cytokine TNFα Promotes the Long-Term Expansion of Primary Hepatocytes in 3D Culture. *Cell* 2018; **175**: 1607-1619.e15 [PMID: 30500539 DOI: 10.1016/j.cell.2018.11.012]

5 **Hu C**, Li L. In vitro culture of isolated primary hepatocytes and stem cell-derived hepatocyte-like cells for liver regeneration. *Protein Cell* 2015; **6**: 562-574 [PMID: 26088193 DOI: 10.1007/s13238-015-0180-2]
6 **Baharvand H**, Hashemi SM, Kazemi Ashtiani S, Farrokhi A. Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro. *Int J Dev Biol* 2006; **50**: 645-652 [PMID: 16892178 DOI: 10.1387/ijdb.052072hb]
7 **Basma H**, Soto-Gutiérrez A, Yannam GR, Liu L, Ito R, Yamamoto T, Ellis E, Carson SD, Sato S, Chen Y, Muirhead D, Navarro-Alvarez N, Wong RJ, Roy-Chowdhury J, Platt JL, Mercer DF, Miller JD, Strom SC, Kobayashi N, Fox IJ. Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. *Gastroenterology* 2009; **136**: 990-999 [PMID: 19026649 DOI: 10.1053/j.gastro.2008.10.047]
8 **Tam PP**, Kanai-Azuma M, Kanai Y. Early endoderm development in vertebrates: Lineage differentiation and morphogenetic function. *Curr Opin Genet Dev* 2003; **13**: 393-400 [PMID: 12888013]
9 **Whitman M**. Nodal signaling in early vertebrate embryos: themes and variations. *Dev Cell* 2001; **1**: 605-617 [PMID: 11709181]
10 **Schier AF**. Nodal signaling in vertebrate development. *Annu Rev Cell Dev Biol* 2003; **19**: 589-621 [PMID: 14570583 DOI: 10.1146/annurev.cellbio.19.041603.094522]
11 **de Caestecker M**. The transforming growth factor-β superfamily of receptors. *Cytokine Growth Factor Rev* 2004; **15**: 1-11 [PMID: 14746809]
12 **D'Amour KA**, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* 2005; **23**: 1534-1541 [PMID: 16258519 DOI: 10.1038/nbt1163]
13 **Han S**, Bourdon A, Hamou W, Dziedzic N, Goldman O, Gouon-Evans V. Generation of functional hepatic cells from pluripotent stem cells. *J Stem Cell Res Ther* 2012; **Suppl** 10: 1-7 [PMID: 25364624 DOI: 10.4172/2157-7633.S10-008]
14 **Schier AF**, Neuhauss SC, Helde KA, Talbot WS, Driever W. The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. *Development* 1997; **124**: 327-342 [PMID: 9053309]
15 **Stainier DY**. A glimpse into the molecular entrails of endoderm formation. *Genes Dev* 2002; **16**: 893-907 [PMID: 11959838 DOI: 10.1101/gad.974902]
16 **Thisse B**, Wright CV, Thisse C. Activin- and Nodal-related factors control antero-posterior patterning of the zebrafish embryo. *Nature* 2000; **403**: 425-428 [PMID: 10667793 DOI: 10.1038/35000200]
17 **McLean AB**, D'Amour KA, Jones KL, Krishnamoorthy M, Kulik MJ, Reynolds DM, Sheppard AM, Liu H, Xu Y, Baetge EE, Dalton S. Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. *Stem Cells* 2007; **25**: 29-38 [PMID: 17204604 DOI: 10.1634/stemcells.2006-0219]
18 **Cai J**, Zhao Y, Liu Y, Ye F, Song Z, Qin H, Meng S, Chen Y, Zhou R, Song X, Guo Y, Ding M, Deng H. Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* 2007; **45**: 1229-1239 [PMID: 17464996 DOI: 10.1002/hep.21582]
19 **Hay DC**, Fletcher J, Payne C, Terrace JD, Gallagher RC, Snoeys J, Black JR, Wojtacha D, Samuel K, Hannoun Z, Pryde A, Filippi C, Currie IS, Forbes SJ, Ross JA, Newsome PN, Iredale JP. Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. *Proc Natl Acad Sci USA* 2008; **105**: 12301-12306 [PMID: 18719101 DOI: 10.1073/pnas.0806522105]
20 **Song Z**, Cai J, Liu Y, Zhao D, Yong J, Duo S, Song X, Guo Y, Zhao Y, Qin H, Yin X, Wu C, Che J, Lu S, Ding M, Deng H. Efficient generation of hepatocyte-like cells from human induced pluripotent stem cells. *Cell Res* 2009; **19**: 1233-1242 [PMID: 19736565 DOI: 10.1038/cr.2009.107]
21 **Si-Tayeb K**, Noto FK, Nagaoka M, Li J, Battle MA, Duris C, North PE, Dalton S, Duncan SA. Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* 2010; **51**: 297-305 [PMID: 19998274 DOI: 10.1002/hep.23354]
22 **Sullivan GJ**, Hay DC, Park IH, Fletcher J, Hannoun Z, Payne CM, Dalgetty D, Black JR, Ross JA, Samuel K, Wang G, Daley GQ, Lee JH, Church GM, Forbes SJ, Iredale JP, Wilmut I. Generation of functional human hepatic endoderm from human induced pluripotent stem cells. *Hepatology* 2010; **51**: 329-335 [PMID: 19877180 DOI: 10.1002/hep.23335]
23 **Touboul T**, Hannan NR, Corbineau S, Martinez A, Martinet C, Branchereau S, Mainot S, Strick-Marchand H, Pedersen R, Di Santo J, Weber A, Vallier L. Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology* 2010; **51**: 1754-1765 [PMID: 20301097 DOI: 10.1002/hep.23506]
24 **Agarwal S**, Holton KL, Lanza R. Efficient differentiation of functional hepatocytes from human embryonic stem cells. *Stem Cells* 2008; **26**: 1117-1127 [PMID: 18292207 DOI: 10.1634/stemcells.2007-1102]
25 **Zaret KS**. Regulatory phases of early liver development: paradigms of organogenesis. *Nat Rev Genet* 2002; **3**: 499-512 [PMID: 12094228 DOI: 10.1038/nrg837]
26 **Rambhatla L**, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. *Cell Transplant* 2003; **12**: 1-11 [PMID: 12693659]
27 **Soto-Gutierrez A**, Navarro-Alvarez N, Rivas-Carrillo JD, Chen Y, Yamatsuji T, Tanaka N, Kobayashi N. Differentiation of human embryonic stem cells to hepatocytes using deleted variant of HGF and poly-amino-urethane-coated nonwoven polytetrafluoroethylene fabric. *Cell Transplant* 2006; **15**: 335-341 [PMID: 16898227]
28 **Kamiya A**, Kinoshita T, Miyajima A. Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways. *FEBS Lett* 2001; **492**: 90-94 [PMID: 11248243]
29 **Schmidt C**, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, Gherardi E, Birchmeier C. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* 1995; **373**: 699-702 [PMID: 7854452 DOI: 10.1038/373699a0]
30 **Kinoshita T**, Sekiguchi T, Xu MJ, Ito Y, Kamiya A, Tsuji K, Nakahata T, Miyajima A. Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. *Proc Natl Acad Sci USA* 1999; **96**: 7265-7270 [PMID: 10377403]
31 **Kinoshita T**, Miyajima A. Cytokine regulation of liver development. *Biochim Biophys Acta* 2002; **1592**: 303-312 [PMID: 12421674]
32 **Kamiya A**, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K, Taga T, Yoshida K, Kishimoto T, Miyajima A. Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. *EMBO J* 1999; **18**: 2127-2136 [PMID: 10205167 DOI: 10.1093/emboj/18.8.2127]
33 **Funakoshi N**, Duret C, Pascussi JM, Blanc P, Maurel P, Daujat-Chavanieu M, Gerbal-Chaloin S.

Comparison of hepatic-like cell production from human embryonic stem cells and adult liver progenitor cells: CAR transduction activates a battery of detoxification genes. *Stem Cell Rev* 2011; **7**: 518-531 [PMID: 21210253 DOI: 10.1007/s12015-010-9225-3]

34 **Ogawa S**, Surapisitchat J, Virtanen C, Ogawa M, Niapour M, Sugamori KS, Wang S, Tamblyn L, Guillemette C, Hoffmann E, Zhao B, Strom S, Laposa RR, Tyndale RF, Grant DM, Keller G. Three-dimensional culture and camp signaling promote the maturation of human pluripotent stem cell-derived hepatocytes. *Development* 2013; **140**: 3285-3296 [PMID: 23861064 DOI: 10.1242/dev.090266]

35 **Borowiak M**, Maehr R, Chen S, Chen AE, Tang W, Fox JL, Schreiber SL, Melton DA. Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. *Cell Stem Cell* 2009; **4**: 348-358 [PMID: 19341624 DOI: 10.1016/j.stem.2009.01.014]

36 **Tahamtani Y**, Azarnia M, Farrokhi A, Sharifi-Zarchi A, Aghdami N, Baharvand H. Treatment of human embryonic stem cells with different combinations of priming and inducing factors toward definitive endoderm. *Stem Cells Dev* 2013; **22**: 1419-1432 [PMID: 23249309 DOI: 10.1089/scd.2012.0453]

37 **Siller R**, Greenhough S, Naumovska E, Sullivan Gareth J. Small-molecule-driven hepatocyte differentiation of human pluripotent stem cells. *Stem Cell Reports* 2015; **4**: 939-952 [PMID: 25937370 DOI: 10.1016/j.stemcr.2015.04.001]

38 **Shan J**, Schwartz RE, Ross NT, Logan DJ, Thomas D, Duncan SA, North TE, Goessling W, Carpenter AE, Bhatia SN. Identification of small molecules for human hepatocyte expansion and iPS differentiation. *Nat Chem Biol* 2013; **9**: 514-520 [PMID: 23728495 DOI: 10.1038/nchembio.1270]

39 **Li S**, Li M, Liu X, Yang Y, Wei Y, Chen Y, Qiu Y, Zhou T, Feng Z, Ma D, Fang J, Ying H, Wang H, Musunuru K, Shao Z, Zhao Y, Ding Q. Genetic and Chemical Screenings Identify HDAC3 as a Key Regulator in Hepatic Differentiation of Human Pluripotent Stem Cells. *Stem Cell Reports* 2018; **11**: 22-31 [PMID: 29861165 DOI: 10.1016/j.stemcr.2018.05.001]

40 **Rashid ST**, Corbineau S, Hannan N, Marciniak SJ, Miranda E, Alexander G, Huang-Doran I, Griffin J, Ahrlund-Richter L, Skepper J, Semple R, Weber A, Lomas DA, Vallier L. Modeling inherited metabolic disorders of the liver using human induced pluripotent stem cells. *J Clin Invest* 2010; **120**: 3127-3136 [PMID: 20739751 DOI: 10.1172/JCI43122]

41 **Zhang S**, Chen S, Li W, Guo X, Zhao P, Xu J, Chen Y, Pan Q, Liu X, Zychlinski D, Lu H, Tortorella MD, Schambach A, Wang Y, Pei D, Esteban MA. Rescue of ATP7B function in hepatocyte-like cells from Wilson's disease induced pluripotent stem cells using gene therapy or the chaperone drug curcumin. *Hum Mol Genet* 2011; **20**: 3176-3187 [PMID: 21593220 DOI: 10.1093/hmg/ddr223]

42 **Cayo MA**, Cai J, DeLaForest A, Noto FK, Nagaoka M, Clark BS, Collery RF, Si-Tayeb K, Duncan SA. JD induced pluripotent stem cell-derived hepatocytes faithfully recapitulate the pathophysiology of familial hypercholesterolemia. *Hepatology* 2012; **56**: 2163-2171 [PMID: 22653811 DOI: 10.1002/hep.25871]

43 **Ding Q**, Lee YK, Schaefer EA, Peters DT, Veres A, Kim K, Kuperwasser N, Motola DL, Meissner TB, Hendriks WT, Trevisan M, Gupta RM, Moisan A, Banks E, Friesen M, Schinzel RT, Xia F, Tang A, Xia Y, Figueroa E, Wann A, Ahfeldt T, Daheron L, Zhang F, Rubin LL, Peng LF, Chung RT, Musunuru K, Cowan CA. A TALEN genome-editing system for generating human stem cell-based disease models. *Cell Stem Cell* 2013; **12**: 238-251 [PMID: 23246482 DOI: 10.1016/j.stem.2012.11.011]

44 **Ding Q**, Regan SN, Xia Y, Oostrom LA, Cowan CA, Musunuru K. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. *Cell Stem Cell* 2013; **12**: 393-394 [PMID: 23561441 DOI: 10.1016/j.stem.2013.03.006]

45 **Cayo MA**, Mallanna SK, Di Furio F, Jing R, Tolliver LB, Bures M, Urick A, Noto FK, Pashos EE, Greseth MD, Czarnecki M, Traktman P, Yang W, Morrisey EE, Grompe M, Rader DJ, Duncan SA. A Drug Screen using Human iPSC-Derived Hepatocyte-like Cells Reveals Cardiac Glycosides as a Potential Treatment for Hypercholesterolemia. *Cell Stem Cell* 2017; **20**: 478-489.e5 [PMID: 28388428 DOI: 10.1016/j.stem.2017.01.011]

46 **Yoshida T**, Takayama K, Kondoh M, Sakurai F, Tani H, Sakamoto N, Matsuura Y, Mizuguchi H, Yagi K. Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection. *Biochem Biophys Res Commun* 2011; **416**: 119-124 [PMID: 22093821 DOI: 10.1016/j.bbrc.2011.11.007]

47 **Zhou XL**, Sullivan GJ, Sun P, Park IH. Humanized murine model for HBV and HCV using human induced pluripotent stem cells. *Arch Pharm Res* 2012; **35**: 261-269 [PMID: 22370780 DOI: 10.1007/s12272-012-0206-8]

48 **Pashos EE**, Park Y, Wang X, Raghavan A, Yang W, Abbey D, Peters DT, Arbelaez J, Hernandez M, Kuperwasser N, Li W, Lian Z, Liu Y, Lv W, Lytle-Gabbin SL, Marchadier DH, Rogov P, Shi J, Slovik KJ, Stylianou IM, Wang L, Yan R, Zhang X, Kathiresan S, Duncan SA, Mikkelsen TS, Morrisey EE, Rader DJ, Brown CD, Musunuru K. Large, Diverse Population Cohorts of hiPSCs and Derived Hepatocyte-like Cells Reveal Functional Genetic Variation at Blood Lipid-Associated Loci. *Cell Stem Cell* 2017; **20**: 558-570.e10 [PMID: 28388432 DOI: 10.1016/j.stem.2017.03.017]

49 **Warren CR**, O'Sullivan JF, Friesen M, Becker CE, Zhang X, Liu P, Wakabayashi Y, Morningstar JE, Shi X, Choi J, Xia F, Peters DT, Florido MHC, Tsankov AM, Duberow E, Comisar L, Shay J, Jiang X, Meissner A, Musunuru K, Kathiresan S, Daheron L, Zhu J, Gerszten RE, Deo RC, Vasan RS, O'Donnell CJ, Cowan CA. Induced Pluripotent Stem Cell Differentiation Enables Functional Validation of GWAS Variants in Metabolic Disease. *Cell Stem Cell* 2017; **20**: 547-557.e7 [PMID: 28388431 DOI: 10.1016/j.stem.2017.01.010]

50 **Carcamo-Orive I**, Hoffman GE, Cundiff P, Beckmann ND, D'Souza SL, Knowles JW, Patel A, Papatsenko D, Abbasi F, Reaven GM, Whalen S, Lee P, Shahbazi M, Henrion MYR, Zhu K, Wang S, Roussos P, Schadt EE, Pandey G, Chang R, Quertermous T, Lemischka I. Analysis of Transcriptional Variability in a Large Human iPSC Library Reveals Genetic and Non-genetic Determinants of Heterogeneity. *Cell Stem Cell* 2017; **20**: 518-532.e9 [PMID: 28017796 DOI: 10.1016/j.stem.2016.11.005]

51 **DeBoever C**, Li H, Jakubosky D, Benaglio P, Reyna J, Olson KM, Huang H, Biggs W, Sandoval E, D'Antonio M, Jepsen K, Matsui H, Arias A, Ren B, Nariai N, Smith EN, D'Antonio-Chronowska A, Farley EK, Frazer KA. Large-Scale Profiling Reveals the Influence of Genetic Variation on Gene Expression in Human Induced Pluripotent Stem Cells. *Cell Stem Cell* 2017; **20**: 533-546.e7 [PMID: 28388430 DOI: 10.1016/j.stem.2017.03.009]

52 **Kilpinen H**, Goncalves A, Leha A, Afzal V, Alasoo K, Ashford S, Bala S, Bensaddek D, Casale FP, Culley OJ, Danecek P, Faulconbridge A, Harrison PW, Kathuria A, McCarthy D, McCarthy SA, Meleckyte R, Memari Y, Moens N, Soares F, Mann A, Streeter I, Agu CA, Alderton A, Nelson R, Harper S, Patel M, White A, Patel SR, Clarke L, Halai R, Kirton CM, Kolb-Kokocinski A, Beales P, Birney E,

Danovi D, Lamond AI, Ouwehand WH, Vallier L, Watt FM, Durbin R, Stegle O, Gaffney DJ. Common genetic variation drives molecular heterogeneity in human iPSCs. *Nature* 2017; **546**: 370-375 [PMID: 28489815 DOI: 10.1038/nature22403]

53 **Warren CR**, Cowan CA. Humanity in a Dish: Population Genetics with iPSCs. *Trends Cell Biol* 2018; **28**: 46-57 [PMID: 29054332 DOI: 10.1016/j.tcb.2017.09.006]

54 **Huch M**, Bonfanti P, Boj SF, Sato T, Loomans CJ, van de Wetering M, Sojoodi M, Li VS, Schuijers J, Gracanin A, Ringnalda F, Begthel H, Hamer K, Mulder J, van Es JH, de Koning E, Vries RG, Heimberg H, Clevers H. Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. *EMBO J* 2013; **32**: 2708-2721 [PMID: 24045232 DOI: 10.1038/emboj.2013.204]

55 **Huch M**, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MM, Ellis E, van Wenum M, Fuchs SA, de Ligt J, van de Wetering M, Sasaki N, Boers SJ, Kemperman H, de Jonge J, Ijzermans JN, Nieuwenhuis EE, Hoekstra R, Strom S, Vries RR, van der Laan LJ, Cuppen E, Clevers H. Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell* 2015; **160**: 299-312 [PMID: 25533785 DOI: 10.1016/j.cell.2014.11.050]

56 **Ogawa M**, Ogawa S, Bear CE, Ahmadi S, Chin S, Li B, Grompe M, Keller G, Kamath BM, Ghanekar A. Directed differentiation of cholangiocytes from human pluripotent stem cells. *Nat Biotechnol* 2015; **33**: 853-861 [PMID: 26167630 DOI: 10.1038/nbt.3294]

57 **Sampaziotis F**, de Brito MC, Madrigal P, Bertero A, Saeb-Parsy K, Soares FAC, Schrumpf E, Melum E, Karlsen TH, Bradley JA, Gelson WT, Davies S, Baker A, Kaser A, Alexander GJ, Hannan NRF, Vallier L. Cholangiocytes derived from human induced pluripotent stem cells for disease modeling and drug validation. *Nat Biotechnol* 2015; **33**: 845-852 [PMID: 26167629 DOI: 10.1038/nbt.3275]

58 **Takebe T**, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, Zhang RR, Ueno Y, Zheng YW, Koike N, Aoyama S, Adachi Y, Taniguchi H. Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* 2013; **499**: 481-484 [PMID: 23823721 DOI: 10.1038/nature12271]

59 **Takebe T**, Zhang RR, Koike H, Kimura M, Yoshizawa E, Enomura M, Koike N, Sekine K, Taniguchi H. Generation of a vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nat Protoc* 2014; **9**: 396-409 [PMID: 24457331 DOI: 10.1038/nprot.2014.020]

60 **Patsch C**, Challet-Meylan L, Thoma EC, Urich E, Heckel T, O'Sullivan JF, Grainger SJ, Kapp FG, Sun L, Christensen K, Xia Y, Florido MH, He W, Pan W, Prummer M, Warren CR, Jakob-Roetne R, Certa U, Jagasia R, Freskgård PO, Adatto I, Kling D, Huang P, Zon LI, Chaikof EL, Gerszten RE, Graf M, Iacone R, Cowan CA. Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. *Nat Cell Biol* 2015; **17**: 994-1003 [PMID: 26214132 DOI: 10.1038/ncb3205]

61 **Coll M**, Perea L, Boon R, Leite SB, Vallverdú J, Mannaerts I, Smout A, El Taghdouini A, Blaya D, Rodrigo-Torres D, Graupera I, Aguilar-Bravo B, Chesne C, Najimi M, Sokal E, Lozano JJ, van Grunsven LA, Verfaillie CM, Sancho-Bru P. Generation of Hepatic Stellate Cells from Human Pluripotent Stem Cells Enables In Vitro Modeling of Liver Fibrosis. *Cell Stem Cell* 2018; **23**: 101-113.e7 [PMID: 30049452 DOI: 10.1016/j.stem.2018.05.027]

62 **Sun Y**, Ding Q. Genome engineering of stem cell organoids for disease modeling. *Protein Cell* 2017; **8**: 315-327 [PMID: 28102490 DOI: 10.1007/s13238-016-0368-0]

63 **Solanas E**, Pla-Palacín I, Sainz-Arnal P, Almeida M, Lue A, Serrano T, Baptista PM. Tissue organoids: Liver. *Soker S and Skardal A Tumor organoids*. Cham: Springer International Publishing 2018; 17-33

64 **Mito M**. Hepatic assist: present and future. *Artif Organs* 1986; **10**: 214-218 [PMID: 3741195]

65 **Sakiyama R**, Blau BJ, Miki T. Clinical translation of bioartificial liver support systems with human pluripotent stem cell-derived hepatic cells. *World J Gastroenterol* 2017; **23**: 1974-1979 [PMID: 28373763 DOI: 10.3748/wjg.v23.i11.1974]

66 **Struecker B**, Raschzok N, Sauer IM. Liver support strategies: cutting-edge technologies. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 166-176 [PMID: 24166083 DOI: 10.1038/nrgastro.2013.204]

67 **Shi XL**, Gao Y, Yan Y, Ma H, Sun L, Huang P, Ni X, Zhang L, Zhao X, Ren H, Hu D, Zhou Y, Tian F, Ji Y, Cheng X, Pan G, Ding YT, Hui L. Improved survival of porcine acute liver failure by a bioartificial liver device implanted with induced human functional hepatocytes. *Cell Res* 2016; **26**: 206-216 [PMID: 26768767 DOI: 10.1038/cr.2016.6]

68 **Mavri-Damelin D**, Damelin LH, Eaton S, Rees M, Selden C, Hodgson HJ. Cells for bioartificial liver devices: the human hepatoma-derived cell line C3A produces urea but does not detoxify ammonia. *Biotechnol Bioeng* 2008; **99**: 644-651 [PMID: 17680661 DOI: 10.1002/bit.21599]

69 **Allen KJ**, Soriano HE. Liver cell transplantation: the road to clinical application. *J Lab Clin Med* 2001; **138**: 298-312 [PMID: 11709654 DOI: 10.1067/mlc.2001.119148]

70 **Dhawan A**, Mitry RR, Hughes RD. Hepatocyte transplantation for liver-based metabolic disorders. *J Inherit Metab Dis* 2006; **29**: 431-435 [PMID: 16763914 DOI: 10.1007/s10545-006-0245-8]

71 **Fisher RA**, Strom SC. Human hepatocyte transplantation: worldwide results. *Transplantation* 2006; **82**: 441-449 [PMID: 16926585 DOI: 10.1097/01.tp.0000231689.44266.ac]

72 **Roy-Chowdhury N**, Wang X, Guha C, Roy-Chowdhury J. Hepatocyte-like cells derived from induced pluripotent stem cells. *Hepatol Int* 2017; **11**: 54-69 [PMID: 27530815 DOI: 10.1007/s12072-016-9757-y]

73 **Carpentier A**, Tesfaye A, Chu V, Nimgaonkar I, Zhang F, Lee SB, Thorgeirsson SS, Feinstone SM, Liang TJ. Engrafted human stem cell-derived hepatocytes establish an infectious HCV murine model. *J Clin Invest* 2014; **124**: 4953-4964 [PMID: 25295540 DOI: 10.1172/JCI75456]

74 **Nagamoto Y**, Takayama K, Tashiro K, Tateno C, Sakurai F, Tachibana M, Kawabata K, Ikeda K, Tanaka Y, Mizuguchi H. Efficient Engraftment of Human Induced Pluripotent Stem Cell-Derived Hepatocyte-Like Cells in uPA/SCID Mice by Overexpression of FNK, a Bcl-xL Mutant Gene. *Cell Transplant* 2015; **24**: 1127-1138 [PMID: 24806294 DOI: 10.3727/096368914X681702]

75 **Azuma H**, Paulk N, Ranade A, Dorrell C, Al-Dhalimy M, Ellis E, Strom S, Kay MA, Finegold M, Grompe M. Robust expansion of human hepatocytes in Fah-/-/Rag2-/-/Il2rg-/- mice. *Nat Biotechnol* 2007; **25**: 903-910 [PMID: 17664939 DOI: 10.1038/nbt1326]

76 **Asgari S**, Moslem M, Bagheri-Lankarani K, Pournasr B, Miryounesi M, Baharvand H. Differentiation and transplantation of human induced pluripotent stem cell-derived hepatocyte-like cells. *Stem Cell Rev* 2013; **9**: 493-504 [PMID: 22076752 DOI: 10.1007/s12015-011-9330-y]

77 **Liu H**, Kim Y, Sharkis S, Marchionni L, Jang YY. In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. *Sci Transl Med* 2011; **3**: 82ra39 [PMID: 21562231 DOI: 10.1126/scitranslmed.3002376]

78 **Nagamoto Y**, Takayama K, Ohashi K, Okamoto R, Sakurai F, Tachibana M, Kawabata K, Mizuguchi H. Transplantation of a human iPSC-derived hepatocyte sheet increases survival in mice with acute liver failure. *J Hepatol* 2016; **64**: 1068-1075 [PMID: 26778754 DOI: 10.1016/j.jhep.2016.01.004]

79 **Song W**, Lu YC, Frankel AS, An D, Schwartz RE, Ma M. Engraftment of human induced pluripotent stem cell-derived hepatocytes in immunocompetent mice via 3D co-aggregation and encapsulation. *Sci Rep* 2015; **5**: 16884 [PMID: 26592180 DOI: 10.1038/srep16884]

80 **Lemaigre FP**. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. *Gastroenterology* 2009; **137**: 62-79 [PMID: 19328801 DOI: 10.1053/j.gastro.2009.03.035]

81 **Okita K**, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 2008; **322**: 949-953 [PMID: 18845712 DOI: 10.1126/science.1164270]

82 **Takayama K**, Akita N, Mimura N, Akahira R, Taniguchi Y, Ikeda M, Sakurai F, Ohara O, Morio T, Sekiguchi K, Mizuguchi H. Generation of safe and therapeutically effective human induced pluripotent stem cell-derived hepatocyte-like cells for regenerative medicine. *Hepatol Commun* 2017; **1**: 1058-1069 [PMID: 29404442 DOI: 10.1002/hep4.1111]

83 **Ben-David U**, Gan QF, Golan-Lev T, Arora P, Yanuka O, Oren YS, Leikin-Frenkel A, Graf M, Garippa R, Boehringer M, Gromo G, Benvenisty N. Selective elimination of human pluripotent stem cells by an oleate synthesis inhibitor discovered in a high-throughput screen. *Cell Stem Cell* 2013; **12**: 167-179 [PMID: 23318055 DOI: 10.1016/j.stem.2012.11.015]

84 **Tang C**, Lee AS, Volkmer JP, Sahoo D, Nag D, Mosley AR, Inlay MA, Ardehali R, Chavez SL, Pera RR, Behr B, Wu JC, Weissman IL, Drukker M. An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. *Nat Biotechnol* 2011; **29**: 829-834 [PMID: 21841799 DOI: 10.1038/nbt.1947]

85 **Peters DT**, Henderson CA, Warren CR, Friesen M, Xia F, Becker CE, Musunuru K, Cowan CA. Asialoglycoprotein receptor 1 is a specific cell-surface marker for isolating hepatocytes derived from human pluripotent stem cells. *Development* 2016; **143**: 1475-1481 [PMID: 27143754 DOI: 10.1242/dev.132209]

86 **Kiuru M**, Boyer JL, O'Connor TP, Crystal RG. Genetic control of wayward pluripotent stem cells and their progeny after transplantation. *Cell Stem Cell* 2009; **4**: 289-300 [PMID: 19341619 DOI: 10.1016/j.stem.2009.03.010]

87 **Choi SH**, Kim YH, Quinti L, Tanzi RE, Kim DY. 3D culture models of Alzheimer's disease: a road map to a "cure-in-a-dish". *Mol Neurodegener* 2016; **11**: 75 [PMID: 27938410 DOI: 10.1186/s13024-016-0139-7]

88 **Giacomelli E**, Bellin M, Sala L, van Meer BJ, Tertoolen LG, Orlova VV, Mummery CL. Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells. *Development* 2017; **144**: 1008-1017 [PMID: 28279973 DOI: 10.1242/dev.143438]

89 **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, Laflamme 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]

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REVIEW

Human umbilical cord mesenchymal stem cells ameliorate liver fibrosis *in vitro* and *in vivo*: From biological characteristics to therapeutic mechanisms

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Abstract

Liver fibrosis is a wound-healing response to chronic injuries, characterized by the excessive accumulation of extracellular matrix or scar tissue within the liver; in addition, its formation is associated with multiple cytokines as well as several cell types and a variety of signaling pathways. When liver fibrosis is not well controlled, it can progress to liver cirrhosis, but it is reversible in principle. Thus far, no efficient therapy is available for treatment of liver fibrosis. Although liver transplantation is the preferred strategy, there are many challenges remaining in this approach, such as shortage of donor organs, immunological rejection, and surgical complications. Hence, there is a great need for an alternative therapeutic strategy. Currently, mesenchymal stem cell (MSC) therapy is considered a promising therapeutic strategy for the treatment of liver fibrosis; advantageously, the characteristics of MSCs are continuous self-renewal, proliferation, multipotent differentiation, and immunomodulatory activities. The human umbilical cord-derived (hUC)-MSCs possess not only the common attributes of MSCs but also more stable biological characteristics, relatively easy accessibility, abundant source, and no ethical issues (*e.g.*, bone marrow being the adult source), making hUC-MSCs a good choice for treatment of liver fibrosis. In this review, we summarize the biological characteristics of hUC-MSCs and their paracrine effects, exerted by secretion of various cytokines, which ultimately promote liver repair through several signaling pathways. Additionally, we discuss the capacity of hUC-MSCs to differentiate into hepatocyte-like cells for compensating the function of existing hepatocytes, which may aid in amelioration of liver fibrosis. Finally, we discuss the current status of the research field and its future prospects.

Key words: Human umbilical cord mesenchymal stem cells; Liver fibrosis; Hepatocyte-like cells; Mechanism; Cell therapy; Paracrine effect; Exosome; Transdifferentiation

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Core tip: Liver fibrosis is a major global health problem, for which no efficient therapy is available. Cell therapy, particularly involving human umbilical cord mesenchymal stem cells (known as hUC-MSCs), represents a promising therapeutic strategy, based mainly on the cells' paracrine effects, transdifferentiation capacity and immunomodulatory function. In this review, we discuss the characteristics of hUC-MSCs, focusing on the possible mechanisms of these cells to ameliorate liver fibrosis, based upon evidence from *in vitro* and *in vivo* studies as well as ongoing clinical trials. This review also includes a discussion of the current status of the field and its future prospects.

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INTRODUCTION

Liver fibrosis is a common outcome of severe chronic liver injuries, characterized by imbalance in the production and degradation of extracellular matrix (ECM). It can be triggered by viruses, alcohol abuse, drug abuse, and autoimmunity[1]. In the early stages of liver fibrosis[1], the ECM deposition can be hydrolyzed by proteolytic enzymes, such as matrix metalloproteinases. However, continuous damage will lead to the accumulation of matrix components, such as collagen I and collagen III, leading to scar tissue deposition and the onset of an inflammatory process[2-6]. Notably, several studies have shown that hepatic stellate cells (HSCs) play a critical role in liver fibrosis (Figure 1).

When the liver is exposed to various injuries, quiescent HSCs change into activated HSCs, which are the major source of collagen and ECM proteins. Under the action of various cytokines, such as inflammatory mediators, released by activated Kupffer cells, the activated HSCs then differentiate into myofibroblasts[7]. Furthermore, activated HSCs promote the activation of peripheral static HSCs and promote the development of liver fibrosis through paracrine and autocrine modes. A variety of cytokines[8-12] (*i.e.*, transforming growth factor-beta (TGF-β), platelet-derived growth factor, connective tissue growth factor, fibroblast growth factor (FGF), interferon-gamma, and leptin) are involved in the activation of HSCs through multiple signaling pathways[8] (*e.g.*, TGF-β/Smad, Ras/ERK, Notch[13], and Wnt/β-catenin[14]). As such, liver fibrosis is associated with multiple cytokines, several cell types, and a variety of signaling pathways. Without efficient treatment, liver cirrhosis or liver failure can occur, and the risk of hepatocellular carcinoma is increased[3].

Although numerous drugs have been shown to exhibit antifibrotic activity both *in vitro* and in animal models, none have been effective for clinical use. Until now, liver transplantation remains the only effective therapy for end-stage liver disease[15]. The primary limitation of this treatment, however, is a shortage of donor organs. Moreover, adverse effects (*e.g.*, rejection) as well as the high cost of the procedure and inevitable side effects associated with long-term postoperative use of immunosuppressants make liver transplantation unfavorable for many patients[16,17]. These ongoing challenges highlight the need for new therapeutic strategies to treat liver fibrosis in effective, safe and convenient ways.

Currently, MSCs are a focus of research regarding treatment of liver diseases[18-21] because they exhibit the following characteristics: multidifferentiation potential, strong proliferative ability, immune regulation, and self-replication[15,22,23]. MSCs can be obtained from a variety of tissues, including bone marrow, adipose tissue, umbilical cord tissue, placenta, umbilical cord blood, amniotic fluid, peripheral blood, liver, lung, endodontic pulp, skeletal muscle[24], and hair follicles. Several researchers have cited that human umbilical cord-derived (hUC)-MSCs are a superior choice over the other types of MSCs, as they have no substantial ethical challenges, exhibit a low risk of viral transmission[25] and low immunogenicity, are readily available, and are more primitive[26].

In this review, the biological characteristics of hUC-MSCs are discussed, as are their possible underlying therapeutic mechanisms, clinical applications, and future

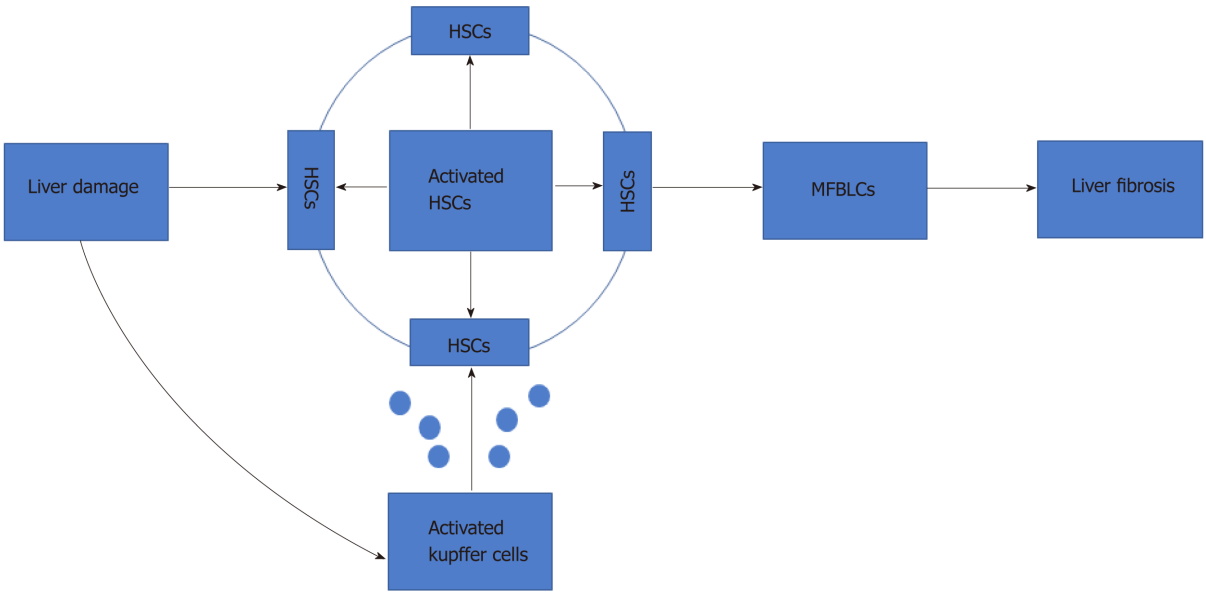

Figure 1 Process of hepatic stellate cells' activation. HSC: Hepatic stellate cell; MFBLC: Myo-fibroblast-like cell.

prospects for amelioration of liver fibrosis.

CHARACTERISTICS OF hUC-MSCs

During the 5th wk of gestation, the human umbilical cord begins to develop, and it can grow up to 50 cm in length[27]. The hUC itself can be obtained after delivery, as it is considered medical waste; this convenient acquirement dovetails with the recognition of it as an ideal source of MSCs[28,29].

Sources of hUC-MSCs

Recently, researchers have isolated six types of MSCs from different portions of the hUC (Figure 2)[30]; these include the amnion, subamnion, perivascular, Wharton's jelly, mixed cord, and cord blood. Notably, Wharton's jelly[31-33] has become the major source of MSCs[34-38].

Isolation of hUC-MSCs

There are three main methods for isolation of hUC-MSCs, these being explant, enzymatic digestion, and a so-called "mixed" method[39].

The explant method is also known as "tissue block adherence". Mennan *et al*[38] reported that explants could be obtained from Wharton's jelly that had been minced into small pieces and then cultured in basal medium supplemented with 100 mL/L fetal calf serum. This explant method is relatively simple and inexpensive, but requires extended time for subculturing (typically 20-30 d) because of the low adhesion efficiency of tissue explants and the slow migration of cells out of tissue explants (approximately 5-10 d). To overcome these disadvantages, explant reculture[40] and multiple adherence methods[41] have been devised to obtain larger numbers of primary culture cells. According to a report by Lu *et al*[40], unattached tissues could be recovered and subsequently recultured to generate more stable hUC-MSCs, which were found to express similar biomarkers and differentiation capacity. hUC-MSCs can also be obtained by an enzymatic digestion method[32]; For this, the whole cord is cut into small pieces and digested with collagenase, followed by centrifugation of the supernatant, resuspension of the resulting pellet in medium, and seeding in a tissue culture flask. Other approaches to digest the tissues[42-44] include the use of collagenase with or without trypsin, or a combination of collagenase or hyaluronidase with or without trypsin. In 2007, Ding *et al*[32] reported a mixed method to obtain hUC-MSCs. For this, the Wharton's jelly is cut into small pieces, treated with collagenase type I for 14–18 h, and then explant-cultured using the above-described tissue block adherence method.

Overall, the explant method is conducive for maintenance of cell quality, avoids contamination, involves a simple procedure, and is inexpensive. Therefore, this method has been widely adopted, although it requires additional time for

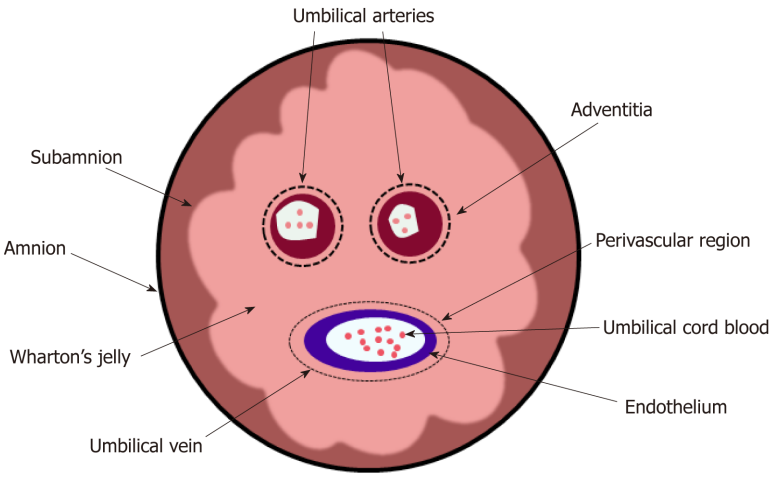

Figure 2 Cross-sectional diagram of human umbilical cord showing anatomical compartments, including Wharton's jelly, as a source of mesenchymal stem cells.

subculturing of the hUC-MSCs. The enzymatic digestion method can provide more homogenous cell populations in a shorter time[45] but the cell quality and functions can be degraded compared with the former method[46,47]. Additionally, this method is difficult to control the digestion time and it is expensive. The mixed method procedure is more complicated and can lead to cell contamination more easily. Thus, hUC-MSCs' isolation protocols need to be improved to obtain a greater number of primary cells in a short time.

Cultivation and expansion of hUC-MSCs

Many factors influence the growth of hUC-MSCs, including basal medium, serum, and substitutes of fetal bovine serum (FBS).

The various types of basal medium used to culture hUC-MSCs include DMEM-LG[48-50], DMEM/F12[51-53], α-MEM[54-56], and MSC-qualified medium[57]. In our culture strategy[58], we have used DMEM/F12 (1:1) as our basal medium, supplemented with 100 mL/L FBS, to culture and proliferate hUC-MSCs. The resulting cells exhibit typical features of MSCs in morphology, proliferation capacity, and the surface antigen profiles.

Though FBS is always used in cell culture of hUC-MSCs, some inherent factors can elicit negative effects in clinical treatment. Fortunately, blood components of autologous or allogeneic donors (*e.g.*, human serum, plasma, platelet lysate, and cord blood serum) have been confirmed by Bieback *et al*[59] as alternative media supplements for clinical use. Wu *et al*[60] cultured and cryopreserved hUC-MSCs with hUC blood plasma. The resulting phenotype and differentiation potential (osteogenic and adipogenic) were almost indistinguishable from those of cells cultured with FBS; moreover, cells cultured with cord blood plasma demonstrated significantly greater proliferation rates than those cultured with FBS. In a separate study by Sun *et al*[61], when hUC-MSCs were cultured *in vitro* using a human platelet lysate culture system, the stemness was maintained in a more consistent manner. Additional findings regarding proteinaceous medium additives have been reported by Hatlapatka *et al*[62], specifically being that human serum appears to support optimal growth conditions and efficient cell expansion.

Furthermore, the addition of a subset of growth factors into the medium, such as epithelial growth factor (EGF), FGF, platelet-derived growth factor, TGF-β and insulin growth factor-1, is conducive to the maintenance of stemness among stem cells[63].

In brief, a serum-free culture system can maintain the growth and propagation of hUC-MSCs *in vitro* through the addition of nutrients and growth factors. This approach avoids the negative effects of FBS, maintains hUC-MSCs stemness, and improves hUC-MSCs' proliferation efficiency. However, in a serum-free culture system, cells tend to lose their stemness characteristics and exhibit reduced proliferation efficiency as the number of passages increases[61]. Therefore, optimal nutrients and growth factors must be selected to maintain the biological characteristics of hUC-MSCs.

Biomarkers of hUC-MSCs

Thus far, no surface markers have been found that are characteristic of MSCs, likely because cell phenotype is influenced by medium composition, cell seeding density,

and oxygen partial pressure.

Although a variety of markers have been described, the International Society for Cellular Therapy has proposed the following set of minimum criteria to define MSCs: (1) Plastic adherence; (2) Presence of a specific set of cell surface markers (CD73, CD90, CD105) and concomitant absence of other markers (CD14, CD34, CD45, and human leukocyte antigen-DR); and (3) Ability to differentiate into adipocytes, chondrocytes, and osteoblasts *in vitro*[64]. Importantly, hUC-MSCs, like other MSCs, meet these criteria.

Gene expression analyses[65,66] have shown hUC-MSCs to be a primitive type of cell, with a phenotype between that of adult stem cells and embryonic stem cells, expressing unique molecular markers of various embryonic stem cells, such as OCT4, SOX2, and NANOG.

Furthermore, hUC-MSCs express many biomarkers that are beneficial for transplantation. Notably, hUC-MSCs do not express the major histocompatibility complex-II or costimulatory molecules CD80 and CD86; this may facilitate allogeneic and heterogeneic transplantation. Moreover, hUC-MSCs produce moderate amounts of major histocompatibility complex-I[67] and constitutively express B7 homolog 1, a negative regulator of T cell activation.

Overall, regardless of the method used for culture, some cell phenotypes remain consistent among the hUC-MSCs. For example, surface markers of interstitial cells, endothelial cells, and epidermal cells are expressed, whereas hematopoietic stem cell markers are not. The hUC-MSCs are more primitive than other sources of MSCs and beneficial for transplantation.

Bioactive molecules secreted by hUC-MSCs

The hUC-MSCs can secrete multiple bioactive molecules that exert specific physiological functions in a variety of processes. Wang[68] reported the identification of 236 proteins within hUC-MSCs-conditioned medium, of which 114 were known; these proteins included cytokines, transporters, receptors, and binding proteins that participated in 15 biological processes and 14 molecular functions (*e.g.*, growth, proliferation, differentiation, inflammatory responses, immune responses, maintenance of homeostasis[69], angiogenesis, and apoptosis[70]). Notably, some of the identified proteins are involved in amelioration of liver fibrosis.

Three-dimensional (3D) culture system

The conventional adherent monolayer culture system for growth of stem cells has many limitations. For example, it provides insufficient yield to meet clinical demands, requires frequent digestion and subculturing of cells, and carries a high risk for contamination. More importantly, this method fails to recapitulate the *in vivo* native 3D cellular microenvironment, and may thus result in phenotypic changes as well as impairment of homing and migration capacities[54]. Therefore, to ensure high-quality and high-quantity preparation of hUC-MSCs, 3D culture systems have been explored as an ideal preparatory and delivery method.

Li *et al*[48] established a 3D culture system for hUC-MSCs, in which primary hUC-MSCs were isolated and grown in serum-free medium on a suspension rocker system for 3 d. Compared with monolayer culture, the 3D culture system yielded more hUC-MSCs within the same volume, in a spheroid morphology. The spheroids expressed higher levels of stem cell markers and more robust multipotency. After transplantation into carbon tetrachloride (CCl_4)-injured mice, the 3D culture-grown hUC-MSCs alleviated liver necrosis and promoted regeneration significantly, as compared with monolayer-cultured hUC-MSCs. In a study by Zhou *et al*[54], hUC-MSCs were seeded in a 3D culture system with porcine acellular dermal matrix, which led to increased expression of Toll-like receptors, C-X-C chemokine receptor type 4, and CD34 and CD271 surface markers and decreased expression of CD105. Moreover, the numbers of migratory 3D-grown hUC-MSCs in the liver were significantly greater than the numbers of monolayer-grown hUC-MSCs.

In general, the characteristics of hUC-MSCs in 3D culture resemble those of hUC-MSCs more closely in their native environments, whereas characteristics of hUC-MSCs in two-dimensional (2D) culture do not exhibit this similarity. Thus, 3D culture enables reversal of changes in certain phenotypic markers and chemokine receptors as well as restoration[71] of some functional loss (*e.g.*, homing, migration, and immune regulation) which occur in the 2D system. Therefore, this approach represents a promising strategy for hUC-MSCs expansion on an industrial scale, with great potential for cell therapy and biotechnology.

Potential for differentiation into hepatocyte-like cells in vitro

The hUC-MSCs have been demonstrated to be multipotent, and can differentiate into endodermal cell types as well as most mesodermal and ectodermal cell types[72].

Several studies have shown that hUC-MSCs could differentiate into hepatocyte-like cells *in vitro*. Below, we summarize some of the protocols reported for hepatic differentiation of hUC-MSCs, such as the addition of cell growth factors or small molecules, induction by conditioned medium (except liver fibrosis-conditioned medium), coculture with normal hepatocytes, as well as genetic manipulation.

Addition of cell growth factors and small molecules into the medium: Among the existing induction methods, the cytokine combination method has been widely studied, due to the need for exact components and doses, as well as its benefit of easy repeatability. Campard *et al*[31] differentiated hUC-MSCs into hepatocyte-like cells using a three-stage protocol. First, hUC-MSCs were seeded in IMDM containing EGF and basic (b)FGF, and cultured for 2 d. Second, hepatocyte growth factor (HGF), bFGF, nicotinamide, and insulin-transferrin-selenium premix was added and the culture continued for 10 d. Third, oncostatin M (OSM), dexamethasone, and insulin-transferrin-selenium premix was added and the culture continued for another 10 d. The resulting hUC-MSCs exhibited hepatocyte-like morphology, having up-regulation of several hepatic markers, presence of stored glycogen, functional urea production, and inducible CYP3A4 activity. However, the absence of some hepatic markers [*e.g.*, HepPar1 or hepatocyte nuclear factor 4 (HNF4)] in the differentiated hUC-MSCs implied that differentiation did not reach the level of mature hepatocytes. Similarly, Zhao *et al*[73] used a medium containing HGF, bFGF, dexamethasone, insulin, and sodium selenite to culture hUC-MSCs for 16 d, after which the cells were transferred into OSM-containing medium. The resulting hUC-MSCs exhibited a high hepatic differentiation ability and hepatocyte-specific functions.

To resolve the low differentiation efficiency, Su *et al*[57] investigated the effect of valproic acid (VPA), a histone deacetylase inhibitor, on hepatic differentiation of hUC-MSCs. In that study, hUC-MSCs were treated with VPA for 6 h and then differentiated for 15 d in insulin-transferrin-selenium medium containing 20 ng/mL HGF, 10 ng/mL OSM, and 10^{-6} mol/L dexamethasone. The resulting cells showed expression of endodermal genes and secretion of urea; in addition, the number of albumin (ALB)-positive hUC-MSCs was profoundly increased in response to the VPA pretreatment. Moreover, the expression levels of phospho-AKT1 and ERK1/2 proteins were increased in these hUC-MSCs. Together, the results suggest that VPA promotes hepatic differentiation of hUC-MSCs by up-regulating expression of endodermal genes through AKT and ERK activation.

Overall, many cytokines can induce hepatic differentiation of hUC-MSCs but problems persist in the form of low induction efficiency as well as in the presence of immature, heterogeneous hepatocyte-like cells. Therefore, a standard induction protocol should be established; this will, however, depend upon discoveries of specific key factors and a consistent mechanism for hepatic differentiation.

Conditioned medium: Previous findings have suggested that, under defined conditions (*e.g.*, a simulated normal liver microenvironment), hUC-MSCs can differentiate into functional hepatocytes. Yan *et al*[74] explored the use of fetal liver-conditioned medium for hepatic differentiation of hUC-MSCs. The expression of MSC-specific markers decreased, while hepatocyte-specific gene expression was increased. Urea production, ALB secretion, glycogen storage, and CYP3A4 activity were significantly enhanced in the fetal liver-conditioned medium-treated cells. Furthermore, the protein expression levels of P-ERK, P-Raf, and P-MEK increased significantly in fetal liver-conditioned medium-treated hUC-MSCs.

Similarly, Xue *et al*[75] simulated a liver microenvironment *in vitro* by using liver homogenate supernatant, which induced differentiation of hUC-MSCs into hepatocyte-like cells expressing the hepatocyte markers α-fetoprotein (AFP), cytokeratin (CK)18, and tryptophan 2,3-dioxygenase (TPH2). Moreover, CYP3A enzyme activity, as well as ALB and urea secretion, indicative of hepatocyte function, were also significantly increased by the liver homogenate supernatant induction. Thus, the liver microenvironment is a key factor in the differentiation of hUC-MSCs into hepatocytes.

Overall, hepatic differentiation of hUC-MSCs can be promoted through coculture with conditioned medium. Thus, interactions between stem cells and their microenvironment are considered to be the primary mechanism regulating stem cell self-renewal and differentiation. This is a feasible method to easily differentiate hUC-MSCs into functional hepatocyte-like cells.

Coculture with normal hepatocytes: In a study by Li *et al*[76], hUC-MSCs were cocultured with liver LO2 cells for 7, 14 and 21 d. In that study, AFP mRNA was found on the 7th d after coculture, and the expression of ALB and CK19 mRNA, both hepatocyte-specific markers, reached detectable levels at 7 d, remaining at d 14 and

21, after the coculture. In particular, the mRNA expression levels of ALB and human CK19 were increased at 14 d, and glycogen staining was positive after the coculture for 21 d. However, at 21 d, AFP expression was no longer detectable in the cocultured cells. These results indicated that hUC-MSCs can differentiate into hepatocyte-like cells after coculture with normal hepatocytes; notably, increased induction time leads to more mature differentiated cells.

Gene transfection in hUC-MSCs: The hepatic differentiation status of hepatocyte-like cells derived from stem cells is inadequate for clinical use because of relatively low expression levels of functional proteins and lack of full induction of metabolic activity[77]; therefore, genetic manipulation may be an ideal strategy to resolve this problem.

There have been many reports regarding overexpression of HNF4α and some micro (mi)RNAs in several types of MSCs to facilitate differentiation into hepatocyte-like cells[78-80]. HNF4α is a dominant transcriptional regulator of hepatocyte differentiation and hepatocellular carcinogenesis. Moreover, HNF4α-overexpressing hUC-MSCs have been established[81], and hepatocyte-specific proteins and genes have been significantly enhanced by the activation of several target genes, including liver-enriched transcription factors[82].

However, transcription factors are not the only molecules that can promote cell transdifferentiation; miRNAs can also be used for this purpose[83-85]. Transfection with several miRNAs has been reported to induce the transdifferentiation of hUC-MSCs into hepatocyte-like cells, or even mature hepatocytes.

Cui *et al*[86] transfected six miRNAs (miR-1246, miR-1290, miR-148a, miR-30a, miR-424, miR-542-5p) and the liver-enriched miR-122 together, and observed stimulation of hMSCs' conversion into functionally mature induced hepatocytes (known as iHeps). Additionally, after transplantation of the iHeps into mice with CCl_4-induced liver injury, the iHeps not only were able to improve liver function but also restored injured liver tissue. The findings from that study indicate that miRNAs have the capability to directly convert hMSCs to a hepatocyte phenotype *in vitro*. In order to screen out the optimal miRNA candidates for hepatic differentiation of hUC-MSCs, Zhou *et al*[87] sequentially removed individual miRNAs from the seven-member pool of miR-1246, miR-1290, miR-148a, miR-30a, miR-424, miR-542-5p and miR-122, performing transfection with the remainder. Examination of the relevant indices (*e.g.*, hepatic markers, glycogen storage, low density lipoprotein uptake, and urea production) showed that five of the miRNAs (miR-122, miR-148a, miR-424, miR-542-5p and miR-1246) were essential for this process. Similarly, Khosravi *et al*[88] transfected miR-106a, miR-574-3p, and miR-451 into hUC-MSCs to study hepatic differentiation. The up-regulation of any of these three miRs alone did not induce expression of all hepatocyte-specific genes, but led to Sox17 and FoxA2 expression, both being factors involved in the initiation of hepatic differentiation. Furthermore, through concurrent ectopic overexpression of the three miRs (miR-106a, miR-574-3p, and miR-451), hUC-MSCs could be induced into functionally mature hepatocytes with the mRNA expression of the hepatocyte-specific genes HNF4α, ALB, and CK18, which began to increase significantly only in the terminal phases of differentiation.

Notably, gene transfection methods exhibit greater induction efficiency and shorter intervals for target cell production, compared with previous methods, but their safety and ethical issues must be considered carefully. Furthermore, the key molecules and mechanism must be explored in detail.

Promotion of hepatic differentiation of hUC-MSCs by 3D culture system: Recent studies have found that 3D scaffolds contain the main ECM components, such as heparin, which may provide an appropriate microenvironment for hepatocyte function. Aleahmad *et al*[89] revealed that 3D culture can prevent loss of hepatocyte function and improve efficiency of hepatocyte differentiation. When hUC-MSCs were cultured within 3D heparinized collagen scaffolds, the cells expressed early liver-specific markers, at both the gene and protein levels, including HNF4α, ALB, CK18 and CK19, as well as late liver-specific markers, such as G6P and CYP2B. In addition, the cells stored more glycogen than cells cultured in 2D collagen gels. Different from the above-described results, Khodabandeh *et al*[90] studied hUC-MSCs cultured in 3D collagen scaffolds for 21 d; they showed that the mRNA expression levels of ALB, AFP, CK18, CK19, G6P, and CYP2B did not significantly differ between cells cultured in 3D collagen scaffolds, 2D collagen films, or conventional monolayer culture. However, claudin expression was significantly increased in the cells cultured in 3D collagen scaffolds, compared with those cultured in 2D collagen films or conventional monolayer culture; this finding indicated that 3D collagen scaffolds provided adequate ECM for induction of cellular interconnection.

In general, 3D culture can better mimic the *in vivo* microenvironment, thereby

improving stem cell functions, such as differentiation, proliferation, response to stimulation, and gene expression. However, there remain many issues to be explored to facilitate differentiation of hUC-MSCs into hepatocyte-like cells or mature hepatocytes; for example, further optimization is needed for exogenous small molecules to mimic the *in vivo* microenvironment, and greater understanding is needed regarding the mechanism of action between components of the ECM/scaffold materials and stem cells.

EFFECTS AND MECHANISMS OF hUC-MSCs FOR AMELIORATION OF LIVER FIBROSIS

Recently, several studies have provided encouraging results regarding the use of hUC-MSCs in the treatment of various hepatic injuries[31,91,92], such as liver fibrosis. The known mechanisms of hUC-MSCs' ability to ameliorate liver fibrosis include paracrine effects, transdifferentiation into hepatocyte-like cells, and immunomodulatory functions[93]. This section of the review focuses on the first two mechanisms, as determined through *in vitro* and *in vivo* studies.

Paracrine effects of hUC-MSCs for amelioration of liver fibrosis

MSCs from sources other than hUC can secrete various cytokines, HGF, and interluekin-10[94], which can promote liver repair; other cytokines or growth factors can inhibit the occurrence of liver cirrhosis[95]. Furthermore, researchers have reported that hUC-MSCs exert antifibrosis functions in a paracrine manner through the TGF-β1/Smad signaling pathway[22,96].

In the development of liver fibrosis, TGF-β1 (Figure 3) plays a critical role in hepatic fibrogenesis and the development of cirrhosis[97]. Several studies have shown that the TGF-β/Smad pathway is one of the most important signaling pathways related to liver fibrosis[49]. The Smad protein family participates in a downstream signaling pathway of TGF-β. The Smad proteins are classified according to their receptor activation and inhibitory functions. The former group includes Smad3, which can transfer signals from the cytoplasm to the nucleus, thereby promoting onset of liver fibrosis. Inhibitory Smad proteins include Smad7, which can block the formation of Smad complexes and the resulting signal transduction process, thereby inhibiting the onset of fibrosis[98].

Bioactive factors and cytokines released from hUC-MSCs: In the past, most studies have focused on indirect coculture of hUC-MSCs and HSCs *in vitro*, and/or transplantation of hUC-MSCs into fibrotic animal models *in vivo*. The subsequent assessment of changes in liver fibrosis indices (with the exception of transdifferentiation into ALB-expressing or AFP-expressing hepatocytes) served to illustrate the antifibrotic effect of hUC-MSCs.

Using an *in vitro* approach, Zhang *et al*[22] reported that indirect coculture of hUC-MSCs and LX2 cells could determine the paracrine effect of hUC-MSCs on the proliferation of HSCs. In that study, the proliferation of LX2 cells was inhibited and the apoptosis level was increased in the coculture condition. Furthermore, the expression levels of TGF-β1 and Smad3 (both mRNA and protein) were reduced, whereas expression of Smad7 (both mRNA and protein) was increased in the coculture condition. These results indicated that the paracrine effect of hUC-MSCs inhibited proliferation of HSCs, possibly by inhibition of TGF-β1 and Smad3 expression and enhancement of Smad7 protein expression.

In vivo studies by Tsai *et al*[92] revealed that hUC-MSCs injected into CCl_4-induced rats did not differentiate into ALB-expressing or AFP-expressing hepatocytes. Thus, the effect of hUC-MSCs on reducing fibrogenesis most likely relies on release of bioactive factors or cytokines from grafted hUC-MSCs to trigger liver regeneration, rather than on differentiation of these cells into hepatocytes. In the same study, human cytokine assay analysis revealed that the amounts of human cutaneous T cell-attracting chemokine, leukemia inhibitory factor, and prolactin were substantially increased in livers of CCl_4-induced rats after transplantation of hUC-MSCs; prolactin is widely considered to be a hepatotrophic hormone[99]. Additionally, Elmahdy *et al*[100] studied the antifibrotic potential of hUC mononuclear cells in CCl_4-induced liver fibrosis in mice. The levels of alanine aminotransferase, aspartate transaminase, malondialdehyde, hydroxyproline, laminin, TGF-β1, and tumor necrosis factor-α were reduced, whereas the level of glutathione was significantly increased, when the rats were treated with the hUC mononuclear cells. These results indicated a potential therapeutic effect of hUC mononuclear cells in ameliorating liver fibrosis through reduction of oxidative stress and inflammatory mediators.

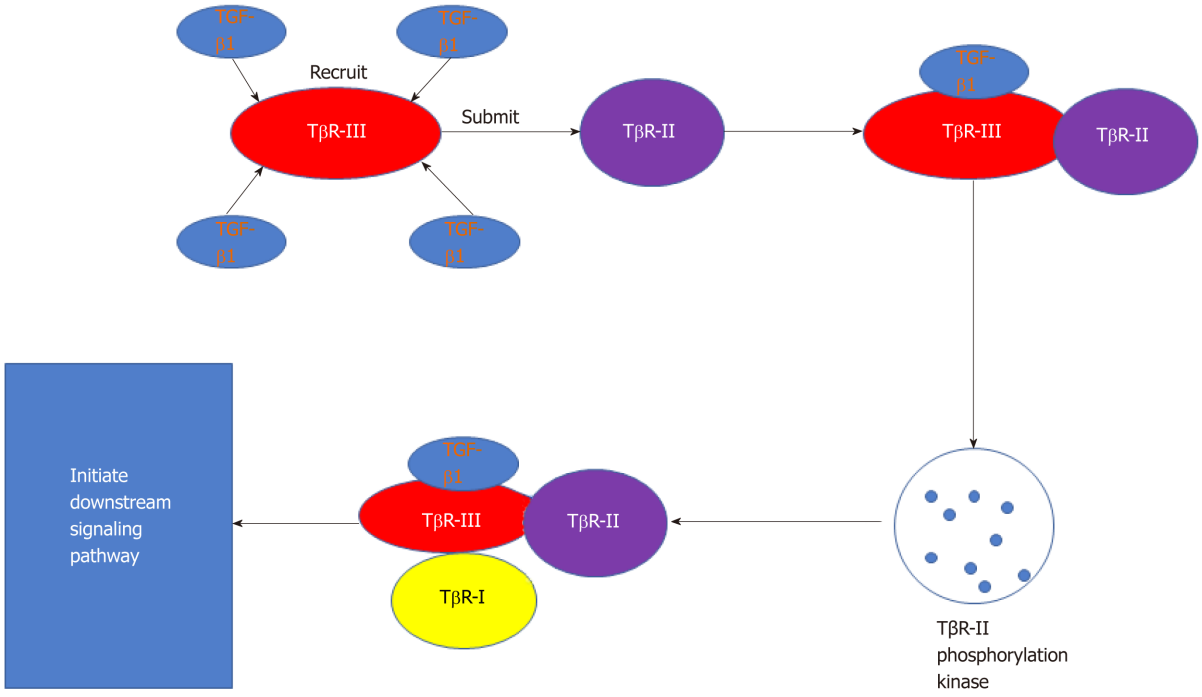

Figure 3 TGF-β1 receptors and intracellular signal transmission. TβR type I, type II and type III have high affinity with TGF-β1. The TβR-III recruits TGF-β1 and submits it to TβR-II, activating TβR-II phosphorylation kinase when it combines to type II receptors. Then, they are recognized and combined by TβR-I, forming a tetramer ligand receptor complex and initiating downstream signaling pathways. TβR: TGF-β receptor; TGF: Transforming growth factor.

The milk fat globule-EGF factor 8 (MFGE8) was identified by Su *et al*[101] as a novel key antifibrotic factor, based on its role in modulation of TGFβ signaling. In that study, the secretome of hUC-MSCs (referred to here as the 'hUC-MSC-scrtm') showed strong antifibrogenic activity. In an *in vivo* analysis of mice, the hUC-MSC-scrtm was injected into mice with fibrosis; this led to a significant reduction in expression of α-smooth muscle actin and fibrosis-related genes. Furthermore, *in vitro* analyses of human HSC lines (hTert-HSC and LX2) and human primary HSCs revealed that TGFβ1-induced up-regulation of α-smooth muscle actin and phosphorylation of Smad2 (which modulates expression of α-smooth muscle actin and procollagen I in liver fibrosis) were significantly inhibited by treatment with the hUC-MSC-scrtm. Regarding MFGE8 suppression of TGFβ-induced HSCs' activation, MFGE8 has been shown to be involved in diverse cellular events, *via* binding to the αvβ3 integrin on cell surfaces through its RGD domain[102]. Furthermore, they also found that the injection of recombinant human MFGE8 also produced antifibrotic effects, such as reduction of ECM deposition and HSC activation.

Although hUC-MSCs can secrete hundreds of cytokines or bioactive factors, few have been identified as antifibrotic factors. Other antifibrotic cytokines and mechanisms involved in the antifibrotic effect must be clarified in the future.

Exosomes released from hUC-MSCs: Exosomes comprise a primary subclass of extracellular vesicles[103], which are small biological membrane vesicles secreted by various cells. Exosomes contain a cargo of genetic materials (mRNA, miRNA, premiRNA, and other noncoding RNA) and proteins that are transferred to and released into target cells; notably, exosomes are critical for cell-to-cell communication. MSC-secreted exosomes are increasingly regarded as a novel cell-free therapy, in that they have many advantages over the use of corresponding MSCs. In particular, exosomes are smaller and less complex than their parent cells, easier to produce and store, devoid of viable cells, exhibit no risk of tumor formation, and are less immunogenic than their parent cells because of their lower membrane-bound protein content[104].

Several studies have focused on the therapeutic effects of hUC-MSC-derived exosomes (hUC-MSC-Ex) in liver fibrosis. Li *et al*[49] found that 3 wk after injection of 250 μg hUC-MSC-Ex into the livers of CCl_4-induced liver injury mice led to reduction of fibrous surface capsules and softened textures; moreover, this procedure alleviated hepatic inflammation and collagen deposition. It also significantly restored serum aspartate transaminase activity and reduced the expression of TGF-β1 and collagen types I and III as well as the phosphorylation of Smad2. Moreover, expression of the

epithelial-mesenchymal transition-associated marker E-cadherin increased, whereas the expression levels of N-cadherin and vimentin (also epithelial-mesenchymal transition-associated markers) decreased after hUC-MSC-Ex transplantation. *In vitro* experiments showed that, in TGF-β1-induced HL7702 cells, 3 d of treatment with 100 μg/mL hUC-MSC-Ex led to increased E-cadherin mRNA expression as well as decreased N-cadherin and Twist mRNA expression. Taken together, these results suggest that hUC-MSC-Ex could ameliorate CCl_4-induced liver fibrosis through inactivation of the TGF-β/Smad signaling pathway and protection of hepatocytes by inhibiting the epithelial-mesenchymal transition.

Jiang *et al*[105] investigated the effects and underlying mechanism of hUC-MSC-Ex treatment on liver fibrosis. Mice with CCl_4-induced liver fibrosis were treated with 6.4 × 10^9 particles of hUC-MSC-Ex by tail vein injection. When the mice were sacrificed 1 mo later, hematoxylin-eosin and Masson staining showed that the hUC-MSC-Ex treatment had inhibited infiltration of inflammatory cells, hepatocyte apoptosis, and lobule destruction; moreover, the treatment had led to decreased collagen deposition. After the hUC-MSC-Ex injection, the expression levels of collagen I and III had decreased remarkably, as did the level of activated caspase 3 and production of 8-hydroxy-2 deoxyguanosine in liver fibrosis. The levels of malondialdehyde and TGFβ were also reduced. Thus, the hUC-MSC-Ex may facilitate oxidation resistance and antiapoptotic functions in liver fibrosis. Overall, as carriers involved in intercellular signal transduction and substance transfer, the hUC-MSC-Ex enable substantial amelioration of liver fibrosis.

Transdifferentiation into hepatocyte-like cells within liver fibrosis conditions

Differentiation of stem cells may be influenced by their microenvironment, as noted earlier in this review. It is speculated that, upon differentiation into hepatocytes in the microenvironment of liver fibers, hUC-MSCs can be used as an option to inhibit liver fibrosis. Whether this differentiation can be performed, either *in vitro* or *in vivo*, is an important current topic of research.

Lin and colleagues[91] explored the effect of thioacetamide (TAA)-injured mouse liver tissue on hepatic differentiation of hUC-MSCs. Reverse transcription-PCR analysis showed increases in expression of liver-specific genes, including CK18, ALB, TPH2, AFP, and CYP7A1. Further, the expression levels of stem cell genes were decreased, including those of NANOG, OCT4, and CKIT. These results suggest that the hUC-MSCs had the potential to differentiate into hepatocyte-like cells when exposed to TAA-injured mouse liver tissue. Furthermore, an *in vitro* study by Yan *et al*[106] showed that, by mimicking the microenvironment of liver fibrosis using 50 g/L rat fibrotic liver tissue extracts, hUC-MSCs could be stimulated to differentiate into hepatocyte-like cells in a shorter period of time. The hepatocyte biomarkers AFP and CK18 were found to be expressed at the protein level, as was the critical metabolic protein CYP3A4; moreover, urea production, ALB secretion, and glycogen storage were increased. However, urea production and ALB secretion were relatively lower than in normal hepatocytes, suggesting that these cells could replace the function of existing hepatocytes only partially.

In 2016, Yan *et al*[107] investigated the effect on and underlying mechanisms of the blood microenvironment of rats with TAA-induced hepatic fibrosis for the differentiation of hUC-MSCs into hepatocytes. The expression levels of human hepatocyte biomarkers (AFP and CK18) and hepatocyte-specific proteins (ALB, TPH2, and CYP3A4) were found to increase significantly, as did the blood urea nitrogen concentration. Additionally, the levels of HGF and p-ERK significantly increased, while those of EGF and OSM significantly decreased. Thus, it was concluded that the hepatic fibrosis blood microenvironment induced hepatic differentiation of hUC-MSCs through activation of the MAPK/ERK signaling pathway. In an *in vivo* study, Lin *et al*[91] also transplanted 1 × 10^6 hUC-MSCs into rats with TAA-damaged livers (*via* portal vein injection). The transplanted hUC-MSCs were found to be distributed in the fibrotic area as well as around blood vessels; moreover, the effects of accelerated hepatic recovery and significantly restored serum prothrombin time were observed. In addition to human serum ALB, human ALB-positive hUC-MSCs were increased at 21 d posttransplantation. These findings indicate that hUC-MSCs can differentiate into hepatocyte-like cells *in vivo*. Similarly, Ren *et al*[108] transplanted hUC-MSCs into NOD/SCID mice with CCl_4-induced liver fibrosis. They found that human-specific AFP and ALB mRNA and protein were present in livers of CCl_4-treated mice that had received transplanted human hUC-MSCs.

In 2017, Zhang *et al*[53] reported that hUC-MSCs transplanted through the tail vein into rats with CCl_4-induced liver fibrosis could differentiate into functional hepatocytes. The hUC-MSCs treatment led to improved liver transaminase and synthetase functions, reduced liver histopathology, and reversed hepatic fibrosis. Moreover, the expression of human hepatic markers ALB, CK18, AFP, and CK19

gradually increased in the rat liver tissues, coincident with the additional time of hUC-MSC transplantation. The differentiation of hUC-MSCs into hepatocyte-like cells *in vivo* through the mesenchymal-to-epithelial transition process was confirmed by the significantly decreased expression of the mesenchymal marker human vimentin and the significantly increased expression levels of the epithelial markers human E-cadherin and α-catenin, all of which occurred in a time-dependent manner.

The above findings indicate that hUC-MSCs can differentiate into hepatocyte-like cells through exposure to the liver fibrosis microenvironment, both *in vitro* and *in vivo*. However, the influence of liver fibrosis tissue volume, induction duration, and liver fibrosis animal model type needs further exploration. Collectively, the studies described above have suggested that hUC-MSCs can migrate to the damaged liver and that paracrine activities of various growth factors and cytokines can reverse liver fibrosis (*e.g.*, inactivating HSCs, and degrading excessive ECM) through several signaling pathways; then, the cells can differentiate into hepatocyte-like cells for promotion of the function of existing hepatocytes. However, the exact mechanisms by which hUC-MSCs repair liver fibrosis and undergo hepatic differentiation remain to be elucidated.

CLINICAL TRIALS

The establishment of practical applications of MSCs involve clinical trials to investigate their therapeutic potential for treatment of decompensated liver cirrhosis or end-stage liver disease. Indeed, there have been some measures of progress in hUC-MSC therapy (Table 1), according to ClinicalTrials.gov[109]. All of the trials have involved allogeneic transplantation, with no side effects reported.

To date, findings from the trials have indicated that transplantation of hUC-MSCs could contribute to marked recovery in patients with liver injuries; both liver function and survival rate have been improved. The therapeutic effect of liver fibrosis treatment is exciting, and has broad clinical application. However, the protocol for hUC-MSC treatment needs further refinement, and its efficacy should be further assessed in randomized trials with large cohorts.

CONCLUSION AND FUTURE PROSPECTS

Transplantation of hUC-MSCs is an effective and promising treatment for liver fibrosis. This method has the unique characteristics of presenting no major ethical problems and having lower risk of viral transmission, low immunogenicity, abundant availability, and more primitive cell type; thus, the hUC-MSCs are outstanding in comparison to other sources of MSCs. Regarding the application of hUC-MSCs in treatment of liver fibrosis, although the specific mechanism is not yet known, many studies have begun to elucidate the probable mechanisms, including paracrine effects, transdifferentiation into hepatocyte-like cells, and regulation of immunomodulatory function. Yet, some important issues with these studies remain to be resolved.

First, the method of acquisition of sufficient hUC-MSCs in a short period of time is not yet defined. Second, improvement of the efficiency of induction into hepatocyte-like cells or mature hepatocytes *in vitro* and *in vivo* still requires more detailed exploration of the hepatic transdifferentiation mechanism of hUC-MSCs. Third, more effective techniques for large-scale exosome production are needed. Fourth, the paracrine mechanism of hUC-MSCs to alleviate liver fibrosis must be discerned, including specific cytokine signaling pathways to prevent the progress of liver fibrosis. Fifth, further data are needed regarding the oncogenic potential and risks of using hUC-MSCs. Although hUC-MSCs are not tumorigenic, these safety concerns must be explored intensively, especially the long-term effects on the immune response and tumorigenesis. Sixth, the frequency and stage of engraftment, the dose of hUC-MSCs, and standardized protocols for hUC-MSC transplantation are not yet established. Ultimately, large-sample, randomized, placebo-controlled clinical trials are needed to verify the therapeutic potential of hUC-MSCs in liver fibrosis.

Overall, although a large number of challenges remain in the application of hUC-MSCs for ameliorating liver fibrosis, these issues will be resolved by assessment of the mechanisms of liver fibrosis, liver genesis, and liver development, as well as further research regarding stem cells and genetic tissue engineering.

Table 1 Clinical trials in liver diseases using human umbilical cord mesenchymal stem cells

Ref.	Administration route	Number of cells infused	Etiology or disease	Study design, *n* for total; groups	Follow-up period	Trial number	Results
[110]	Intravenous	(4.0-4.5) × 10^8	Decompensated liver cirrhosis	103; 50 hUC-MSCs, 53 control	52 wk	ChiCTR-ONC-12002103	Decrease in AST, increase in ALB, TBIL, PT Improvement in MELD and Child-Pugh scores
[56]	Intravenous	0.5 × 10^6/kg Every 4 wk, 3 times	ACLF (HBV cirrhosis)	43; 24 hUC-MSCs, 19 control	72 wk	NCT01218464	Improvement in MELD Increase in ALB, PT
[111]	Intravenous	0.5 × 10^6/kg Every 4 wk, 3 times	Cirrhosis (HBV)	45; 30 hUC-MSCs, 15 control	48 wk	NCT01220492	Decrease in ascites. Increase in ALB, TBIL. Improvement in MELD.
[112]	Intravenous	0.5 × 10^6/kg Every 4 wk, 3 times	UDCA-resistant PBC	7	48 wk	NCT01662973	Decrease in ALP, γ-GT, fatigue, pruritus Improvement quality of life
[113]	Intravenous	1 × 10^6/kg; wk 1, 2, 4, 8, 12 and 16	Ischemic-type biliary lesions	82; 12 hUC-MSCs, 70 control	96 wk	NCT02223897	Decrease in BIL, ALP, γ-GT Improvement in graft survival
[114]	Intravenous	1 × 10^6/kg; Every 4 wk, 3 times	Liver transplant patients with acute graft rejection	27; 14 hUC-MSCs, 13 control	12 wk	NCT01690247	Decrease in ALT, AST, TBIL, acute rejection Improvement in liver allograft histology
	Peripheral vein	4.0 × 10^7/patient, 4 times	LC	320	144 wk	NCT01573923	Unknown
	Unknown	Unknown	LC	20	48 wk	NCT01342250	Unknown
	Peripheral vein	1.0 × 10^5/kg, 4 times	Liver failure (HBV)	120	48 wk	NCT01724398	Unknown
	Portal vein or hepatic artery	Unknown	LC	200	48 wk	NCT01233102	Unknown
	Hepatic artery	Unknown	LC	50	4 wk	NCT01224327	Unknown
	Hepatic artery	1.0 × 10^6/kg	LC (HBV)	240	48 wk	NCT01728727	Unknown
	Peripheral vein			210	72 wk	NCT01844063	Unknown
	Peripheral vein	Unknown	ACLF (HBV)	261	52 wk	NCT02812121	Unknown
	Portal vein or hepatic artery	2.0 × 10^7/patient, 4 times	LC	20	48 wk	NCT02652351	Unknown
	Peripheral vein	1.0 × 10^6/kg, 3 times	Liver failure (AIH)	100	96 wk	NCT01661842	Unknown
	Lobe	5.0 × 10^8/patient	LC	40	96 wk	NCT02786017	Unknown

ACLF: Acute-on-chronic liver failure; AIH: Autoimmune hepatitis; ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; BIL: Bilirubin; γ-GTP: Gamma-glutamyl transpeptidase; HBV: Hepatitis B virus; LC: Liver cirrhosis; MELD: Model for end-stage liver disease; PBC: Primary biliary cholangitis; PT: Prothrombin time; TBIL: Total bilirubin; UCDA: Ursodeoxycholic acid.

REFERENCES

1 **Guo Y**, Chen B, Chen LJ, Zhang CF, Xiang C. Current status and future prospects of mesenchymal stem cell therapy for liver fibrosis. *J Zhejiang Univ Sci B* 2016; **17**: 831-841 [PMID: 27819130 DOI: 10.1631/jzus.B1600101]

2 **van Dijk F**, Olinga P, Poelstra K, Beljaars L. Targeted Therapies in Liver Fibrosis: Combining the Best Parts of Platelet-Derived Growth Factor BB and Interferon Gamma. *Front Med (Lausanne)* 2015; **2**: 72 [PMID: 26501061 DOI: 10.3389/fmed.2015.00072]

3 **Tacke F**, Trautwein C. Mechanisms of liver fibrosis resolution. *J Hepatol* 2015; **63**: 1038-1039 [PMID: 26232376 DOI: 10.1016/j.jhep.2015.03.039]

4 **Zhang CY**, Yuan WG, He P, Lei JH, Wang CX. Liver fibrosis and hepatic stellate cells: Etiology, pathological hallmarks and therapeutic targets. *World J Gastroenterol* 2016; **22**: 10512-10522 [PMID:

28082803 DOI: 10.3748/wjg.v22.i48.10512]
5 **Weiskirchen R**, Tacke F. Liver Fibrosis: From Pathogenesis to Novel Therapies. *Dig Dis* 2016; **34**: 410-422 [PMID: 27170396 DOI: 10.1159/000444556]
6 **Pinzani M**. Pathophysiology of Liver Fibrosis. *Dig Dis* 2015; **33**: 492-497 [PMID: 26159264 DOI: 10.1159/000374096]
7 **Li D**, He L, Guo H, Chen H, Shan H. Targeting activated hepatic stellate cells (aHSCs) for liver fibrosis imaging. *EJNMMI Res* 2015; **5**: 71 [PMID: 26650603 DOI: 10.1186/s13550-015-0151-x]
8 **Higashi T**, Friedman SL, Hoshida Y. Hepatic stellate cells as key target in liver fibrosis. *Adv Drug Deliv Rev* 2017; **121**: 27-42 [PMID: 28506744 DOI: 10.1016/j.addr.2017.05.007]
9 **Zhao YL**, Zhu RT, Sun YL. Epithelial-mesenchymal transition in liver fibrosis. *Biomed Rep* 2016; **4**: 269-274 [PMID: 26998262 DOI: 10.3892/br.2016.578]
10 **Borkham-Kamphorst E**, Herrmann J, Stoll D, Treptau J, Gressner AM, Weiskirchen R. Dominant-negative soluble PDGF-beta receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis. *Lab Invest* 2004; **84**: 766-777 [PMID: 15077122 DOI: 10.1038/labinvest.3700094]
11 **Saxena NK**, Titus MA, Ding X, Floyd J, Srinivasan S, Sitaraman SV, Anania FA. Leptin as a novel profibrogenic cytokine in hepatic stellate cells: mitogenesis and inhibition of apoptosis mediated by extracellular regulated kinase (Erk) and Akt phosphorylation. *FASEB J* 2004; **18**: 1612-1614 [PMID: 15319373 DOI: 10.1096/fj.04-1847fje]
12 **Lang T**, Ikejima K, Yoshikawa M, Enomoto N, Iijima K, Kitamura T, Takei Y, Sato N. Leptin facilitates proliferation of hepatic stellate cells through up-regulation of platelet-derived growth factor receptor. *Biochem Biophys Res Commun* 2004; **323**: 1091-1095 [PMID: 15381111 DOI: 10.1016/j.bbrc.2004.08.192]
13 **Wang Y**, Shen RW, Han B, Li Z, Xiong L, Zhang FY, Cong BB, Zhang B. Notch signaling mediated by TGF-β/Smad pathway in concanavalin A-induced liver fibrosis in rats. *World J Gastroenterol* 2017; **23**: 2330-2336 [PMID: 28428712 DOI: 10.3748/wjg.v23.i13.2330]
14 **Perumal N**, Perumal M, Halagowder D, Sivasithamparam N. Morin attenuates diethylnitrosamine-induced rat liver fibrosis and hepatic stellate cell activation by co-ordinated regulation of Hippo/Yap and TGF-β1/Smad signaling. *Biochimie* 2017; **140**: 10-19 [PMID: 28552397 DOI: 10.1016/j.biochi.2017.05.017]
15 **Liu WH**, Song FQ, Ren LN, Guo WQ, Wang T, Feng YX, Tang LJ, Li K. The multiple functional roles of mesenchymal stem cells in participating in treating liver diseases. *J Cell Mol Med* 2015; **19**: 511-520 [PMID: 25534251 DOI: 10.1111/jcmm.12482]
16 **Dutkowski P**, Linecker M, DeOliveira ML, Müllhaupt B, Clavien PA. Challenges to liver transplantation and strategies to improve outcomes. *Gastroenterology* 2015; **148**: 307-323 [PMID: 25224524 DOI: 10.1053/j.gastro.2014.08.045]
17 **Dutkowski P**, Clavien PA. Solutions to shortage of liver grafts for transplantation. *Br J Surg* 2014; **101**: 739-741 [PMID: 24817650 DOI: 10.1002/bjs.9540]
18 **Kwak KA**, Cho HJ, Yang JY, Park YS. Current Perspectives Regarding Stem Cell-Based Therapy for Liver Cirrhosis. *Can J Gastroenterol Hepatol* 2018; **2018**: 4197857 [PMID: 29670867 DOI: 10.1155/2018/4197857]
19 **Lee CW**, Chen YF, Wu HH, Lee OK. Historical Perspectives and Advances in Mesenchymal Stem Cell Research for the Treatment of Liver Diseases. *Gastroenterology* 2018; **154**: 46-56 [PMID: 29107021 DOI: 10.1053/j.gastro.2017.09.049]
20 **Fiore EJ**, Domínguez LM, Bayo J, García MG, Mazzolini GD. Taking advantage of the potential of mesenchymal stromal cells in liver regeneration: Cells and extracellular vesicles as therapeutic strategies. *World J Gastroenterol* 2018; **24**: 2427-2440 [PMID: 29930465 DOI: 10.3748/wjg.v24.i23.2427]
21 **Zhao L**, Chen S, Shi X, Cao H, Li L. A pooled analysis of mesenchymal stem cell-based therapy for liver disease. *Stem Cell Res Ther* 2018; **9**: 72 [PMID: 29562935 DOI: 10.1186/s13287-018-0816-2]
22 **Zhang LT**, Peng XB, Fang XQ, Li JF, Chen H, Mao XR. Human umbilical cord mesenchymal stem cells inhibit proliferation of hepatic stellate cells in vitro. *Int J Mol Med* 2018; **41**: 2545-2552 [PMID: 29484382 DOI: 10.3892/ijmm.2018.3500]
23 **Secunda R**, Vennila R, Mohanashankar AM, Rajasundari M, Jeswanth S, Surendran R. Isolation, expansion and characterisation of mesenchymal stem cells from human bone marrow, adipose tissue, umbilical cord blood and matrix: a comparative study. *Cytotechnology* 2015; **67**: 793-807 [PMID: 24798808 DOI: 10.1007/s10616-014-9718-z]
24 **Williams JT**, Southerland SS, Souza J, Calcutt AF, Cartledge RG. Cells isolated from adult human skeletal muscle capable of differentiating into multiple mesodermal phenotypes. *Am Surg* 1999; **65**: 22-26 [PMID: 9915526]
25 **Wu KH**, Liu YL, Zhou B, Han ZC. Cellular therapy and myocardial tissue engineering: the role of adult stem and progenitor cells. *Eur J Cardiothorac Surg* 2006; **30**: 770-781 [PMID: 16963271 DOI: 10.1016/j.ejcts.2006.08.003]
26 **Jo CH**, Kim OS, Park EY, Kim BJ, Lee JH, Kang SB, Lee JH, Han HS, Rhee SH, Yoon KS. Fetal mesenchymal stem cells derived from human umbilical cord sustain primitive characteristics during extensive expansion. *Cell Tissue Res* 2008; **334**: 423-433 [PMID: 18941782 DOI: 10.1007/s00441-008-0696-3]
27 **Henry G**, William P, Bannister L. Gray's anatomy. London, UK: ELBS Churchill Livingstone, 1995
28 **Kestendjieva S**, Kyurkchiev D, Tsvetkova G, Mehandjiev T, Dimitrov A, Nikolov A, Kyurkchiev S. Characterization of mesenchymal stem cells isolated from the human umbilical cord. *Cell Biol Int* 2008; **32**: 724-732 [PMID: 18396423 DOI: 10.1016/j.cellbi.2008.02.002]
29 **Troyer DL**, Weiss ML. Wharton's jelly-derived cells are a primitive stromal cell population. *Stem Cells* 2008; **26**: 591-599 [PMID: 18065397 DOI: 10.1634/stemcells.2007-0439]
30 **Kim DW**, Staples M, Shinozuka K, Pantcheva P, Kang SD, Borlongan CV. Wharton's jelly-derived mesenchymal stem cells: phenotypic characterization and optimizing their therapeutic potential for clinical applications. *Int J Mol Sci* 2013; **14**: 11692-11712 [PMID: 23727936 DOI: 10.3390/ijms140611692]
31 **Campard D**, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology* 2008; **134**: 833-848 [PMID: 18243183 DOI: 10.1053/j.gastro.2007.12.024]
32 **Ding DC**, Shyu WC, Chiang MF, Lin SZ, Chang YC, Wang HJ, Su CY, Li H. Enhancement of neuroplasticity through upregulation of beta1-integrin in human umbilical cord-derived stromal cell implanted stroke model. *Neurobiol Dis* 2007; **27**: 339-353 [PMID: 17651977 DOI: 10.1016/j.nbd.2007.06.010]
33 **Weiss ML**, Anderson C, Medicetty S, Seshareddy KB, Weiss RJ, VanderWerff I, Troyer D, McIntosh KR.

Immune properties of human umbilical cord Wharton's jelly-derived cells. *Stem Cells* 2008; **26**: 2865-2874 [PMID: 18703664 DOI: 10.1634/stemcells.2007-1028]
34 **Fu YS**, Cheng YC, Lin MY, Cheng H, Chu PM, Chou SC, Shih YH, Ko MH, Sung MS. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons in vitro: potential therapeutic application for Parkinsonism. *Stem Cells* 2006; **24**: 115-124 [PMID: 16099997 DOI: 10.1634/stemcells.2005-0053]
35 **Karahuseyinoglu S**, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, Tukun A, Uckan D, Can A. Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys. *Stem Cells* 2007; **25**: 319-331 [PMID: 17053211 DOI: 10.1634/stemcells.2006-0286]
36 **Sarugaser R**, Lickorish D, Baksh D, Hosseini MM, Davies JE. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. *Stem Cells* 2005; **23**: 220-229 [PMID: 15671145 DOI: 10.1634/stemcells.2004-0166]
37 **Wang HS**, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, Fu YS, Lai MC, Chen CC. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells* 2004; **22**: 1330-1337 [PMID: 15579650 DOI: 10.1634/stemcells.2004-0013]
38 **Mennan C**, Wright K, Bhattacharjee A, Balain B, Richardson J, Roberts S. Isolation and characterisation of mesenchymal stem cells from different regions of the human umbilical cord. *Biomed Res Int* 2013; **2013**: 916136 [PMID: 23984420 DOI: 10.1155/2013/916136]
39 **Nagamura-Inoue T**, He H. Umbilical cord-derived mesenchymal stem cells: Their advantages and potential clinical utility. *World J Stem Cells* 2014; **6**: 195-202 [PMID: 24772246 DOI: 10.4252/wjsc.v6.i2.195]
40 **Lu HY**, Du WJ, Liu CX, Meng QX, Fu YS, Shi JN, Zhang TT, Zhang Y. Efficient generation of human umbilical cord derived mensenchymal stem cells by explant re-culture method. *Chin J Biol* 2017; **30**: 649-654 [DOI: 10.13200/j.cnki.cjb.001774]
41 **He J**, Zhao J, Wang JX, Cai XM, Pang RQ, Pan XH. Efficient preparation of human umbilical cord mesenchymal stem cells by multiple adherence method. *Military Med J Southeast China* 2015; **25**: 828-831 [DOI: 10.3969/j.issn.1004-0188.2015.08.006]
42 **Ding DC**, Shyu WC, Lin SZ, Liu HW, Chiou SH, Chu TY. Human umbilical cord mesenchymal stem cells support nontumorigenic expansion of human embryonic stem cells. *Cell Transplant* 2012; **21**: 1515-1527 [PMID: 22732188 DOI: 10.3727/096368912X647199]
43 **Salehinejad P**, Alitheen NB, Ali AM, Omar AR, Mohit M, Janzamin E, Samani FS, Torshizi Z, Nematollahi-Mahani SN. Comparison of different methods for the isolation of mesenchymal stem cells from human umbilical cord Wharton's jelly. *In Vitro Cell Dev Biol Anim* 2012; **48**: 75-83 [PMID: 22274909 DOI: 10.1007/s11626-011-9480-x]
44 **Tsagias N**, Koliakos I, Karagiannis V, Eleftheriadou M, Koliakos GG. Isolation of mesenchymal stem cells using the total length of umbilical cord for transplantation purposes. *Transfus Med* 2011; **21**: 253-261 [PMID: 21623971 DOI: 10.1111/j.1365-3148.2011.01076.x]
45 **Nekanti U**, Rao VB, Bahirvani AG, Jan M, Totey S, Ta M. Long-term expansion and pluripotent marker array analysis of Wharton's jelly-derived mesenchymal stem cells. *Stem Cells Dev* 2010; **19**: 117-130 [PMID: 19619003 DOI: 10.1089/scd.2009.0177]
46 **Mitchell KE**, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T, Troyer D, Medicetty S. Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells* 2003; **21**: 50-60 [PMID: 12529551 DOI: 10.1634/stemcells.21-1-50]
47 **Ishige I**, Nagamura-Inoue T, Honda MJ, Harnprasopwat R, Kido M, Sugimoto M, Nakauchi H, Tojo A. Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. *Int J Hematol* 2009; **90**: 261-269 [PMID: 19657615 DOI: 10.1007/s12185-009-0377-3]
48 **Li Y**, Guo G, Li L, Chen F, Bao J, Shi YJ, Bu H. Three-dimensional spheroid culture of human umbilical cord mesenchymal stem cells promotes cell yield and stemness maintenance. *Cell Tissue Res* 2015; **360**: 297-307 [PMID: 25749992 DOI: 10.1007/s00441-014-2055-x]
49 **Li T**, Yan Y, Wang B, Qian H, Zhang X, Shen L, Wang M, Zhou Y, Zhu W, Li W, Xu W. Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem Cells Dev* 2013; **22**: 845-854 [PMID: 23002959 DOI: 10.1089/scd.2012.0395]
50 **Zhang B**, Shen L, Shi H, Pan Z, Wu L, Yan Y, Zhang X, Mao F, Qian H, Xu W. Exosomes from Human Umbilical Cord Mesenchymal Stem Cells: Identification, Purification, and Biological Characteristics. *Stem Cells Int* 2016; **2016**: 1929536 [PMID: 28105054 DOI: 10.1155/2016/1929536]
51 **Shuai H**, Shi C, Lan J, Chen D, Luo X. Double labelling of human umbilical cord mesenchymal stem cells with Gd-DTPA and PKH26 and the influence on biological characteristics of hUCMSCs. *Int J Exp Pathol* 2015; **96**: 63-72 [PMID: 25649907 DOI: 10.1111/iep.12111]
52 **Hong J**, Jin H, Han J, Hu H, Liu J, Li L, Huang Y, Wang D, Wu M, Qiu L, Qian Q. Infusion of human umbilical cordderived mesenchymal stem cells effectively relieves liver cirrhosis in DENinduced rats. *Mol Med Rep* 2014; **9**: 1103-1111 [PMID: 24481983 DOI: 10.3892/mmr.2014.1927]
53 **Zhang GZ**, Sun HC, Zheng LB, Guo JB, Zhang XL. In vivo hepatic differentiation potential of human umbilical cord-derived mesenchymal stem cells: Therapeutic effect on liver fibrosis/cirrhosis. *World J Gastroenterol* 2017; **23**: 8152-8168 [PMID: 29290652 DOI: 10.3748/wjg.v23.i46.8152]
54 **Zhou P**, Liu Z, Li X, Zhang B, Wang X, Lan J, Shi Q, Li D, Ju X. Migration ability and Toll-like receptor expression of human mesenchymal stem cells improves significantly after three-dimensional culture. *Biochem Biophys Res Commun* 2017; **491**: 323-328 [PMID: 28734835 DOI: 10.1016/j.bbrc.2017.07.102]
55 **Talaei-Khozani T**, Khodabandeh Z, Jaberipour M, Hosseini A, Bahmanpour S, Vojdani Z. Comparison of hepatic nuclear factor-4 expression in two- and three-dimensional culture of Wharton's jelly-derived cells exposed to hepatogenic medium. *Rom J Morphol Embryol* 2015; **56**: 1365-1370 [PMID: 26743282]
56 **Shi M**, Zhang Z, Xu R, Lin H, Fu J, Zou Z, Zhang A, Shi J, Chen L, Lv S, He W, Geng H, Jin L, Liu Z, Wang FS. Human mesenchymal stem cell transfusion is safe and improves liver function in acute-on-chronic liver failure patients. *Stem Cells Transl Med* 2012; **1**: 725-731 [PMID: 23197664 DOI: 10.5966/sctm.2012-0034]
57 **An SY**, Han J, Lim HJ, Park SY, Kim JH, Do BR, Kim JH. Valproic acid promotes differentiation of hepatocyte-like cells from whole human umbilical cord-derived mesenchymal stem cells. *Tissue Cell* 2014; **46**: 127-135 [PMID: 24472423 DOI: 10.1016/j.tice.2013.12.006]
58 **Yang LM**, Liu Y, Zhao J, Hao LM, Huang KX, Jiang WH. Characterization of human umbilical cord mesenchymal stem cells following tissue mass culture. *Cell Mol Biol (Noisy-le-grand)* 2014; **60**: 12-18 [PMID: 24606723]

59 **Bieback K**, Hecker A, Kocaömer A, Lannert H, Schallmoser K, Strunk D, Klüter H. Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. *Stem Cells* 2009; **27**: 2331-2341 [PMID: 19544413 DOI: 10.1002/stem.139]

60 **Wu JY**, Lu Y, Chen JS, Zhu L, GanWT. Human umbilical cord blood plasma can replace fetal bovine serum for primary culture, proliferation and cryopreservation of umbilical cord mesenchymal stem cell. *Chin J Tissue Engineering Research* 2014; **18**: 5947-5954 [DOI: 10.3969/j.issn.2095-4344.2014.37.008]

61 **Sun YR**, Zhang BQ, Wang FB, Xun P, Wang EP, Li CC. Effect of cultivation systems on the maintenance of human umbilical cord mesenchymal stem cell characteristics and their proliferation rate in vitro. *Chin J Tissue Engineering Research* 2017; **21**: 2023-2028 [DOI: 10.3969/j.issn.2095-4344.2017.13.010]

62 **Hatlapatka T**, Moretti P, Lavrentieva A, Hass R, Marquardt N, Jacobs R, Kasper C. Optimization of culture conditions for the expansion of umbilical cord-derived mesenchymal stem or stromal cell-like cells using xeno-free culture conditions. *Tissue Eng Part C Methods* 2011; **17**: 485-493 [PMID: 21166520 DOI: 10.1089/ten.TEC.2010.0406]

63 **Priya N**, Sarcar S, Majumdar AS, SundarRaj S. Explant culture: a simple, reproducible, efficient and economic technique for isolation of mesenchymal stromal cells from human adipose tissue and lipoaspirate. *J Tissue Eng Regen Med* 2014; **8**: 706-716 [PMID: 22837175 DOI: 10.1002/term.1569]

64 **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]

65 **Can A**, Karahuseyinoglu S. Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. *Stem Cells* 2007; **25**: 2886-2895 [PMID: 17690177 DOI: 10.1634/stemcells.2007-0417]

66 **Weiss ML**, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, Luo Y, Rao MS, Velagaleti G, Troyer D. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells* 2006; **24**: 781-792 [PMID: 16223852 DOI: 10.1634/stemcells.2005-0330]

67 **Liu S**, Yuan M, Hou K, Zhang L, Zheng X, Zhao B, Sui X, Xu W, Lu S, Guo Q. Immune characterization of mesenchymal stem cells in human umbilical cord Wharton's jelly and derived cartilage cells. *Cell Immunol* 2012; **278**: 35-44 [PMID: 23121974 DOI: 10.1016/j.cellimm.2012.06.010]

68 **Wang LM**. The research for Proteins Expression Profile of Umbilical Cord Mesenchymal Stem Cells. M.Sc. Thesis, Anhui Medical University. 2014. Available from: URL: http://www.wanfangdata.com.cn/details/detail.do?_type=degreeid=D522929

69 **Stenken JA**, Poschenrieder AJ. Bioanalytical chemistry of cytokines--a review. *Anal Chim Acta* 2015; **853**: 95-115 [PMID: 25467452 DOI: 10.1016/j.aca.2014.10.009]

70 **Bai L**, Li D, Li J, Luo Z, Yu S, Cao S, Shen L, Zuo Z, Ma X. Bioactive molecules derived from umbilical cord mesenchymal stem cells. *Acta Histochem* 2016; **118**: 761-769 [PMID: 27692875 DOI: 10.1016/j.acthis.2016.09.006]

71 **Follin B**, Juhl M, Cohen S, Pedersen AE, Kastrup J, Ekblond A. Increased Paracrine Immunomodulatory Potential of Mesenchymal Stromal Cells in Three-Dimensional Culture. *Tissue Eng Part B Rev* 2016; **22**: 322-329 [PMID: 26861485 DOI: 10.1089/ten.TEB.2015.0532]

72 **Bieback K**, Brinkmann I. Mesenchymal stromal cells from human perinatal tissues: From biology to cell therapy. *World J Stem Cells* 2010; **2**: 81-92 [PMID: 21607124 DOI: 10.4252/wjsc.v2.i4.81]

73 **Zhao Q**, Ren H, Li X, Chen Z, Zhang X, Gong W, Liu Y, Pang T, Han ZC. Differentiation of human umbilical cord mesenchymal stromal cells into low immunogenic hepatocyte-like cells. *Cytotherapy* 2009; **11**: 414-426 [PMID: 19513901 DOI: 10.1080/14653240902849754]

74 **Yan Y**, Zhu Y, Sun F, Zhang B, Li L, Sun Z, Li W, Qian H, Zhu W, Xu W. Extracellular regulated protein kinases 1/2 phosphorylation is required for hepatic differentiation of human umbilical cord-derived mesenchymal stem cells. *Exp Biol Med (Maywood)* 2015; **240**: 534-545 [PMID: 25576343 DOI: 10.1177/1535370214548996]

75 **Xue G**, Han X, Ma X, Wu H, Qin Y, Liu J, Hu Y, Hong Y, Hou Y. Effect of Microenvironment on Differentiation of Human Umbilical Cord Mesenchymal Stem Cells into Hepatocytes In Vitro and In Vivo. *Biomed Res Int* 2016; **2016**: 8916534 [PMID: 27088093 DOI: 10.1155/2016/8916534]

76 **Li H**, Wen F, Qi ZC, Zhou JJ, Zhu YJ, Cheng P, Wei D, Su XM, Tan Y, Peng JJ, Luo QL, Li D, Zhang T. Human umbilical cord-derived mesenchymal stem cells co-cultured with hepatocytes can differentiate into hepatocyte-like cells. *Chin J Tissue Engineering Research* 2013; **17**: 5772-5777 [DOI: 10.3969/j.issn.2095-4344.2013.32.005]

77 **Ek M**, Söderdahl T, Küppers-Munther B, Edsbagge J, Andersson TB, Björquist P, Cotgreave I, Jernström B, Ingelman-Sundberg M, Johansson I. Expression of drug metabolizing enzymes in hepatocyte-like cells derived from human embryonic stem cells. *Biochem Pharmacol* 2007; **74**: 496-503 [PMID: 17568565 DOI: 10.1016/j.bcp.2007.05.009]

78 **Dai K**, Chen R, Ding Y, Niu Z, Fan J, Xu C. Induction of Functional Hepatocyte-Like Cells by Overexpression of FOXA3 and HNF4α in Rat Bone Marrow Mesenchymal Stem Cells. *Cells Tissues Organs* 2014; **200**: 132-140 [PMID: 25896100 DOI: 10.1159/000380762]

79 **Alizadeh E**, Akbarzadeh A, Eslaminejad MB, Barzegar A, Hashemzadeh S, Nejati-Koshki K, Zarghami N. Up regulation of liver-enriched transcription factors HNF4a and HNF6 and liver-specific microRNA (miR-122) by inhibition of let-7b in mesenchymal stem cells. *Chem Biol Drug Des* 2015; **85**: 268-279 [PMID: 25059576 DOI: 10.1111/cbdd.12398]

80 **Hu X**, Xie P, Li W, Li Z, Shan H. Direct induction of hepatocyte-like cells from immortalized human bone marrow mesenchymal stem cells by overexpression of HNF4α. *Biochem Biophys Res Commun* 2016; **478**: 791-797 [PMID: 27501760 DOI: 10.1016/j.bbrc.2016.08.026]

81 **Hang H**, Yu Y, Wu N, Huang Q, Xia Q, Bian J. Induction of highly functional hepatocytes from human umbilical cord mesenchymal stem cells by HNF4α transduction. *PLoS One* 2014; **9**: e104133 [PMID: 25137413 DOI: 10.1371/journal.pone.0104133]

82 **Watt AJ**, Garrison WD, Duncan SA. HNF4: a central regulator of hepatocyte differentiation and function. *Hepatology* 2003; **37**: 1249-1253 [PMID: 12774000 DOI: 10.1053/jhep.2003.50273]

83 **Shenoy A**, Blelloch R. microRNA induced transdifferentiation. *F1000 Biol Rep* 2012; **4**: 3 [PMID: 22312415 DOI: 10.3410/B4-3]

84 **Yoo AS**, Sun AX, Li L, Shcheglovitov A, Portmann T, Li Y, Lee-Messer C, Dolmetsch RE, Tsien RW, Crabtree GR. MicroRNA-mediated conversion of human fibroblasts to neurons. *Nature* 2011; **476**: 228-231 [PMID: 21753754 DOI: 10.1038/nature10323]

85 **Jayawardena TM**, Egemnazarov B, Finch EA, Zhang L, Payne JA, Pandya K, Zhang Z, Rosenberg P, Mirotsou M, Dzau VJ. MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. *Circ Res* 2012; **110**: 1465-1473 [PMID: 22539765 DOI: 10.1161/CIRCRESAHA.112.269035]

86 **Cui L**, Shi Y, Zhou X, Wang X, Wang J, Lan Y, Wang M, Zheng L, Li H, Wu Q, Zhang J, Fan D, Han Y. A set of microRNAs mediate direct conversion of human umbilical cord lining-derived mesenchymal stem cells into hepatocytes. *Cell Death Dis* 2013; **4**: e918 [PMID: 24232094 DOI: 10.1038/cddis.2013.429]

87 **Zhou X**, Cui L, Zhou X, Yang Q, Wang L, Guo G, Hou Y, Cai W, Han Z, Shi Y, Han Y. Induction of hepatocyte-like cells from human umbilical cord-derived mesenchymal stem cells by defined microRNAs. *J Cell Mol Med* 2017; **21**: 881-893 [PMID: 27874233 DOI: 10.1111/jcmm.13027]

88 **Khosravi M**, Azarpira N, Shamdani S, Hojjat-Assari S, Naserian S, Karimi MH. Differentiation of umbilical cord derived mesenchymal stem cells to hepatocyte cells by transfection of miR-106a, miR-574-3p, and miR-451. *Gene* 2018; **667**: 1-9 [PMID: 29763649 DOI: 10.1016/j.gene.2018.05.028]

89 **Aleahmad F**, Ebrahimi S, Salmannezhad M, Azarnia M, Jaberipour M, Hoseini M, Talaei-Khozani T. Heparin/Collagen 3D Scaffold Accelerates Hepatocyte Differentiation of Wharton's Jelly-Derived Mesenchymal Stem Cells. *Tissue Eng Regen Med* 2017; **14**: 443-452 [PMID: 30603500 DOI: 10.1007/s13770-017-0048-z]

90 **Khodabandeh Z**, Vojdani Z, Talaei-Khozani T, Jaberipour M, Hosseini A, Bahmanpour S. Comparison of the Expression of Hepatic Genes by Human Wharton's Jelly Mesenchymal Stem Cells Cultured in 2D and 3D Collagen Culture Systems. *Iran J Med Sci* 2016; **41**: 28-36 [PMID: 26722142]

91 **Lin SZ**, Chang YJ, Liu JW, Chang LF, Sun LY, Li YS, Luo GH, Liao CH, Chen PH, Chen TM, Lee RP, Yang KL, Harn HJ, Chiou TW. Transplantation of human Wharton's Jelly-derived stem cells alleviates chemically induced liver fibrosis in rats. *Cell Transplant* 2010; **19**: 1451-1463 [PMID: 20587139 DOI: 10.3727/096368910X514198]

92 **Tsai PC**, Fu TW, Chen YM, Ko TL, Chen TH, Shih YH, Hung SC, Fu YS. The therapeutic potential of human umbilical mesenchymal stem cells from Wharton's jelly in the treatment of rat liver fibrosis. *Liver Transpl* 2009; **15**: 484-495 [PMID: 19399744 DOI: 10.1002/lt.21715]

93 **Liu M**, Wang J, Liu M, Hu X, Xu J. [Study of immunomodulatory function of exosomes derived from human umbilical cord mesenchymal stem cells]. *Zhonghua Yi Xue Za Zhi* 2015; **95**: 2630-2633 [PMID: 26711615]

94 **Berardis S**, Lombard C, Evraerts J, El Taghdouini A, Rosseels V, Sancho-Bru P, Lozano JJ, van Grunsven L, Sokal E, Najimi M. Gene expression profiling and secretome analysis differentiate adult-derived human liver stem/progenitor cells and human hepatic stellate cells. *PLoS One* 2014; **9**: e86137 [PMID: 24516514 DOI: 10.1371/journal.pone.0086137]

95 **Pan RL**, Wang P, Xiang LX, Shao JZ. Delta-like 1 serves as a new target and contributor to liver fibrosis down-regulated by mesenchymal stem cell transplantation. *J Biol Chem* 2011; **286**: 12340-12348 [PMID: 21239501 DOI: 10.1074/jbc.M110.194498]

96 **Xuan J**, Feng W, An ZT, Yang J, Xu HB, Li J, Zhao ZF, Wen W. Anti-TGFβ-1 receptor inhibitor mediates the efficacy of the human umbilical cord mesenchymal stem cells against liver fibrosis through TGFβ-1/Smad pathway. *Mol Cell Biochem* 2017; **429**: 113-122 [PMID: 28181132 DOI: 10.1007/s11010-017-2940-1]

97 **Argentou N**, Germanidis G, Hytiroglou P, Apostolou E, Vassiliadis T, Patsiaoura K, Sideras P, Germenis AE, Speletas M. TGF-β signaling is activated in patients with chronic HBV infection and repressed by SMAD7 overexpression after successful antiviral treatment. *Inflamm Res* 2016; **65**: 355-365 [PMID: 26856334 DOI: 10.1007/s00011-016-0921-6]

98 **Hata A**, Chen YG. TGF-β Signaling from Receptors to Smads. *Cold Spring Harb Perspect Biol* 2016; **8**: a022061 [PMID: 27449815 DOI: 10.1101/cshperspect.a022061]

99 **Buckley AR**, Russell DH, Montgomery DW, Putnam CW. Prolactin as a hepatotrophic hormone. *Transplant Proc* 1988; **20**: 706-709 [PMID: 3347958]

100 **Elmahdy NA**, Sokar SS, Salem ML, Sarhan NI, Abou-Elela SH. Anti-fibrotic potential of human umbilical cord mononuclear cells and mouse bone marrow cells in CCl_4- induced liver fibrosis in mice. *Biomed Pharmacother* 2017; **89**: 1378-1386 [PMID: 28320105 DOI: 10.1016/j.biopha.2017.03.007]

101 **An SY**, Jang YJ, Lim HJ, Han J, Lee J, Lee G, Park JY, Park SY, Kim JH, Do BR, Han C, Park HK, Kim OH, Song MJ, Kim SJ, Kim JH. Milk Fat Globule-EGF Factor 8, Secreted by Mesenchymal Stem Cells, Protects Against Liver Fibrosis in Mice. *Gastroenterology* 2017; **152**: 1174-1186 [PMID: 27956229 DOI: 10.1053/j.gastro.2016.12.003]

102 **Raymond A**, Ensslin MA, Shur BD. SED1/MFG-E8: a bi-motif protein that orchestrates diverse cellular interactions. *J Cell Biochem* 2009; **106**: 957-966 [PMID: 19204935 DOI: 10.1002/jcb.22076]

103 **Keshtkar S**, Azarpira N, Ghahremani MH. Mesenchymal stem cell-derived extracellular vesicles: novel frontiers in regenerative medicine. *Stem Cell Res Ther* 2018; **9**: 63 [PMID: 29523213 DOI: 10.1186/s13287-018-0791-7]

104 **Lou G**, Chen Z, Zheng M, Liu Y. Mesenchymal stem cell-derived exosomes as a new therapeutic strategy for liver diseases. *Exp Mol Med* 2017; **49**: e346 [PMID: 28620221 DOI: 10.1038/emm.2017.63]

105 **Jiang W**, Tan Y, Cai M, Zhao T, Mao F, Zhang X, Xu W, Yan Z, Qian H, Yan Y. Human Umbilical Cord MSC-Derived Exosomes Suppress the Development of CCl4-Induced Liver Injury through Antioxidant Effect. *Stem Cells Int* 2018; **2018**: 6079642 [PMID: 29686713 DOI: 10.1155/2018/6079642]

106 **Yan C**, Xue G, Wu L, Liu J, Hou Y. [Differentiation of Human Umbilical Cord Mesenchymal Stem Cells into Hepatocytes Induced by Rat Fibrotic Liver Tissue Extracts]. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2015; **29**: 878-883 [PMID: 26540984]

107 **Yan C**, Xue G, Zhang W, Han X, Liu J, Hou Y. [Effect of Blood Microenvironment of Rats with Hepatic Fibrosis on Differentiation of Human Umbilical Cord Mesenchymal Stem Cells into Hepatocytes and Its Mechanisms]. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2016; **30**: 754-760 [PMID: 29786272 DOI: 10.7507/1002-1892.20160154]

108 **Ren H**, Zhao Q, Cheng T, Lu S, Chen Z, Meng L, Zhu X, Yang S, Xing W, Xiao Y, Ren Q, Chi Y, Gu D, Yang R, Han ZC. No contribution of umbilical cord mesenchymal stromal cells to capillarization and venularization of hepatic sinusoids accompanied by hepatic differentiation in carbon tetrachloride-induced mouse liver fibrosis. *Cytotherapy* 2010; **12**: 371-383 [PMID: 20184502 DOI: 10.3109/14653241003596661]

109 **Tsuchiya A**, Kojima Y, Ikarashi S, Seino S, Watanabe Y, Kawata Y, Terai S. Clinical trials using mesenchymal stem cells in liver diseases and inflammatory bowel diseases. *Inflamm Regen* 2017; **37**: 16 [PMID: 29259715 DOI: 10.1186/s41232-017-0045-6]

110 **Fang X**, Liu L, Dong J, Zhang J, Song H, Song Y, Huang Y, Cui X, Lin J, Chen C, Liu B, Chen Z, Pan J, Chen X. A study about immunomodulatory effect and efficacy and prognosis of human umbilical cord mesenchymal stem cells in patients with chronic hepatitis B-induced decompensated liver cirrhosis. *J Gastroenterol Hepatol* 2018; **33**: 774-780 [PMID: 29293276 DOI: 10.1111/jgh.14081]

111 **Zhang Z**, Lin H, Shi M, Xu R, Fu J, Lv J, Chen L, Lv S, Li Y, Yu S, Geng H, Jin L, Lau GK, Wang FS. Human umbilical cord mesenchymal stem cells improve liver function and ascites in decompensated liver cirrhosis patients. *J Gastroenterol Hepatol* 2012; **27** Suppl 2: 112-120 [PMID: 22320928 DOI: 10.1111/j.1440-1746.2011.07024.x]

112 **Wang L**, Li J, Liu H, Li Y, Fu J, Sun Y, Xu R, Lin H, Wang S, Lv S, Chen L, Zou Z, Li B, Shi M, Zhang Z, Wang FS. Pilot study of umbilical cord-derived mesenchymal stem cell transfusion in patients with primary biliary cirrhosis. *J Gastroenterol Hepatol* 2013; **28** Suppl 1: 85-92 [PMID: 23855301 DOI: 10.1111/jgh.12029]

113 **Zhang YC**, Liu W, Fu BS, Wang GY, Li HB, Yi HM, Jiang N, Wang G, Zhang J, Yi SH, Li H, Zhang Q, Yang Y, Chen GH. Therapeutic potentials of umbilical cord-derived mesenchymal stromal cells for ischemic-type biliary lesions following liver transplantation. *Cytotherapy* 2017; **19**: 194-199 [PMID: 27964826 DOI: 10.1016/j.jcyt.2016.11.005]

114 **Shi M**, Liu Z, Wang Y, Xu R, Sun Y, Zhang M, Yu X, Wang H, Meng L, Su H, Jin L, Wang FS. A Pilot Study of Mesenchymal Stem Cell Therapy for Acute Liver Allograft Rejection. *Stem Cells Transl Med* 2017; **6**: 2053-2061 [PMID: 29178564 DOI: 10.1002/sctm.17-0134]

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