ISSN 1948-0210 (online)

World Journal of *Stem Cells*

World J Stem Cells 2019 November 26; 11(11): 904-1019

Published by Baishideng Publishing Group Inc

WJSC

World Journal of Stem Cells

Contents

Monthly Volume 11 Number 11 November 26, 2019

REVIEW

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

SYSTEMATIC REVIEW

World Journal of Stem Cells
Volume 11 Number 11 November 26, 2019

Contents

NAME OF JOURNAL
World Journal of Stem Cells

ISSN
ISSN 1948-0210 (online)

LAUNCH DATE
December 31, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Tong Cao, Shengwen Calvin Li, Carlo Ventura

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-0210/editorialboard.htm

EDITORIAL OFFICE
Jin-Lei Wang, Director

PUBLICATION DATE
November 26, 2019

INSTRUCTIONS TO AUTHORS
https://www.wjgnet.com/bpg/gerinfo/204

GUIDELINES FOR ETHICS DOCUMENTS
https://www.wjgnet.com/bpg/GerInfo/287

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
https://www.wjgnet.com/bpg/gerinfo/240

PUBLICATION MISCONDUCT
https://www.wjgnet.com/bpg/gerinfo/208

ARTICLE PROCESSING CHARGE
https://www.wjgnet.com/bpg/gerinfo/242

STEPS FOR SUBMITTING MANUSCRIPTS
https://www.wjgnet.com/bpg/GerInfo/239

ONLINE SUBMISSION
https://www.f6publishing.com

World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2019 November 26; 11(11): 904-919

DOI: 10.4252/wjsc.v11.i11.904

ISSN 1948-0210 (online)

REVIEW

Unexpected encounter of the parasitic kind

Holly Matthews, Florian Noulin

ORCID number: Holly Matthews (0000-0002-3890-9098); Florian Noulin (0000-0001-7430-8073).

Author contributions: Matthews H and Noulin F performed the literature search, and wrote and edited the review.

Conflict-of-interest statement: The authors declare no conflicts of interest for this article.

Manuscript source: Invited manuscript

Received: March 22, 2019
Peer-review started: March 22, 2019
First decision: August 1, 2019
Revised: August 10, 2019
Accepted: September 13, 2019
Article in press: September 13, 2019
Published online: November 26, 2019

P-Reviewer: Liu L
S-Editor: Zhang L
L-Editor: Filipodia
E-Editor: Xing YX

Holly Matthews, Florian Noulin, Centre for Applied Entomology and Parasitology, School of Life Sciences, Keele University, Keele ST5 5BG, United Kingdom

Corresponding author: Florian Noulin, PhD, Research Associate, Centre for Applied Entomology and Parasitology, School of Life Sciences, Keele University, Keele ST5 5BG, United Kingdom. f.noulin@keele.ac.uk
Telephone: +44-17-82733026
Fax: +44-17-82733516

Abstract

Both parasitology and stem cell research are important disciplines in their own right. Parasites are a real threat to human health causing a broad spectrum of diseases and significant annual rates morbidity and mortality globally. Stem cell research, on the other hand, focuses on the potential for regenerative medicine for a range of diseases including cancer and regenerative therapies. Though these two topics might appear distant, there are some "unexpected encounters". In this review, we summarise the various links between parasites and stem cells. First, we discuss how parasites' own stem cells represent interesting models of regeneration that can be translated to human stem cell regeneration. Second, we explore the interactions between parasites and host stem cells during the course of infection. Third, we investigate from a clinical perspective, how stem cell regeneration can be exploited to help circumvent the damage induced by parasitic infection and its potential to serve as treatment options for parasitic diseases in the future. Finally, we discuss the importance of screening for pathogens during organ transplantation by presenting some clinical cases of parasitic infection following stem cell therapy.

Key words: Stem cells; Parasites; Transplantation; Therapeutic; Host pathogen interactions

Core tip: The aim of this review was to bring together two important research disciplines: parasitology and stem cell biology. Parasites are the caustic agents of numerous diseases that have a huge impact on human health. The regeneration properties of stem cells are remarkable and the exploitation of such biology for clinical applications is an exciting area of research that may provide future treatment options for a wide range of human diseases. These apparently independent fields of research have multiple areas of overlap which we cover highlighting that these interactions, particularly from a clinical perspective, should be more seriously considered and studied.

Citation: Matthews H, Noulin F. Unexpected encounter of the parasitic kind. *World J Stem Cells* 2019; 11(11): 904-919
URL: https://www.wjgnet.com/1948-0210/full/v11/i11/904.htm
DOI: https://dx.doi.org/10.4252/wjsc.v11.i11.904

INTRODUCTION

Stem cells are defined by their self-renewal properties, and their ability to differentiate into adult cells[1]. They can be classified into two groups according to their biological properties: Pluripotent and multipotent stem cells. Pluripotent stem cells are able to differentiate into any cell type, and are found in the blastocyst, and early embryonic developmental stage[2]. Pluripotent stem cells give rise to three germ layers (endoderm, mesoderm and ectoderm) that ultimately produce different organs: cardiac, skeletal muscle or red blood cells for the mesoderm, pancreatic or lung cells for the endoderm, and neuron or skin cells for the ectoderm (Figure 1). Multipotent stem cells are more limited with regards to their differentiating capabilities. While they can differentiate into multiple cell types, they are already polarised down a specific route and can be found in most adult tissues where they replace aged or damaged cells throughout the lifespan of the multicellular organism[3].

Parasites are organisms that survive by taking nutrients from another organism: their host[4]. They represent more than 50% of all animal species[5] and can be broadly divided into three groups: Protozoa, helminths, and ectoparasites (Figure 2). The diseases they cause inflict huge health and socio-economic burden on low income countries[6]. Indeed, parasitic infections were considered responsible for 7.2% of deaths globally in 2016[7].

The relationship between parasites and stem cells is becoming increasingly studied (Figure 3). The purpose of this review is to further examine the relationship between stem cells and parasites, specifically looking at parasitic stem cells, how the parasites influence the host's stem cells, and how these interactions may be exploited clinically.

PARASITIC STEM CELLS

Although unicellular protozoan parasites do not have stem cells, the stems cells of other multi-cellular parasites such as helminths have been studied. Interestingly, one of the earliest references to the term "stem cell" was in relation to the parasitic helminth *Ascaris megalocephala* in the late 19th century[8]. Thus, it is perhaps unsurprising that some parasite's stem cells have been used to better understand the regeneration system.

Echinococcus

The tapeworm *Echinococcus* is one such parasite. This organism presents primarily as a zoonosis but can infect humans through animal transmission[9]. While the infection can manifest in four distinct forms, only two are relevant to human health: cystic and alveolar. Cystic *Echinococcus* infection is caused by *Echinococcus granulosus* and is characterised by the development of hydatid cysts, typically in the liver and lungs. Alveolar *Echinococcus* infection is caused by *E. multilocularis* and is initially asymptomatic, but a primary tumour-like lesion develops in the liver. This form is fatal if untreated. The *Echinococcus* life cycle begins when the adult (located in the intestine of the definitive Canidae host) releases eggs that exit the host in the faeces. Once ingested by an intermediate host, *i.e*. sheep, the eggs hatch and release oncospheres that pass through the intestinal wall and migrate into different organs. There, the larval forms develop into cysts containing protoscolices: the form that is ingested by the definitive host that evolves into protoscolex. Following ingestion, the protoscolices attach to the intestinal mucosa where they develop into adults.

Recently, Koziol *et al*[10] proposed to identify germinal cells in *E. granulosus*. For this, they treated the parasites cells with 5-Ethynyl-2´-deoxyuridine (EDU), a synthetic nucleotide that is incorporated into newly synthesised DNA, to identify cells in proliferation. They noticed that the germinal cells were less proliferative during the protoscolex stage but were reactivated when ingestion by a host was mimicked, highlighting the ability of these cells to remain quiescent while not in optimal conditions. To better characterise this cell population, they proposed to identify potential specific marker genes using the whole mount *in situ* hybridisation

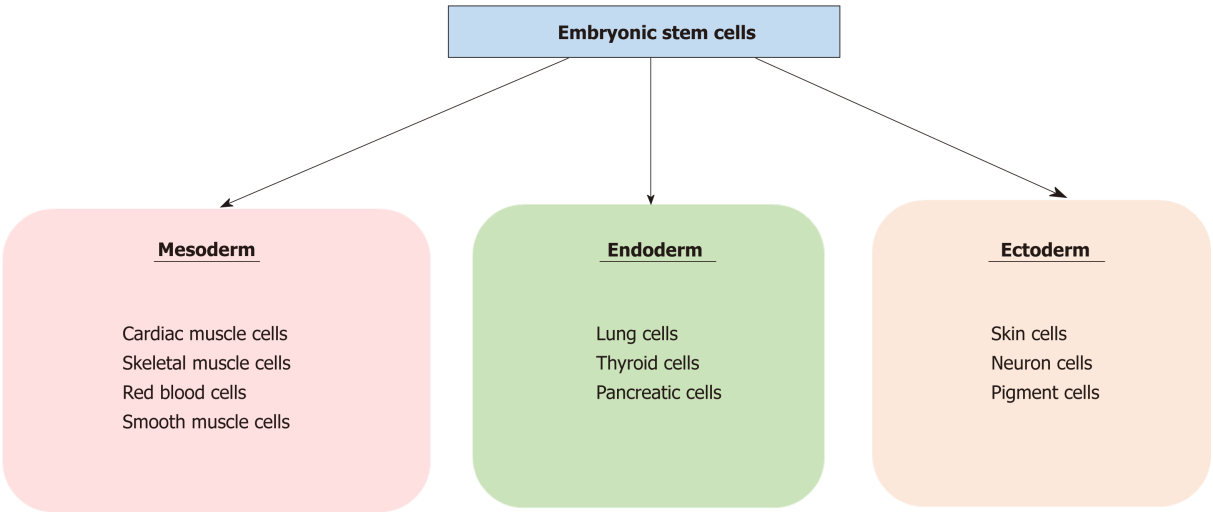

Figure 1 Diagram of the three human germ layers and lineage fates. This diagram shows the different organs' fate according to their original germ layer.

(commonly known as WMISH) technique but unfortunately were unsuccessful in this attempt. Notably, germinative cells could not be fully eliminated after gamma radiation treatment and the parasite only showed a delayed growth defect. From all these observations, they concluded that some parasite cells are capable of self-renewal and differentiation into proliferative competent cells.

In further work focusing on mobile genetic elements, Koziol *et al*[11], identified a novel family of terminal-repeat retrotransposons in miniature (known as TRIMs) as potential germline cell markers. Using a computer modelling approach, they identified putative Taeniid (Ta-)TRIMs and confirmed, by using the WMISH technic, that their expression was strongly restricted to proliferative germinative cells. They concluded that Ta-TRIMs could be a good marker of germinative cells in *E. granulosus*.

Schistosoma

Schistosoma spp are trematode worms that infect mammalian hosts. Eggs are released into a water source in the faeces or urine of the definitive host. The eggs hatch, releasing miracidia that infect aquatic snails. Once there, the parasite develops into a sporozoite and produces cercariae. These are released into the water and penetrate the skin of the definitive host. The parasite then sheds its characteristic forked tail to become schistosomulae and migrates to the veins. The final venule location of the adult *Schistosoma* is dependant of the species. The females lay eggs that migrate through the intestines to be excreted by either urination or defecation[12].

Collins *et al*[13] in 2013 produced the first report on adult somatic stem cells in *Schistosoma mansoni*. Comparing *S. mansoni* to already documented worms (*Planarian* and *Echinococcus*), they investigated the possible presence of neoblast-like cells in the parasite. Using EDU labelling, they observed a proliferating population in the mesenchyme of male and female parasites. Similar genes to the ones observed in planarian neoblasts were downregulated after gamma irradiation, which were correlated with a potential stem cell population in the parasite. They confirmed the self-renewal and differentiation potential of these cells using EDU/Bromodeoxyuridine labelling studies and noted that the *Sm-fgfrA* gene seemed to promote the long-term maintenance of neoblast-like cells in *S. mansoni* following RNA interference experiments. In order to better characterise these cell populations, they investigated gene expression following gamma radiation and performed RNA interference[14]. They identified 135 downregulated genes, most of which were involved in parasite's surface cell populations. By focusing in more detail on a specific gene (tetraspanin, *TSP-2*), they observed that its expression disappeared a couple of days after stem cell population depletion. They proposed that neoblast differentiation was biased towards tegument cells, especially those expressing *TSP-2+*.

Recently, Wang *et al*[15] investigated the role of *S. mansoni* stem cells throughout the different parasite stages, including the snail hosting period (Figure 4). Using single RNA sequencing (RNA-seq) studies, they identified three distinct stem cell populations in the sporozoite stage based on the main expression of *kfl nanos 2* and *fgfrA, B genes*: κ cells expressing *kfl+ nanos 2+*, cells expressing fgfrA, B^+ and δ cells expression both *kfl+ nanos 2+* and *fgfrA, B+*. During the asexual stages in the snail, δ cells were found in the embryo, as well as κ cells that were also present in extra-embryonic tissues, while the cells were mainly in the parasite's outer layer and not in

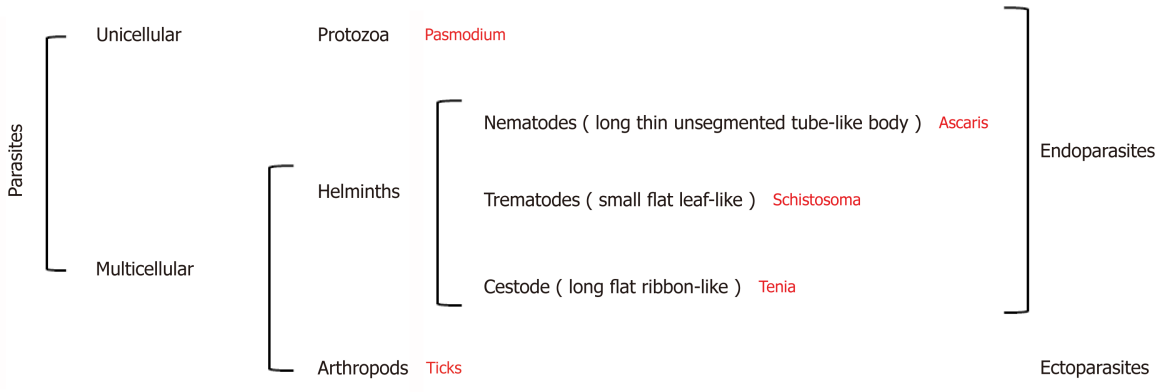

Figure 2 Diagram of parasite classification. Parasite classification is based on their cellular properties. Some species examples are provided.

the daughter embryo. Notably, a fixed number of κ and δ cells seem to be kept by the parasite while in the mammalian host where they form the source of the previously described stem cells. Another subpopulation of stem cell derived from κ cells and expressing *eledh* gene (a *Schistosome*-specific factor) was found in juvenal parasites and named ε cells. These cells can give rise to germline or posterior somatic cells depending on the nanos-1 expression (germline if active).

Parasite and stem cell models

In addition to the study of parasite's own stem cells, two worm species, *Planaria* and *Caenorhabditis elegans*, although they are not or are only rarely parasitic, have served as important models for parasite stem cell and general stem cell biology, respectively, and will therefore be discussed further.

Planaria are flatworms that are only rarely parasitic. They are typically hermaphroditic but can reproduce by fission[16]. These flatworms can be compared to the parasitic trematode *Schistosoma*. One example of this is from Collins *et al*[17], who studied a bioactive peptide in the planarian *Schmidtea mediterranea* and used this to identify novel pro-hormones in *S. mansoni*.

Planarian are able to fully regenerate as they have a population of stem cells named neoblasts[18]. Rossi *et al*[19] identified a group of 60 genes specific to planarian stem cells using microarrays and WMISH, many of which are involved in epigenetics and regulation of transcription. Interestingly, the planarian seems to be a good model for human nervous system regeneration[20] due to the its high degree of conservation with vertebrates. Moreover, Onal *et al*[21] found that pluripotent genes in human stem cells were conserved in the planarian. Using fluorescence-activated cell sorting coupled with RNA-seq studies, they specifically identified octamer-binding transcription factor 4, NANOG, and sex determining region Y-box 2. This later study proved that planarian organisms are valuable models to better understand human stem cells.

C. elegans is a roundworm belonging to the nematode family. This organism is well known by scientists as it is one of the most studied and best models for fundamental research as summarised in Kevin Strange's review[22]. It has been extensively used as a parasite model[23,24]. A better understanding of *C. elegans* stem cell biology would allow a better understanding of stem cell biology in general[25]. Among the many studies involving *C. elegans* stem cells, we can cite the work from Seidel *et al*[26] who studied stem cell quiescence following starvation, or Noormohammadi *et al*[27] who showed that TRiC/CCT assembly is linked to pluripotency of human stem cells and resistance to proteotoxic stress in *C. elegans*.

Parasite stem cells are evidently an interesting topic that is becoming increasingly better studied. Although their strong regeneration capacity is beyond that of their human counterparts, they serve to facilitate our understanding of stem cell capabilities and biology in general.

INTERACTIONS BETWEEN HOST STEM CELLS AND PARASITES

Parasites invade and occupy a number of different niches within their host species. Consequently, they interact with stems cells in a variety of ways. Many of the interactions are indirect due to tissue damage caused by the parasite, which trigger host immunity and damage repair mechanisms. Other interactions can be more direct

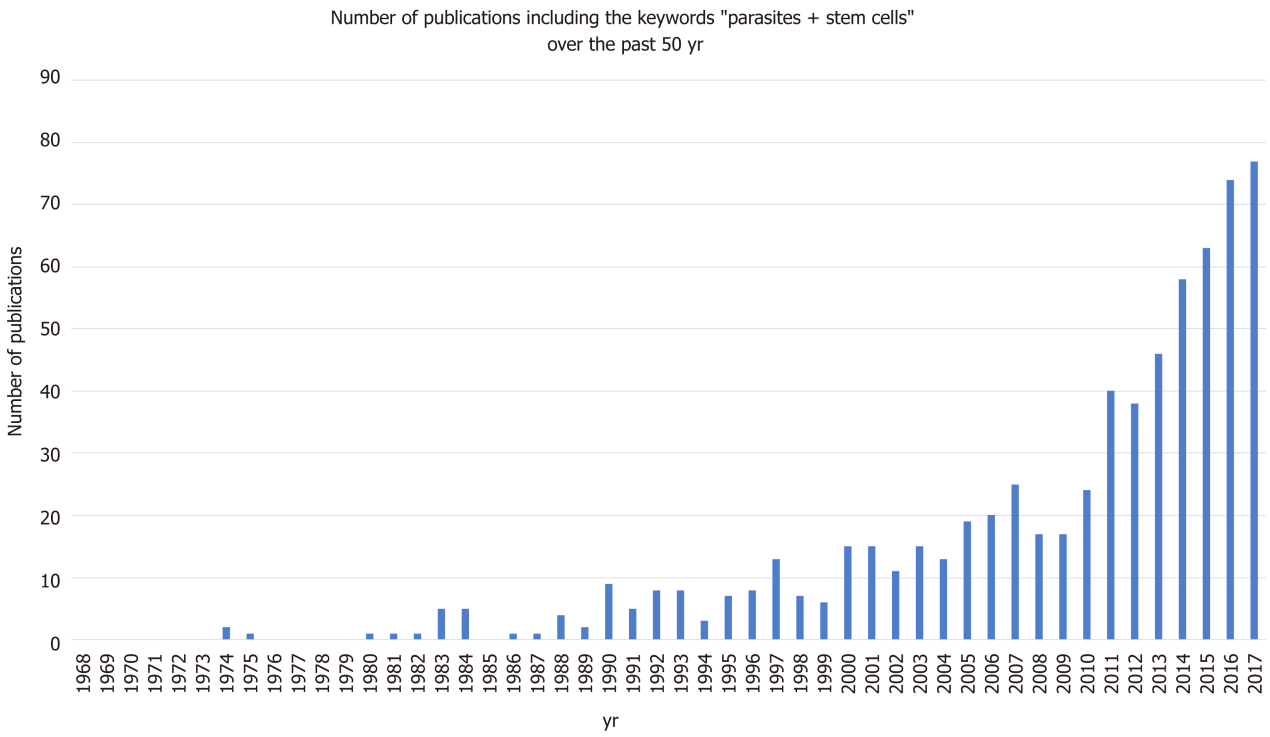

Figure 3 Chart of the yearly number of scientific literature publications including the keywords "stem cells + parasites" over the last 50 years. This diagram shows the numbers corresponding to scientific literature on PubMed found using the inclusive keywords "stem cells" and "parasites".

whereby the parasite or soluble parasitic factors directly affect stem cell proliferation. An additional layer of complexity is the ability of certain parasites to manipulate their host by not only inducing stem cell proliferation, but also stimulating differentiation into the parasite's preferred cell type. Evidence of such interactions will be discussed below.

Insect hosts

As well as infecting humans and other mammals, various insect species are subject to parasitism. In many cases, insects serve as disease vectors transmitting the parasite from one human to another. The parasites often go through developmental stages within their host insect and therefore impact insect fitness and insect stem cell biology.

Mosquitos: The malaria parasite *Plasmodium* uses female *Anopheles* mosquitos, which feed on mammalian blood as an intermediate host. Upon consuming a blood meal, the parasite enters the mosquito midgut. Following transversal of the midgut epithelium, the parasite undergoes a multiplication phase and the progeny migrate to the mosquito salivary gland. These parasites are then injected into another human during a subsequent blood meal. During invasion and intracellular migration of the mosquito midgut epithelium, the parasite triggers apoptosis and necrosis of invaded cells[28]. Surprisingly, this is not detrimental to mosquito survival. During infection of *Anopheles stephensi* with *Plasmodium falciparum*, Baton and Ranford-Cartwright (2007) showed that midgut regenerative cells underwent proliferation and differentiation to replace columnar midgut cells. Moreover, the number of proliferating and differentiating cells correlated with the level of midgut cell destruction. This indicates that the mosquito is able to compensate for parasitic damage through proliferative regeneration, inadvertently permitting parasite transmission[29].

Honey bees: A similar protective mechanism to that described for the mosquito was predicted in the honey bee, *Apis mellifera*. The honey bee endures enterocyte damage during infection with the parasitic microsporidian, *Nosema ceranae*[30]. Despite reports elsewhere that G1/S phase genes were upregulated during *N. ceranae* infection[31], Panek *et al*[32] recently showed a reduction in the replicative capacity of intestinal stem cells during infection. *Drosophila* infection with the parasitic wasp *Leptopilina boulardi* induces haematopoiesis through ROS-dependent induction of the Toll/NF-KB and EGFR pathways. *L. boulardi* also stimulates the preferential differentiation of lamellocytes, increasing their proportion to nearly 50% of all haemocytes. These cells are specifically involved in pathogen encapsulation as well as killing and implicate

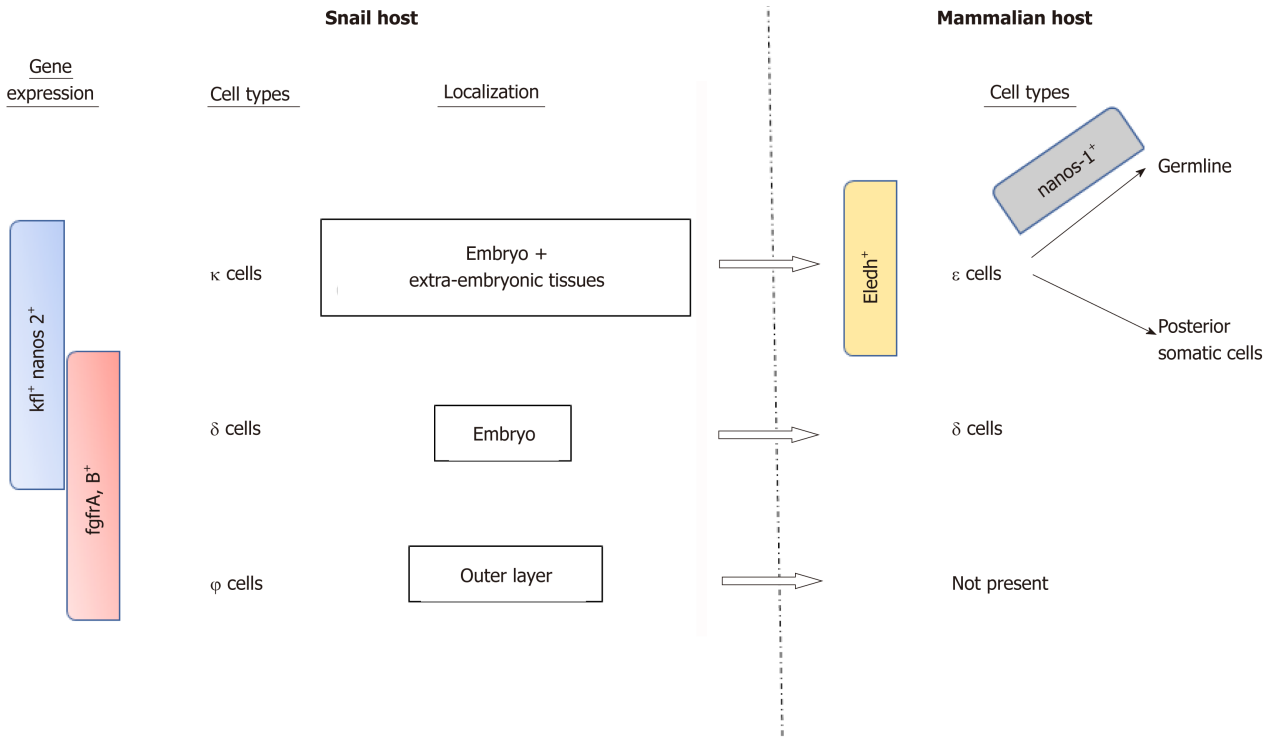

Figure 4 Gene expression and localisation of the different *S mansoni* stem cell populations in the two main hosts. This diagram explains the different subpopulations of *S. mansoni* stem cells based on specific gene expression and localisation.

the existence of a "do or die" mechanism, as survival depends on the neutralisation of wasp eggs before they hatch[33]. Evidently, insects have utilised their stem cell response when necessary to permit endurance or destruction of the parasite insult.

Mammalian hosts

Helminths: *Trichinella spiralis* is a nematode parasite that infects many carnivorous and omnivorous animals, including humans, and can be acquired by ingesting cysts from contaminated/undercooked meat[34]. Infection of Balb/c mice with parasitic worms such as *T. spiralis* or *Heligmosomoides polygyrus* (a mouse model for human helminth infections) have been shown to induce changes in stem cell proliferation within intestinal crypts of their hosts. *T. spiralis* leads to small intestine inflammation and an initial increase in epithelial stems cells (Ki-67-positive) and/or transient daughter cells (day 2 post infection). This alters the architecture of the intestinal crypt and causes an upward shift in the proliferative zone[35]. In another nematode parasite, *Trichuris muris*, such alterations are considered to facilitate expulsion of the parasite from its inter-epithelial niche[36]. However, during *T. spiralis* infection, the proliferative response is not sustained. The Ki-67-positive population decreased by days 6-12 post-infection, while the number of secretory Paneth cells increased (possibly due to differentiation). This may be advantageous as secretory products from Paneth cells, such as antimicrobial peptides and proteins, are considered important for host protection against luminal microorganisms[35]. Conversely, adult intestinal stem cells were shown to be repressed in Balb/c mice infected with *H. polygyrus*. During infection, granuloma associated crypts exhibited loss of the adult intestinal stem cell markers *Lgr5-GFP* and *Olfm4*, while *Ly6a* (which encodes stem cell antigen 1 (Sca-1)), was upregulated. *Nippostrongylus brasiliensis*, a nematode parasite that does not invade intestinal tissue, did not induce Sca-1. This suggests that upregulation of Sca-1 is a specific response to crypt injury induced by *H. polygyrus*. Immune cell-derived interferon gamma (IFNγ) was critical for the granuloma associated crypt response. Interestingly, foetal stem cell markers *Gjal* and *Spp1* were shown to be upregulated in the granuloma associated crypt, implicating a novel mechanism to repair intestinal crypt disruption.

Toxoplasma: *Toxoplasma* is a single-cell, protozoan parasite and is the causative agent of toxoplasmosis. Most human infections are asymptomatic, but the disease is more severe in immune compromised individuals and infants, if the mother were to become infected during pregnancy. Cats are the definitive host and shed the oocysts in their faeces which can be accidentally ingested by humans and other mammals or

birds[37]. *T. gondii* induces apoptosis and inhibits the differentiation of neuronal stem cells. These interactions are considered major contributors to congenital neuropathology. Various markers of apoptosis were found to be upregulated during the co-culture of *T. gondii* tachyzoites and neuronal stem cells. These experiments used a trans-well culture system, which only permits the passage of parasitic soluble factors, not the parasite itself. Consequently, inhibition was attributed to *T. gondii* excreted-secreted antigens (*Tg*-ESAs). The disruption of endoplasmic reticulum homeostasis occurs in many diseases and can lead to the activation of cell death pathways. Wang *et al*[38] showed decreased apoptosis following pre-treatment with the endoplasmic reticulum stress inhibitor TUDCA, implicating the involvement of the endoplasmic reticulum stress pathway in the interaction between *Tg*-ESAs and neuronal stem cell apoptosis. *Tg*-ESAs were also found to inhibit the differentiation of neuronal stem cells into neurones and astrocytes. This was determined by measuring the concentration of cell markers, specifically βIII-tubulin and glial fibrillary acidic protein. These decreased in a dose-dependent manner. A reduction in β-catenin and interactivity effects between *Tg*-ESAs and wnt3a (an activator of the Wnt/β-catenin pathway) implicated the involvement of the Wnt/β-catenin pathway, an important differentiation pathway in neuronal stem cells[39]. Further work implicated the virulence factor Rhoptry protein 18 as a partial mediator of the endoplasmic reticulum stress-induced apoptosis and cell differentiation (Wnt/β-catenin) pathways[40].

Plasmodium: Once injected into the human host *via* the bite of the female *Anopheles* mosquito, the parasites invade hepatocytes and multiply, forming liver schizonts[41]. These burst releasing merozoites, which invade erythrocytes, and begin the asexual reproductive cycle. During early malarial infection, both MSCs and HSCs are produced in the bone marrow (BM) and subsequently migrate to the spleen. Interestingly, adoptive transfer of *P. berghei*-induced MSCs conferred resistance to *P. berghei* in naive mice. MSCs were shown to migrate to the spleen, enhance pro-inflammatory cytokine production (specifically interleukin 12 [IL-12]), inhibit anti-inflammatory cytokines (specifically IL-10), and inhibit the accumulation of T-regulatory cells, which are induced by malaria as an immune evasion mechanism. Adoptive transfer of MSCs in late stage infections, however, did not alter disease progression[42]. An early haematopoiesis response is considered protective to the host, to counteract erythrocyte loss caused by parasite maturation in red blood cells. Unfortunately, the response cannot sustained during the course of malaria infection leading to the common disease-associated pathophysiology of anaemia[43]. One suggestion is that there is competition for the production of erythropoietic and immune cell, both derived from pluripotent stem cells[44]. Interestingly, during low oxygen conditions the kidneys produce erythropoietin, which in turn stimulates erythropoiesis. During mouse infection with *P. vinckei* or *P. chabaudi*, but not *P. berghei*, further erythropoiesis could be stimulated by exposing infected mice to high altitude conditions (hypoxia) in a decompression chamber. This indicated that the infection with *P. berghei* had already saturated the maximal erythropoiesis response. The initial increase in HSCs during early infection may be due to *P. berghei's* preference for invasion of reticulocytes. However, cellularity (total number of nucleated cells) and subsequently erythropoietic capacity in the BM decreased during the course of malaria infection. Despite enlargement and an increase in cellularity, the spleen does not compensate to meet the erythropoietic and hemopoietic needs of the host, which leads to anaemia. Although there are reports that pluripotent stems cells remain in G0 and are therefore protected, the lethality and severity of anaemia during malaria infection may be determined by irreversible damage to the self-renewal capacity of hematopoietic stems cells, as observed with chronic leishmania infection[45].

***Leishmania*:** *Leishmania spp* have a broad spectrum of interactions with various host stem cell lineages. Although macrophages are their primary host, they have been shown to be capable of infecting a variety of cells including different types of stems cells both *in vitro* and *in vivo*[46-49]. Allahverdiyev *et al*[47] showed *in vitro* invasion of adipose tissue-derived mesenchymal stem cells by *L. donovani, L. major, L. tropica*, and *L. infantum* parasites, raising potential concerns about stem cell transplantations and parasite transmission, which is discussed in more detail later. BM-derived MSCs (BM-MSCs) were also shown to be vulnerable to *L. infantum* and *L. donovani* invasion in an *in vivo* mouse model. This was confirmed by confocal microscopy and co-staining with antibodies for parasites and MSCs ($CD271^+/Sca1^+$) isolated from both the BM and spleen of infected mice. Interestingly, the authors not only visualised this invasion events *ex vivo*, but determined the viability of the amastigotes identified in BM-MSC $CD27^+CD45^-$ cells by inoculating the infected cells into an axenic *in vitro* culture system. Then promastigote forms were detected. Since MSCs have potent drug efflux pumps, the proposed purpose of this interaction is the potential avoidance

of drug-induced death. This is considered particularly important for latent infections that occur despite treatment. Such stem cells niches could also serve as a "hide-out" for parasites during asymptomatic infections.

As well as the modulation of host stem cell proliferation, following the direct invasion of stem cells, tissues infected with *Leishmania* parasites have also been reported to display alterations in host cytokine/chemokine expression and stem cell proliferation. Dameshghi *et al*[50] in 2016 isolated macrophages and adipose-derived MSCs from mice that were either sensitive or resistant to *Leishmania*. Isolated cells were placed, in various combinations, in a trans-well system. After 72 h of co-culture the MSC chamber was removed and the macrophage supernatant was replaced before challenge with *L. major* promastigotes. These macrophages, now considered MSC-educated, showed a reduction in phagocytosis, nitric oxide, IL-10, tumour necrosis factor alpha production and an induction in inflammatory cytokines in response to *L. major* infection. This response was independent of prior sensitivity to *Leishmania* (although the magnitude of the response did vary between treatment-group combinations). This implicates MSCs as the sensors and switchers of immune modulation and supports the potential of stem cells as a treatment for leishmaniasis. Local alterations in hematopoietic activity have also been reported in tissues harbouring *L. donovani* amastigotes *in vivo*[51]. Infection leads to an increase in progenitor cell frequency in the blood, and a correlation was observed between hematopoietic activity and parasite proliferation in the spleen and BM. Tissue-specific regulation of cytokine and chemokine expression was also observed, with an increase in those associated with monocyte and granulocyte maturation from multipotential precursors. *Leishmania*-infected stromal cells also display an enhanced capacity *in vitro*, through cytokine regulation, to support the differentiation of regulatory dendritic cells from hematopoietic progenitor cells[52]. The recombinant heat shock protein 70, from a related kinetoplastid parasite *Trypanosoma cruzi*, was also shown to have an immune-stimulatory effect on the maturation of dendritic cells from BM precursors. Remarkably, dendritic cell maturation occurred in the presence of the recombinant protein alone, fused to the KMP11 antigen as well as a 242-amino acid protein fragment[53]. This again could have implications for therapy. As the main source of immune cells, it is not surprising that BM-hematopoietic stem/progenitor cells are activated during *Leishmania* infection. This is consistent with the stress response to other acute infections[54]. However, *L. donovani* was shown to be capable of host manipulation by skewing HSC expansion to the differentiation of non-classical myeloid progenitor cells. Interestingly, cells produced in this manner were not only the preferred target of the parasite but were also more permissive to *Leishmania* infection, providing evidence of parasite manipulation[54]. Acute infections can give rise to such expansion that cannot be sustained during chronic infection. Using adoptive transfer of IFNγ sufficient $CD4^+$ T cells into immune-deficient mice, Pinto *et al*[55] in 2017 showed that chronic stimulation of HSC expansion, by this pathway, leads to hematopoietic exhaustion. In this instance, the reservoir of long-term HSCs irreversibly loses quiescence and enters into an active cell cycle, where they eventually lose their self-renewal capacity. During chronic infections, an effective anti-parasitic response is therefore at the expense of the reservoir of quiescent LT-HSC hematopoietic fitness. The importance of this trade-off for sufferers of life-long chronic infections is yet to be determined.

Evidently, parasites can develop a range of complex interactions with their host's stem cells by modulating their expression and fate. Although the growing body of research into this area in recent years (Figure 3) has meant that we know more now than ever before, the field remains in its infancy. Most of the aforementioned studies were conducted in insect or mouse models, as obvious technical and ethical issues limit human *in vivo* investigations. Due to the complexity of parasite life cycles, often having distinct development stages in multiple host tissues, studies into general parasite biology during human infection are also limited by the *in vitro* culture systems we have available. However, as our *in vitro* capabilities for stem cells production have expended parasitologist can utilise stems cells in order to recreate and study parasite development within a variety of host tissues that were previously inaccessible. Examples of such studies are detailed in Table 1. The availability of these valuable model systems will not only permit a better understanding of parasite biology but may open up new avenues for parasite treatment.

STEM CELL AND PARASITE THERAPIES

Stem cell therapies have become increasingly more applied as potential cures for important diseases such as liver injuries, muscle degeneration or anaemia[56]. These

Table 1 Table of stem cell classes used to model parasite infections

Stem cell type	Parasite	Details	Future purpose/possibilities	Ref.
Hematopoietic	*Plasmodium vivax*	Cluster of differentiation 34$^+$ derived reticulocytes	Growing *Plasmodium* species that have a reticulocytes preference	Grosgogeat *et al*[100]
	Leishmania infantum	BM HSCs-derived white cells were capable of phagocytosing and supporting promastigote development	Better understanding of promastigote development within macrophages	Carvalho-Gontijo *et al*[101]
Neuronal	*Toxoplasma gondii*	Fibroblast-derived and CD34$^+$-derived human neuronal-like cells were capable of supporting *T. gondii* tachyzoite development	Better understanding of parasite behaviour and development within human brain cells	Passeri *et al*[102], Tanaka *et al*[103]
Liver cells	*Plasmodium falciparum*, *vivax*, *yoelii*, and *berghei*	Human iPSC-derived hepatocyte-like cells, expressing important *Plasmodium* entry receptors CD81 and the SRB1, were permissive to the development many *Plasmodium* species	Better understanding of liver stage infection. Particularly for species such as *Plasmodium vivax* that can remain dormant in the liver and recrudesce many years later	Teranishi *et al*[104]

BM: Bone marrow; CD: Cluster of differentiation; HSCs: Hematopoietic stem cells; iPSCs: Induced pluripotent stem cells; SRB1: Scavenger receptor class B type 1.

therapies aim to replace dysfunctional cells or tissues with the transplantation of newly generated and functional stem cells. In addition to these applications, a new field of use for stem cell therapy has recently emerged: Parasitic treatment. The aim of these treatments is not to directly target the parasite itself but to help the patient fight and recover post infection, as we will discuss further in this section.

Trypanosoma

The first parasite infection that we will cover is the kinetoplastid parasite *T. cruzi*. This parasite is responsible for Chagas disease, that affects up to 7 million people, and predominantly occurs in Latin America[57]. The disease is curable if treated during early stage of infection, however treatment efficacy decreases with time. There are two distinct infection stages: Acute and chronic. The former stage can last up to 2 wk and, though the patient may have a high parasitaemia, the symptoms remain relatively mild and non-specific[58]. The latter stage can be subdivided into determinate and indeterminate phases. The determinate phase is marked by an equilibrium stage where host and parasites co-exist with no damage. Following an unknown mechanism, up to 30% of the patients will switch to the indeterminate phase. During this stage, Chagas disease-associated severe complications such as gastrointestinal or chronic heart diseases occur. Chagas heart disease remains the most severe and prevalent complication (45% of the chronic patients), which explains why it has been more extensively described. It is characterised by an extensive fibrosis of the myocardium that can evolve into chronic heart failure[59]. The treatment for Chagas heart disease cases are symptom dependent but can eventually lead to a heart transplant. This has further associated consequences and due to immunosuppression could lead to the reactivation of the parasitic infection[60]. It is thus very important to find an alternative method to repair cardiac tissue affected by the parasite. Soares *et al*[61] in 2004 were the first to use MSCs to treat heart failure following a parasitic infection. They transplanted BM of a healthy mouse into a mouse model of Chagas heart disease and observed a reduction in inflammation and fibrotic areas by 80% and 90% respectively. Interestingly, BMC treatment was shown to affect the myocardium transcriptome by decreasing the expression of upregulated genes involved in inflammation and fibrotic events in *T. cruzi*-infected mice[62]. One striking example is the strong downregulation of Galectin-3. Galectin-3 is a protein that is expressed by macrophages and correlates with inflammation in the mouse model of Chagas heart disease *via* its involvement in the induction of collagen production, proliferation of cardiac fibroblast and T-cell apoptosis suppression. This work implicates Galectin-3 as a potential therapeutic target and highlights the possible use of BMC injection as anti-inflammatory and anti-fibrotic treatment of Chagas's heart disease.

Following the initial success with mouse-models (reviewed by Carvalho *et al*[63]) the next step was to evaluate whether such results could be achieved during human infection. The first clinal trial took place on a 52-year-old man suffering of heart failure due to *T. cruzi* infection[64]. Purified mononuclear cells, isolated from healthy BM, were injected into the patient and an improved ventricular function was observed 30 d post-transplantation. This first success lead to additional studies which further expanded to a multi-centre randomised clinical trial. Over a total of 183 patients were treated with BM mononuclear cells in conjunction with granulocyte-colony stimulating factor. Unfortunately, no improvement in cardiac function was observed[65]. Although the results with the BM-derived mononuclear cells were not as good as expected, the use of a different type of stem cells, MSCs, did yield some interesting results and showed immunomodulatory properties as well as the potential for use in chronic chagasic cardiomyopathies[66]. Notably, only a small fraction of injected MSCs actually migrated to the heart in a Chagas mouse model. Indeed, nearly 70% could be found in liver, lungs, and spleen. Nevertheless, a reduction of the right ventricular dilatation was observed after MSC transplantation in Chagas mouse models. A further study showed that MSC treatment could influence gene expression and more especially the extracellular matrix protein laminin γ1 that was upregulated in infected mice compared to the MSCs treated ones[67]. Interestingly, two antagonist factors (the pro-inflammatory INFγ and the anti-inflammatory IL-10) that were upregulated in infected mice are down-regulated after MSC treatment. The pro-inflammatory stromal cell-derived factor-1 gene was also upregulated after MSC treatment, which could be correlated to the attraction of additional stem cells to the damaged area. More recently, Larocca *et al*[68] did not observe any cardiac function improvements following adipose-derived MSCs but a decrease in fibrosis and inflammatory cells. Evidently, there has been some difficulty in defining effective stem cell therapeutic targets, and further translating these from Chagas heart disease mouse models to the human disease system, but the value of such a treatment warrants further research into this area.

Leishmania spp

Leishmaniasis is a parasitic infection that can take different forms dependent on the parasitic strain. Three distinct forms affect different targets: the cutaneous form (that can be caused by almost any *leishmania spp*) affects the skin with the presence of lesions, the mucocutaneous form (caused by *L. Donovani* and *L. infantum*) leads to a partial or full destruction of mucosal membranes around the mouth, nose or throat while the visceral form affects the liver, BM, and spleen[69]. Leishmaniasis symptoms can also be due to an alteration of the host immune response. Knowing that MSCs can modulate the immune response[70], it seems reasonable to use these cells as therapy against cutaneous leishmania as Pereira *et al*[71] investigated. Unfortunately, they did not observe any protective effect, post-MSC injection, in a mouse model of cutaneous infection and even reported an increase in parasitic burden dependent on the route of MSC administration. This increase was correlated to an increase of IL-10 producing $CD4^+$ and $CD8^+$ T cells in the spleen. Of note, Il-10 is an anti-inflammatory cytokine that can limit the ability of macrophages to kill intracellular organisms and the lack of T-cell-derived IL-10 enhances protection from *L. major* infection in mouse as previously described[72]. Besides cutaneous leishmaniasis, stem cell therapies could be applied to other *Leishmania* strains such as the visceral and mucocutaneous forms of the disease. Studying *L. major* (responsible for the visceral form), Dameshghi *et al*[50] obtained promising immunomodulatory results from a cell-based assay, nevertheless the results still have to be translated to an *in vivo* system.

Schistosoma

Schistosomiasis is caused by a parasitic trematode. These worms remain in the host's blood vessels where the female lays the eggs that are the primary cause of pathology during chronic disease[73]. Proteolytic enzymes released by the eggs trigger a T-helper type 2 response that can lead to the fibrosis of affected tissues[74]. There are two main types of schistosomiasis infection: Urogenital schistosomiasis caused by *S. haematobium* and hepatic schistosomiasis caused by *S. mansoni, S. intercalatum, S. japonicum*, and *S. mekongi*[75]. The urogenital schistosomiasis can lead to fibrosis of the bladder and lower ureters and can even evolve to kidney failure. The hepatic schistosomiasis shows two distinct forms: Fibrotic hepatic disease and early inflammatory. The fibrotic hepatic disease (Symmer's pipestem fibrosis) is caused by the deposition of collagen in the periportal spaces, leading to the tissue's hypoxia. Studies have already been conducted to investigate the potential of MSCs to cure these symptoms and a decrease of liver fibrosis has been observed in treated mouse models of *S. mansoni*[76]. Moreover, El-Shennawy *et al*[77] recently showed that a treatment with BM-MSCs significantly decreased the granulomas size and the expression of alpha-smooth

muscle actin (a fibrosis factor) in hepatic stellate cells. The anti-fibrotic effect of the MSCs was not limited to these features and it included the inhibition of collagen deposition, a downregulation of transforming growth factor β1 and an upregulation of matrix metalloprotease 9, a matrix metalloprotease that leads to hepatic stellate cells apoptosis. As hepatic stellate cells are involved in the hepatic fibrosis process during liver injury[78], early injection of MSCs to trigger their apoptosis may lead to a more efficient cure. MSCs have also been used to treat *S. japonicum* mouse models using single injections or in combination with praziquantel (current drug treatment against schistosomiasis) and a recovery of the spleen and liver was observed due to the inhibition of the collagen deposition[79]. Another stem cell source has been tested by Elkhafif *et al*[80] in 2011 who used $CD133^+$ human umbilical cord blood stem cells to trigger the production of new blood vessels in order to promote vascularisation. This neo-vascularisation created a permissive environment allowing better survival of damaged cells.

Plasmodium

The Plasmodium parasite, the causative agent of malaria, is an important public health threat. Symptoms of the infection are initially non-specific and include headache, fever and fatigue[81]. How the infection progresses will then be defined as uncomplicated or severe (complicated) malaria. The severe form can be fatal with symptoms including consciousness, convulsions, bleeding, anaemia, renal impairment and pulmonary oedema[82]. The erythrocyte is one niche exploited by parasite in the human host and during a 48 or 72 h erythrocytic cycle (dependent on the species) the parasite continuously occupies, destroys and invades yet more red blood cells, thus having a huge impact on blood cell homeostasis. Knowing this feature, the use of HSC could be an interesting therapeutic approach. Indeed, Belyaev *et al*[83] in 2010 identified, in the BM of *P. chabaudi* infected mice, a new subset of haematopoietic progenitors triggered by IFNγ signal. The injection of these healthy atypical HSC progenitors within infected mice showed a greater clearance of infected erythrocytes leading to a decrease in infection-associated anaemia. MSCs isolated from the spleen of *P. berghei* infected mice, could be transplanted to naïve mice prior their exposition to *P. berghei*[42]. Interestingly, these MSCs produced protective cytokines such as tumour necrosis factor alpha and IL-1β that decreased the production of haemozoin (product from the erythrocyte haem degradation). This helps to counteract host evasion mechanisms used by the parasite as hemozoin inhibits macrophages and decreases the number of Treg cells in the spleen[84]. These cells can be used to treat cerebral malaria, a severe complication of *P. falciparum* infection, characterised by neurological symptoms including coma, convulsion, drowsiness and confusion and can affect spleen, kidneys and lungs[85]. Treatment of BM-MSCs improved the clearance of parasitised erythrocytes, increased the regeneration of hepatocytes and Kupffer cells (specific phagocytic cells) and increased the number of astrocytes and oligodendrocytes in the brain. In parallel, lung inflammation was reduced, by limiting collagen deposition, and the number of neutrophils, and amount of malaria pigmentation was decreased while the mesangial architecture of the kidney was restored[86]. Despite all these improvements, no reduction of the brain damage was observed.

Stem cell therapies have the potential to significantly alter the way in which we treat parasitic infections (Figure 5). The transplantation of stem cells to aid the recovery of damaged organs remains a really interesting addition to traditional parasitic cure methods. However, these methods and techniques are still in their infancy, and the affordability of stem cell-based therapies will be a significant factor in their uptake, particularly in developing countries where these neglected tropical diseases predominate[87].

Parasitic infections and transplantation

The future availability of stem cell therapies were predicted to be revolutionary for the cure of multiple disease as emphasised by the recent increase in interest in this research area and the number (7150 with the term "stem cells") of clinical trials registered with clinicaltrials.gov. Nevertheless, only HSCs have been so far approved by United States Food and Drug Administration to serve as cure therapy[88]. They are mainly used, through BM transplantation, to cure blood disorders such as leukaemia or myeloma. Prior to any transplantation, donations are screened for any potential infection. Some screens such as HIV 1+2, hepatitis C, hepatitis B, syphilis, human T-lymphotropic virus 1+2 are mandatory in United Kingdom[89]. However cytomegalovirus, malaria, *Trypanosoma cruzi* and West Nile virus remain simply additional. These additional screens are only performed if a donor risk has been identified. Multiple parasitic infections have been identified in recipient's post-transplantation (as discussed below). However, the immunosuppressive medication

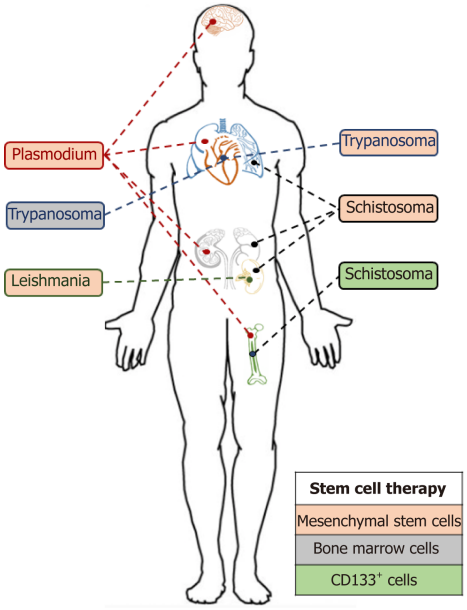

Figure 5 Parasitic infections and stem cell therapies targeting different organs. This diagram shows the different stem cell therapies used for different parasite infections and the targeted organs.

following an allogenic transplantation makes the patient more sensitive to opportunistic parasitic infections and must be considered as a potential source/ route of infection without the availability of donor pre-screens.

Though Leishmaniasis does not appear on the list of infectious pathogens screened prior to transplantation, its ability to survive within an intracellular environment by creating a suitable niche for replication[90] remains a threat. Indeed, although the parasite primarily invades phagocytic cells, *i.e*. macrophages, the amastigote form has been found in fibroblasts[48], hepatocytes[49] and amniotic epithelial cells[91]. The potential of *Leishmania* to survive in MSC has been recently investigated by Allahverdiyev *et al*[47] who were able to observe an *in vitro* infection of adipose-derived MSCs by *L. tropica, L. donovani, L. major* and *L. infantum*.

Of note, several cases of leishmania infections post MSC transplantations have been documented[92,93] though the presence of the parasite in the transplant was not proved. Several hypotheses could be raised, *i.e*. an infectious sand fly bite post transplantation, a latent infection in the patient or a re-activation of the opportunist parasite due to the immunosuppressive treatment. The *Plasmodium* parasite has also been described in the BM of an infected individual[94] and has been reported to be capable of invading HSC progenitors[95]. Moreover, the parasite, once in the human host, remains in the liver for 7 to 10 d and sometimes for a longer period as the hypnozoite form. For these reasons, reports of malaria infection following transplantation are not surprising. Indeed, Martín-Dávila *et al*[96] recently reported patients infected by *P. malariae* and *P. ovale* after liver and kidney transplantations. Parasite transmission can also occur after BM transplantation as described in a recent case even when the donor was not classified at risk[97].

Toxoplasma is a parasite that does not appear on the list of pathogen screens prior to transplantation as they are unlikely to be found in stem cells. Nevertheless, several cases of toxoplasmosis infected patients post-HSC transplantation have been described[98] mostly as an opportunistic infection following the immunosuppression treatment post-transplantation. Another parasite taking advantage of the lower immune defence post stem cell injection is cryptosporidium, which has also been identified in patients post transplantation[99].

It is thus necessary to consider parasites during the screening process pre-transplantation and not only based on donor recent travel history to endemic areas. The follow up post-transplantation monitoring should also not be neglected as opportunistic infections represent an important threat.

CONCLUSION

As can be seen from this review, the two seemingly distinct fields of stem cell research and parasitology are in fact closely entwined. Not only do multicellular parasites have

stem cells of their own, but certain parasites exploit stem cells to create niches either within their host stem cells, or by modulating host stem cell activity to create a more habitable environment. The use of stem cells as therapies to treat either the parasitic diseases or, more commonly, the sequalae associated with the infection, is becoming increasingly more studied. The presented literature allows us to highlight the importance of considering stem cells as important targets for parasites and not underestimate this relationship from a clinical point of view.

REFERENCES

1 **He S**, Nakada D, Morrison SJ. Mechanisms of stem cell self-renewal. *Annu Rev Cell Dev Biol* 2009; **25**: 377-406 [PMID: 19575646 DOI: 10.1146/annurev.cellbio.042308.113248]
2 **Ulloa-Montoya F**, Verfaillie CM, Hu WS. Culture systems for pluripotent stem cells. *J Biosci Bioeng* 2005; **100**: 12-27 [PMID: 16233846 DOI: 10.1263/jbb.100.12]
3 **Costamagna D**, Berardi E, Ceccarelli G, Sampaolesi M. Adult Stem Cells and Skeletal Muscle Regeneration. *Curr Gene Ther* 2015; **15**: 348-363 [PMID: 26122100 DOI: 10.2174/1566523215666150630121024]
4 **Centers for Disease Control and Prevention**. About Parasites. Available from: https://www.cdc.gov/parasites/about.html
5 **Poulin R**, Morand S. The diversity of parasites. *Q Rev Biol* 2000; **75**: 277-293 [PMID: 11008700 DOI: 10.1086/393500]
6 **Shiadeh MN**, Niyyati M, Fallahi S, Rostami A. Human parasitic protozoan infection to infertility: a systematic review. *Parasitol Res* 2016; **115**: 469-477 [PMID: 26573517 DOI: 10.1007/s00436-015-4827-y]
7 **World Health Organization**. Global health estimates 2016: Deaths by cause, age, sex, by country and by region, 2000-2016. 2018; World Health Organization
8 **Maehle AH**. Ambiguous cells: the emergence of the stem cell concept in the nineteenth and twentieth centuries. *Notes Rec R Soc Lond* 2011; **65**: 359-378 [PMID: 22332468 DOI: 10.1098/rsnr.2011.0023]
9 **Centers for Disease Control and Prevention**. Parasites - Echinococcosis. Available from: https://www.cdc.gov/parasites/echinococcosis/index.html
10 **Koziol U**, Rauschendorfer T, Zanon Rodríguez L, Krohne G, Brehm K. The unique stem cell system of the immortal larva of the human parasite Echinococcus multilocularis. *Evodevo* 2014; **5**: 10 [PMID: 24602211 DOI: 10.1186/2041-9139-5-10]
11 **Koziol U**, Radio S, Smircich P, Zarowiecki M, Fernández C, Brehm K. A Novel Terminal-Repeat Retrotransposon in Miniature (TRIM) Is Massively Expressed in Echinococcus multilocularis Stem Cells. *Genome Biol Evol* 2015; **7**: 2136-2153 [PMID: 26133390 DOI: 10.1093/gbe/evv126]
12 **Centers for Disease Control and Prevention**. Parasites - Schistosomiasis. Available from: https://www.cdc.gov/parasites/schistosomiasis/biology.html
13 **Collins JJ**, Wang B, Lambrus BG, Tharp ME, Iyer H, Newmark PA. Adult somatic stem cells in the human parasite Schistosoma mansoni. *Nature* 2013; **494**: 476-479 [PMID: 23426263 DOI: 10.1038/nature11924]
14 **Collins JJ**, Wendt GR, Iyer H, Newmark PA. Stem cell progeny contribute to the schistosome host-parasite interface. *Elife* 2016; **5**: e12473 [PMID: 27003592 DOI: 10.7554/eLife.12473]
15 **Wang B**, Lee J, Li P, Saberi A, Yang H, Liu C, Zhao M, Newmark PA. Stem cell heterogeneity drives the parasitic life cycle of Schistosoma mansoni. *Elife* 2018; 7 [PMID: 29988015 DOI: 10.7554/eLife.35449]
16 **Malinowski PT**, Cochet-Escartin O, Kaj KJ, Ronan E, Groisman A, Diamond PH, Collins ES. Mechanics dictate where and how freshwater planarians fission. *Proc Natl Acad Sci U S A* 2017; **114**: 10888-10893 [PMID: 28973880 DOI: 10.1073/pnas.1700762114]
17 **Collins JJ**, Newmark PA. It's no fluke: the planarian as a model for understanding schistosomes. *PLoS Pathog* 2013; **9**: e1003396 [PMID: 23874195 DOI: 10.1371/journal.ppat.1003396]
18 **Baguñà J**. The planarian neoblast: the rambling history of its origin and some current black boxes. *Int J Dev Biol* 2012; **56**: 19-37 [PMID: 22252540 DOI: 10.1387/ijdb.113463jb]
19 **Rossi L**, Salvetti A, Marincola FM, Lena A, Deri P, Mannini L, Batistoni R, Wang E, Gremigni V. Deciphering the molecular machinery of stem cells: a look at the neoblast gene expression profile. *Genome Biol* 2007; **8**: R62 [PMID: 17445279 DOI: 10.1186/gb-2007-8-4-r62]
20 **Gentile L**, Cebrià F, Bartscherer K. The planarian flatworm: an in vivo model for stem cell biology and nervous system regeneration. *Dis Model Mech* 2011; **4**: 12-19 [PMID: 21135057 DOI: 10.1242/dmm.006692]
21 **Onal P**, Grün D, Adamidi C, Rybak A, Solana J, Mastrobuoni G, Wang Y, Rahn HP, Chen W, Kempa S, Ziebold U, Rajewsky N. Gene expression of pluripotency determinants is conserved between mammalian and planarian stem cells. *EMBO J* 2012; **31**: 2755-2769 [PMID: 22543868 DOI: 10.1038/emboj.2012.110]
22 **Strange K**. An overview of C. elegans biology. *Methods Mol Biol* 2006; **351**: 1-11 [PMID: 16988422 DOI: 10.1385/1-59745-151-7:1]
23 **Gilleard JS**. The use of Caenorhabditis elegans in parasitic nematode research. *Parasitology* 2004; **128** Suppl 1: S49-S70 [PMID: 16454899 DOI: 10.1017/S003118200400647X]
24 **Burns AR**, Luciani GM, Musso G, Bagg R, Yeo M, Zhang Y, Rajendran L, Glavin J, Hunter R, Redman E, Stasiuk S, Schertzberg M, Angus McQuibban G, Caffrey CR, Cutler SR, Tyers M, Giaever G, Nislow C, Fraser AG, MacRae CA, Gilleard J, Roy PJ. Caenorhabditis elegans is a useful model for anthelmintic discovery. *Nat Commun* 2015; **6**: 7485 [PMID: 26108372 DOI: 10.1038/ncomms8485]
25 **Joshi PM**, Riddle MR, Djabrayan NJ, Rothman JH. Caenorhabditis elegans as a model for stem cell biology. *Dev Dyn* 2010; **239**: 1539-1554 [PMID: 20419785 DOI: 10.1002/dvdy.22296]
26 **Seidel HS**, Kimble J. Cell-cycle quiescence maintains Caenorhabditis elegans germline stem cells independent of GLP-1/Notch. *Elife* 2015; 4 [PMID: 26551561 DOI: 10.7554/eLife.10832]
27 **Noormohammadi A**, Khodakarami A, Gutierrez-Garcia R, Lee HJ, Koyuncu S, König T, Schindler C, Saez I, Fatima A, Dieterich C, Vilchez D. Somatic increase of CCT8 mimics proteostasis of human pluripotent stem cells and extends C. elegans lifespan. *Nat Commun* 2016; **7**: 13649 [PMID: 27892468 DOI: 10.1038/ncomms13649]

28 **Aly AS**, Vaughan AM, Kappe SH. Malaria parasite development in the mosquito and infection of the mammalian host. *Annu Rev Microbiol* 2009; **63**: 195-221 [PMID: 19575563 DOI: 10.1146/annurev.micro.091208.073403]

29 **Baton LA**, Ranford-Cartwright LC. Morphological evidence for proliferative regeneration of the Anopheles stephensi midgut epithelium following Plasmodium falciparum ookinete invasion. *J Invertebr Pathol* 2007; **96**: 244-254 [PMID: 17575986 DOI: 10.1016/j.jip.2007.05.005]

30 **Paris L**, El Alaoui H, Delbac F, Diogon M. Effects of the gut parasite Nosema ceranae on honey bee physiology and behavior. *Curr Opin Insect Sci* 2018; **26**: 149-154 [PMID: 29764655 DOI: 10.1016/j.cois.2018.02.017]

31 **Martín-Hernández R**, Higes M, Sagastume S, Juarranz Á, Dias-Almeida J, Budge GE, Meana A, Boonham N. Microsporidia infection impacts the host cell's cycle and reduces host cell apoptosis. *PLoS One* 2017; **12**: e0170183 [PMID: 28152065 DOI: 10.1371/journal.pone.0170183]

32 **Panek J**, Paris L, Roriz D, Mone A, Dubuffet A, Delbac F, Diogon M, El Alaoui H. Impact of the microsporidian Nosema ceranae on the gut epithelium renewal of the honeybee, Apis mellifera. *J Invertebr Pathol* 2018; **159**: 121-128 [PMID: 30268675 DOI: 10.1016/j.jip.2018.09.007]

33 **Louradour I**, Sharma A, Morin-Poulard I, Letourneau M, Vincent A, Crozatier M, Vanzo N. Reactive oxygen species-dependent Toll/NF-κB activation in the Drosophila hematopoietic niche confers resistance to wasp parasitism. *Elife* 2017; 6 [PMID: 29091025 DOI: 10.7554/eLife.25496]

34 **Centers for Disease Control and Prevention**. Parasites - Trichinellosis (also known as Trichinosis). Available from: https://www.cdc.gov/parasites/trichinellosis/biology.html

35 **Walsh R**, Seth R, Behnke J, Potten CS, Mahida YR. Epithelial stem cell-related alterations in Trichinella spiralis-infected small intestine. *Cell Prolif* 2009; **42**: 394-403 [PMID: 19397593 DOI: 10.1111/j.1365-2184.2009.00605.x]

36 **Cliffe LJ**, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK. Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* 2005; **308**: 1463-1465 [PMID: 15933199 DOI: 10.1126/science.1108661]

37 **Centers for Disease Control and Prevention**. Parasites - Toxoplasmosis (Toxoplasma infection). Available from: https://www.cdc.gov/parasites/toxoplasmosis/

38 **Wang T**, Zhou J, Gan X, Wang H, Ding X, Chen L, Wang Y, DU J, Shen J, Yu L. Toxoplasma gondii induce apoptosis of neural stem cells via endoplasmic reticulum stress pathway. *Parasitology* 2014; **141**: 988-995 [PMID: 24612639 DOI: 10.1017/S0031182014000183]

39 **Gan X**, Zhang X, Cheng Z, Chen L, Ding X, Du J, Cai Y, Luo Q, Shen J, Wang Y, Yu L. Toxoplasma gondii inhibits differentiation of C17.2 neural stem cells through Wnt/β-catenin signaling pathway. *Biochem Biophys Res Commun* 2016; **473**: 187-193 [PMID: 27012204 DOI: 10.1016/j.bbrc.2016.03.076]

40 **Zhang X**, Su R, Cheng Z, Zhu W, Li Y, Wang Y, Du J, Cai Y, Luo Q, Shen J, Yu L. A mechanistic study of Toxoplasma gondii ROP18 inhibiting differentiation of C17.2 neural stem cells. *Parasit Vectors* 2017; **10**: 585 [PMID: 29169404 DOI: 10.1186/s13071-017-2529-2]

41 **Centers for Disease Control and Prevention**. Malaria. Available from: https://www.cdc.gov/malaria/about/biology/index.html

42 **Thakur RS**, Tousif S, Awasthi V, Sanyal A, Atul PK, Punia P, Das J. Mesenchymal stem cells play an important role in host protective immune responses against malaria by modulating regulatory T cells. *Eur J Immunol* 2013; **43**: 2070-2077 [PMID: 23670483 DOI: 10.1002/eji.201242882]

43 **Pathak VA**, Ghosh K. Erythropoiesis in Malaria Infections and Factors Modifying the Erythropoietic Response. *Anemia* 2016; **2016**: 9310905 [PMID: 27034825 DOI: 10.1155/2016/9310905]

44 **Silverman PH**, Schooley JC, Mahlmann LJ. Murine malaria decreases hemopoietic stem cells. *Blood* 1987; **69**: 408-413 [PMID: 3801660]

45 **Maggio-Price L**, Brookoff D, Weiss L. Changes in hematopoietic stem cells in bone marrow of mice with plasmodium berghei malaria. *Blood* 1985; **66**: 1080-1085 [PMID: 3902119]

46 **Lopes CS**, Daifalla N, Das B, Dias da Silva V, Campos-Neto A. CD271+ Mesenchymal Stem Cells as a Possible Infectious Niche for Leishmania infantum. *PLoS One* 2016; **11**: e0162927 [PMID: 27622907 DOI: 10.1371/journal.pone.0162927]

47 **Allahverdiyev AM**, Bagirova M, Elcicek S, Koc RC, Baydar SY, Findikli N, Oztel ON. Adipose tissue-derived mesenchymal stem cells as a new host cell in latent leishmaniasis. *Am J Trop Med Hyg* 2011; **85**: 535-539 [PMID: 21896818 DOI: 10.4269/ajtmh.2011.11-0037]

48 **Bogdan C**, Donhauser N, Döring R, Röllinghoff M, Diefenbach A, Rittig MG. Fibroblasts as host cells in latent leishmaniosis. *J Exp Med* 2000; **191**: 2121-2130 [PMID: 10859337 DOI: 10.1084/jem.191.12.2121]

49 **Gangneux JP**, Lemenand O, Reinhard Y, Guiguen C, Guguen-Guillouzo C, Gripon P. In vitro and ex vivo permissivity of hepatocytes for Leishmania donovani. *J Eukaryot Microbiol* 2005; **52**: 489-491 [PMID: 16313440 DOI: 10.1111/j.1550-7408.2005.00055.x]

50 **Dameshghi S**, Zavaran-Hosseini A, Soudi S, Shirazi FJ, Nojehdehi S, Hashemi SM. Mesenchymal stem cells alter macrophage immune responses to Leishmania major infection in both susceptible and resistance mice. *Immunol Lett* 2016; **170**: 15-26 [PMID: 26703818 DOI: 10.1016/j.imlet.2015.12.002]

51 **Cotterell SE**, Engwerda CR, Kaye PM. Enhanced hematopoietic activity accompanies parasite expansion in the spleen and bone marrow of mice infected with Leishmania donovani. *Infect Immun* 2000; **68**: 1840-1848 [PMID: 10722572 DOI: 10.1128/IAI.68.4.1840-1848.2000]

52 **Nguyen Hoang AT**, Liu H, Juaréz J, Aziz N, Kaye PM, Svensson M. Stromal cell-derived CXCL12 and CCL8 cooperate to support increased development of regulatory dendritic cells following Leishmania infection. *J Immunol* 2010; **185**: 2360-2371 [PMID: 20624948 DOI: 10.4049/jimmunol.0903673]

53 **Planelles L**, Thomas M, Pulgar M, Marañón C, Grabbe S, López MC. Trypanosoma cruzi heat-shock protein-70 kDa,alone or fused to the parasite KMP11 antigen, induces functional maturation of murine dendritic cells. *Immunol Cell Biol* 2002; **80**: 241-247 [PMID: 12067411 DOI: 10.1046/j.1440-1711.2002.01081.x]

54 **Abidin BM**, Hammami A, Stäger S, Heinonen KM. Infection-adapted emergency haematopoiesis promotes visceral leishmaniasis. *PLoS Pathog* 2017; **13**: e1006422 [PMID: 28787450 DOI: 10.1371/journal.ppat.1006422]

55 **Pinto AI**, Brown N, Preham O, Doehl JSP, Ashwin H, Kaye PM. TNF signalling drives expansion of bone marrow CD4+ T cells responsible for HSC exhaustion in experimental visceral leishmaniasis. *PLoS Pathog* 2017; **13**: e1006465 [PMID: 28671989 DOI: 10.1371/journal.ppat.1006465]

56 **Mahla RS**. Stem Cells Applications in Regenerative Medicine and Disease Therapeutics. *Int J Cell Biol* 2016; **2016**: 6940283 [PMID: 27516776 DOI: 10.1155/2016/6940283]

57 **World Health Organization**. Chagas disease (American trypanosomiasis). Available from:

https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis)
58 **Malik LH**, Singh GD, Amsterdam EA. The Epidemiology, Clinical Manifestations, and Management of Chagas Heart Disease. *Clin Cardiol* 2015; **38**: 565-569 [PMID: 25993972 DOI: 10.1002/clc.22421]
59 **Bocchi EA**, Bestetti RB, Scanavacca MI, Cunha Neto E, Issa VS. Chronic Chagas Heart Disease Management: From Etiology to Cardiomyopathy Treatment. *J Am Coll Cardiol* 2017; **70**: 1510-1524 [PMID: 28911515 DOI: 10.1016/j.jacc.2017.08.004]
60 **Goldenberg RC**, Jelicks LA, Fortes FS, Weiss LM, Rocha LL, Zhao D, Carvalho AC, Spray DC, Tanowitz HB. Bone marrow cell therapy ameliorates and reverses chagasic cardiomyopathy in a mouse model. *J Infect Dis* 2008; **197**: 544-547 [PMID: 18237267 DOI: 10.1086/526793]
61 **Soares MB**, Lima RS, Rocha LL, Takyia CM, Pontes-de-Carvalho L, de Carvalho AC, Ribeiro-dos-Santos R. Transplanted bone marrow cells repair heart tissue and reduce myocarditis in chronic chagasic mice. *Am J Pathol* 2004; **164**: 441-447 [PMID: 14742250 DOI: 10.1016/S0002-9440(10)63134-3]
62 **Soares MB**, Lima RS, Souza BS, Vasconcelos JF, Rocha LL, Dos Santos RR, Iacobas S, Goldenberg RC, Lisanti MP, Iacobas DA, Tanowitz HB, Spray DC, Campos de Carvalho AC. Reversion of gene expression alterations in hearts of mice with chronic chagasic cardiomyopathy after transplantation of bone marrow cells. *Cell Cycle* 2011; **10**: 1448-1455 [PMID: 21467843 DOI: 10.4161/cc.10.9.15487]
63 **Carvalho AB**, Goldenberg RCDS, Campos de Carvalho AC. Cell therapies for Chagas disease. *Cytotherapy* 2017; **19**: 1339-1349 [PMID: 28887011 DOI: 10.1016/j.jcyt.2017.07.014]
64 **Vilas-Boas F**, Feitosa GS, Soares MB, Pinho-Filho JA, Mota A, Almeida AJ, Carvalho C, de Carvalho HG, de Oliveira AD, dos Santos RR. Bone marrow cell transplantation to the myocardium of a patient with heart failure due to chagas' disease. *Arq Bras Cardiol* 2004; **82**: 185-187, 181-184 [PMID: 15042255 DOI: 10.1590/s0066-782x2004000200010]
65 **Ribeiro Dos Santos R**, Rassi S, Feitosa G, Grecco OT, Rassi A, da Cunha AB, de Carvalho VB, Guarita-Souza LC, de Oliveira W, Tura BR, Soares MB, Campos de Carvalho AC; Chagas Arm of the MiHeart Study Investigators. Cell therapy in Chagas cardiomyopathy (Chagas arm of the multicenter randomized trial of cell therapy in cardiopathies study): a multicenter randomized trial. *Circulation* 2012; **125**: 2454-2461 [PMID: 22523306 DOI: 10.1161/CIRCULATIONAHA.111.067785]
66 **Jasmin**, Jelicks LA, Koba W, Tanowitz HB, Mendez-Otero R, Campos de Carvalho AC, Spray DC. Mesenchymal bone marrow cell therapy in a mouse model of chagas disease. Where do the cells go? *PLoS Negl Trop Dis* 2012; **6**: e1971 [PMID: 23272265 DOI: 10.1371/journal.pntd.0001971]
67 **Jasmin**, Jelicks LA, Tanowitz HB, Peters VM, Mendez-Otero R, de Carvalho ACC, Spray DC. Molecular imaging, biodistribution and efficacy of mesenchymal bone marrow cell therapy in a mouse model of Chagas disease. *Microbes Infect* 2014; **16**: 923-935 [PMID: 25218054 DOI: 10.1016/j.micinf.2014.08.016]
68 **Larocca TF**, Souza BS, Silva CA, Kaneto CM, Alcantara AC, Azevedo CM, Castro MF, Macambira SG, Soares MB, Ribeiro-dos-Santos R. Transplantation of adipose tissue mesenchymal stem cells in experimental chronic chagasic cardiopathy. *Arq Bras Cardiol* 2013; **100**: 460-468 [PMID: 23568098 DOI: 10.5935/abc.20130058]
69 **World Health Organization**. Leishmaniasis. Available from: http://www.who.int/mediacentre/factsheets/fs375/en/
70 **Bifari F**, Lisi V, Mimiola E, Pasini A, Krampera M. Immune Modulation by Mesenchymal Stem Cells. *Transfus Med Hemother* 2008; **35**: 194-204 [PMID: 21547117 DOI: 10.1159/000128968]
71 **Pereira JC**, Ramos TD, Silva JD, de Mello MF, Pratti JES, da Fonseca-Martins AM, Firmino-Cruz L, Kitoko JZ, Chaves SP, Gomes DCO, Diaz BL, Rocco PRM, de Matos Guedes HL. Effects of Bone Marrow Mesenchymal Stromal Cell Therapy in Experimental Cutaneous Leishmaniasis in BALB/c Mice Induced by Leishmania amazonensis. *Front Immunol* 2017; **8**: 893 [PMID: 28848541 DOI: 10.3389/fimmu.2017.00893]
72 **Schwarz T**, Remer KA, Nahrendorf W, Masic A, Siewe L, Müller W, Roers A, Moll H. T cell-derived IL-10 determines leishmaniasis disease outcome and is suppressed by a dendritic cell based vaccine. *PLoS Pathog* 2013; **9**: e1003476 [PMID: 23825956 DOI: 10.1371/journal.ppat.1003476]
73 **Olveda DU**, Li Y, Olveda RM, Lam AK, Chau TN, Harn DA, Williams GM, Gray DJ, Ross AG. Bilharzia: Pathology, Diagnosis, Management and Control. *Trop Med Surg* 2013; 1 [PMID: 25346933 DOI: 10.4172/2329-9088.1000135]
74 **Gryseels B**, Polman K, Clerinx J, Kestens L. Human schistosomiasis. *Lancet* 2006; **368**: 1106-1118 [PMID: 16997665 DOI: 10.1016/S0140-6736(06)69440-3]
75 **Gray DJ**, Ross AG, Li YS, McManus DP. Diagnosis and management of schistosomiasis. *BMJ* 2011; **342**: d2651 [PMID: 21586478 DOI: 10.1136/bmj.d2651]
76 **Fikry H**, Gawad SA, Baher W. Therapeutic Potential of Bone Marrow-Derived Mesenchymal Stem Cells on Experimental Liver Injury Induced by Schistosoma mansoni: A Histological Study. *Int J Stem Cells* 2016; **9**: 96-106 [PMID: 27426091 DOI: 10.15283/ijsc.2016.9.1.96]
77 **El-Shennawy SF**, Abdel Aaty HE, Radwan NA, Abdel-Hameed DM, Alam-Eldin YH, El-Ashkar AM, Abu-Zahra FA. Therapeutic Potential of Mesenchymal Stem Cells on Early and Late Experimental Hepatic Schistosomiasis Model. *J Parasitol* 2015; **101**: 587-597 [PMID: 26010300 DOI: 10.1645/15-754.1]
78 **Puche JE**, Saiman Y, Friedman SL. Hepatic stellate cells and liver fibrosis. *Compr Physiol* 2013; **3**: 1473-1492 [PMID: 24265236 DOI: 10.1002/cphy.c120035]
79 **Xu H**, Qian H, Zhu W, Zhang X, Yan Y, Mao F, Wang M, Xu H, Xu W. Mesenchymal stem cells relieve fibrosis of Schistosoma japonicum-induced mouse liver injury. *Exp Biol Med (Maywood)* 2012; **237**: 585-592 [PMID: 22678013 DOI: 10.1258/ebm.2012.011362]
80 **Elkhafif N**, El Baz H, Hammam O, Hassan S, Salah F, Mansour W, Mansy S, Yehia H, Zaki A, Magdy R. CD133(+) human umbilical cord blood stem cells enhance angiogenesis in experimental chronic hepatic fibrosis. *APMIS* 2011; **119**: 66-75 [PMID: 21143528 DOI: 10.1111/j.1600-0463.2010.02693.x]
81 **World Health Organization**. Malaria. Available from: https://www.who.int/news-room/fact-sheets/detail/malaria
82 **Basu S**, Sahi PK. Malaria: An Update. *Indian J Pediatr* 2017; **84**: 521-528 [PMID: 28357581 DOI: 10.1007/s12098-017-2332-2]
83 **Belyaev NN**, Brown DE, Diaz AI, Rae A, Jarra W, Thompson J, Langhorne J, Potocnik AJ. Induction of an IL7-R(+)c-Kit(hi) myelolymphoid progenitor critically dependent on IFN-gamma signaling during acute malaria. *Nat Immunol* 2010; **11**: 477-485 [PMID: 20431620 DOI: 10.1038/ni.1869]
84 **Hansen DS**, Schofield L. Natural regulatory T cells in malaria: host or parasite allies? *PLoS Pathog* 2010; **6**: e1000771 [PMID: 20442856 DOI: 10.1371/journal.ppat.1000771]
85 **Wang W**, Qian H, Cao J. Stem cell therapy: a novel treatment option for cerebral malaria? *Stem Cell Res Ther* 2015; **6**: 141 [PMID: 26253514 DOI: 10.1186/s13287-015-0138-6]

86 **Souza MC**, Silva JD, Pádua TA, Torres ND, Antunes MA, Xisto DG, Abreu TP, Capelozzi VL, Morales MM, Sá Pinheiro AA, Caruso-Neves C, Henriques MG, Rocco PR. Mesenchymal stromal cell therapy attenuated lung and kidney injury but not brain damage in experimental cerebral malaria. *Stem Cell Res Ther* 2015; **6**: 102 [PMID: 25998168 DOI: 10.1186/s13287-015-0093-2]

87 **Greenwood HL**, Thorsteinsdottir H, Perry G, Renihan J, Singer P, Daar A. Regenerative medicine: New opportunities for developing countries. *International Journal of Biotechnology* 2006; **3**: 60-77 [DOI: 10.1504/IJBT.2006.008964]

88 **Food and Drug Administration**. Approved cellular and gene therapy products. Available from: https://www.fda.gov/biologicsbloodvaccines/cellulargenetherapyproducts/approvedproducts/default.htm

89 **Service NB**. Guidelines for the blood transfusion services in the united kingdom. Norwich, UK: The Stationary Office, 2013

90 **Moradin N**, Descoteaux A. Leishmania promastigotes: building a safe niche within macrophages. *Front Cell Infect Microbiol* 2012; **2**: 121 [PMID: 23050244 DOI: 10.3389/fcimb.2012.00121]

91 **Rittig MG**, Bogdan C. Leishmania-host-cell interaction: Complexities and alternative views. *Parasitol Today* 2000; **16**: 292-297 [PMID: 10858648]

92 **Komitopoulou A**, Tzenou T, Baltadakis J, Apostolidis J, Karakasis D, Harhalakis N. Is leishmaniasis an "unusual suspect" of infection in allogeneic transplantation? *Transpl Infect Dis* 2014; **16**: 1012-1018 [PMID: 25412926 DOI: 10.1111/tid.12316]

93 **Dantas Brito M**, Campilho F, Branca R, Pinho Vaz C, Silva C, Sousa T, Mendes C, Campos A. Visceral leishmaniasis: a differential diagnosis to remember after bone marrow transplantation. *Case Rep Hematol* 2014; **2014**: 587912 [PMID: 25574404 DOI: 10.1155/2014/587912]

94 **Tatura SNN**, Gunawan S, Bernadus J, Sandjoto S. Plasmodium falciparum found in the bone marrow of a child in Manado City, East Indonesia: A case report. *Asian Pac J Trop Med* 2017; **10**: 1015-1017 [PMID: 29111186 DOI: 10.1016/j.apjtm.2017.09.016]

95 **Skoglund CR**. Vasodilatation in human skin induced by low-amplitude high-frequency vibration. *Clin Physiol* 1989; **9**: 361-372 [PMID: 2766680 DOI: 10.1182/blood-2009-07-231894]

96 **Martín-Dávila P**, Norman F, Fortún-Abete J, Píris M, Lovatti R, Rubio JM, Martinez-Pérez A, Graus J, Ta G, Villarubia J, Mahillo B, López-Vélez R. Donor-derived multiorgan transmission of mixed P. malariae and P. ovale infection: Impact of globalization on post-transplant infections. *Transpl Infect Dis* 2018; **20**: e12938 [PMID: 29863799 DOI: 10.1111/tid.12938]

97 **Ladeb S**, Ben Abdejlil N, Fakhfakh N, Lakhal A, Belloumi D, Ben Hamed L, Kallel A, Torjman L, El Fatimi R, Hmida S, Kallel K, Ben Othman T. Plasmodium falciparum infection transmitted by transfusion: A cause of hemophagocytic syndrome after bone marrow tranplantation in a non-endemic country. *Transpl Infect Dis* 2018; **20**: e12887 [PMID: 29573075 DOI: 10.1111/tid.12887]

98 **Martino R**, Maertens J, Bretagne S, Rovira M, Deconinck E, Ullmann AJ, Held T, Cordonnier C. Toxoplasmosis after hematopoietic stem cell transplantation. *Clin Infect Dis* 2000; **31**: 1188-1195 [PMID: 11073751 DOI: 10.1086/317471]

99 **Florescu DF**, Sandkovsky U. Cryptosporidium infection in solid organ transplantation. *World J Transplant* 2016; **6**: 460-471 [PMID: 27683627 DOI: 10.5500/wjt.v6.i3.460]

100 **Grosgogeat Y**, Bessede P, Himbert J. [Myocardial infarct with fatal development in the young patient. Anatomical study]. *Pathol Microbiol (Basel)* 1967; **30**: 727-731 [PMID: 4231068 DOI: 10.1371/journal.pone.0112496]

101 **Carvalho-Gontijo R**, Moreira DR, Resende M, Costa-Silva MF, Peruhype-Magalhães V, Ribeiro CMF, Ribeiro DD, Silvestre R, Cordeiro-da-Silva A, Martins-Filho OA, Teixeira-Carvalho A. Infection of hematopoietic stem cells by Leishmania infantum increases erythropoiesis and alters the phenotypic and functional profiles of progeny. *Cell Immunol* 2018; **326**: 77-85 [PMID: 29248120 DOI: 10.1016/j.cellimm.2017.10.016]

102 **Passeri E**, Jones-Brando L, Bordón C, Sengupta S, Wilson AM, Primerano A, Rapoport JL, Ishizuka K, Kano S, Yolken RH, Sawa A. Infection and characterization of Toxoplasma gondii in human induced neurons from patients with brain disorders and healthy controls. *Microbes Infect* 2016; **18**: 153-158 [PMID: 26432947 DOI: 10.1016/j.micinf.2015.09.023]

103 **Tanaka N**, Ashour D, Dratz E, Halonen S. Use of human induced pluripotent stem cell-derived neurons as a model for Cerebral Toxoplasmosis. *Microbes Infect* 2016; **18**: 496-504 [PMID: 27083472 DOI: 10.1016/j.micinf.2016.03.012]

104 **Teranishi H**. [Infection of Schwann cells along corneal stromal nerve fiber in experimental herpes simplex keratitis]. *Nippon Ganka Gakkai Zasshi* 1974; **78**: 1079-1083 [PMID: 4375936 DOI: 10.1016/j.stemcr.2015.01.002]

World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2019 November 26; 11(11): 920-936

DOI: 10.4252/wjsc.v11.i11.920

ISSN 1948-0210 (online)

REVIEW

Colon cancer stemness as a reversible epigenetic state: Implications for anticancer therapies

Audrey Vincent, Aïcha Ouelkdite-Oumouchal, Mouloud Souidi, Julie Leclerc, Bernadette Neve, Isabelle Van Seuningen

ORCID number: Audrey Vincent (0000-0003-0058-2058); Aïcha Ouelkdite-Oumouchal (0000-0002-7048-5555); Mouloud Souidi (0000-0001-6767-8738); Julie Leclerc (0000-0003-1130-7211); Bernadette Neve (0000-0003-1516-1379); Isabelle Van Seuningen (0000-0002-3131-2694).

Author contributions: Vincent A and Ouelkdite-Oumouchal A equally contributed to this paper with literature review, analysis, and drafting; Neve B contributed to the design of the correlation studies; All authors equally contributed to the critical revision and editing and final approval of the final version.

Supported by "Institut National de la Santé et de la Recherche Médicale"; (Inserm); "Centre National de la Recherche Scientifique" (CNRS); "la Ligue Nationale contre le Cancer" (Committees 59, 60 and 62).

Conflict-of-interest statement: The authors declare no conflicts of interests for this article.

Audrey Vincent, Aïcha Ouelkdite-Oumouchal, Mouloud Souidi, Julie Leclerc, Bernadette Neve, Isabelle Van Seuningen, Lille University, Institut National de la Santé et de la Recherche Médicale, CHU Lille, UMR-S 1172-Jean-Pierre Aubert Research Center, Lille F-59000, France

Julie Leclerc, Department of Biochemistry and Molecular Biology, Lille University Hospital, Lille F-59000, France

Corresponding author: Isabelle Van Seuningen, PhD, Director, Inserm UMR-S 1172-Jean-Pierre Aubert Research Center, Bâtiment Cancer, 1 Rue Michel Polonovski, Lille F-59045, France. isabelle.vanseuningen@inserm.fr
Telephone: +33-320-298867
Fax: +33-320-538562

Abstract

The recent discovery of cancer cell plasticity, *i.e.* their ability to reprogram into cancer stem cells (CSCs) either naturally or under chemotherapy and/or radiotherapy, has changed, once again, the way we consider cancer treatment. If cancer stemness is a reversible epigenetic state rather than a genetic identity, opportunities will arise for therapeutic strategies that remodel epigenetic landscapes of CSCs. However, the systematic use of DNA methyltransferase and histone deacetylase inhibitors, alone or in combination, in advanced solid tumors including colorectal cancers, regardless of their molecular subtypes, does not seem to be the best strategy. In this review, we first summarize the knowledge researchers have gathered on the epigenetic signatures of CSCs with the difficulty of isolating rare populations of cells. We raise questions about the relevant use of currently available epigenetic inhibitors (epidrugs) while the expression of numerous cancer stem cell markers are often repressed by epigenetic mechanisms. These markers include the three cluster of differentiation CD133, CD44 and CD166 that have been extensively used for the isolation of colon CSCs.and . Finally, we describe current treatment strategies using epidrugs, and we hypothesize that, using correlation tools comparing associations of relevant CSC markers with chromatin modifier expression, we could identify better candidates for epienzyme targeting.

Key words: Cancer stem cells; Colon cancer; Epigenetics; Chromatin modifying enzymes; CD44; CD133; CD166

Manuscript source: Invited manuscript

Received: March 26, 2019
Peer-review started: March 28, 2019
First decision: June 3, 2019
Revised: August 29, 2019
Accepted: September 11, 2019
Article in press: September 11, 2019
Published online: November 26, 2019

P-Reviewer: Chivu-Economescu M, Kiselev SL, Miyoshi E
S-Editor: Zhang L
L-Editor: Filipodia
E-Editor: Xing YX

Core tip: The recent discovery of cancer cell plasticity, *i.e.* their ability to reprogram into cancer stem cells either naturally or under chemotherapy and/or radiotherapy, has changed, once again, the way we consider cancer treatment. In this review, we try to understand why current epigenetic treatments have failed to prove their efficacy in solid tumors including colorectal cancer and we hypothesize that, using correlation tools comparing associations of relevant cancer stem cell markers with chromatin modifier expression, we may identify better candidates for epienzyme targeting.

Citation: Vincent A, Ouelkdite-Oumouchal A, Souidi M, Leclerc J, Neve B, Van Seuningen I. Colon cancer stemness as a reversible epigenetic state: Implications for anticancer therapies. *World J Stem Cells* 2019; 11(11): 920-936
URL: https://www.wjgnet.com/1948-0210/full/v11/i11/920.htm
DOI: https://dx.doi.org/10.4252/wjsc.v11.i11.920

INTRODUCTION

Hierarchy of the tumor: turning an old concept into a new dogma

Although only recently upgraded as the keystone of the natural history of tumors, the concept of "cancer stem cells (CSCs)" was anticipated several decades ago as researchers soon discovered that cancer cells possessed unequal capacities when it comes to initiating a new tumor or resisting to therapies[1]. Indeed already during the 1960s, ethically disputed experiments of auto-transplantation that were conducted in human patients demonstrated that numerous cancer cells were necessary to establish cancer transplants, giving hints on the rare nature (1/1000000) of tumor-initiating cells[2].

With the arrival of the first commercially available cell sorters, followed by immunocompromised mouse models that allowed selective xenotransplantation of cancer cells, the interest in this cancer cell subpopulation has then been growing exponentially, with the field of hematologic malignancies as pioneers[1-3]. As early stem or progenitor cells were shown to be involved in leukemias and myelo-proliferative disorders, tumor initiating cells have rapidly been renamed "cancer stem cells", hence creating a link with histological observations from the 1850's when pathologists had first hypothesized that tumors could develop from residual embryonic tissues[1-3]. Indeed, CSCs share numerous characteristics with normal embryonic stem cells, such as rareness, cell cycle arrest and quiescence, unlimited self-renewal through asymmetric division, and addiction to stem cell signaling pathways.

In solid tumors, the cancer stem cell (CSC) model (Figure 1B) was initially considered as a concept that could not be applied to all tumor types and was often opposed to the stochastic clonal evolution hypothesis[4,5], where genetic mutations are the major cause of tumor heterogeneity (Figure 1A)[6,7]. Increasing evidence of cancer plasticity, where cells easily exchange their position in the tumor hierarchy, switching from stem to non-stem states[8,9] and also from non-stem to stem states, reconcile these two models (Figure 1C). Indeed, several studies have demonstrated that cancer cells from different types of tumors, including colon cancer, can naturally convert to CSCs in culture, in total absence of therapeutic agents inducing genetic alteration[8]. Additionally, anti-cancer treatments such as chemotherapies[10] or radiotherapy[9] not only participate in the selection of resistant clones in the bulk of a tumor but also induce stemness characteristics in non-stem cancer cells. These findings are transposable to tumors from patients in whom stemness-related aggressiveness (invasion capacities, release of circulating tumor cells) is either innate or acquired after exposure to hypoxia, metabolic stress, and treatments.

More importantly, the extreme cellular plasticity involving rapid phenotype switches between CSCs and their non-stem counterpart is probably mediated by epigenetic mechanisms that are reversible in nature, rather than newly acquired genetic mutations. Indeed, we (unpublished data) and others have shown a systematic equilibrium between CSC marker expressing and non-expressing cells that spontaneously occurs after cell sorting of negative *vs* positive populations[11]. In accordance with epigenetic mechanisms involved in this balance between stem and non-stem cancer cells, CSCs harbor a permissive epigenetic state[12-14], comparable to normal stem cells, while epigenetic profiles of differentiated cells are locked in order

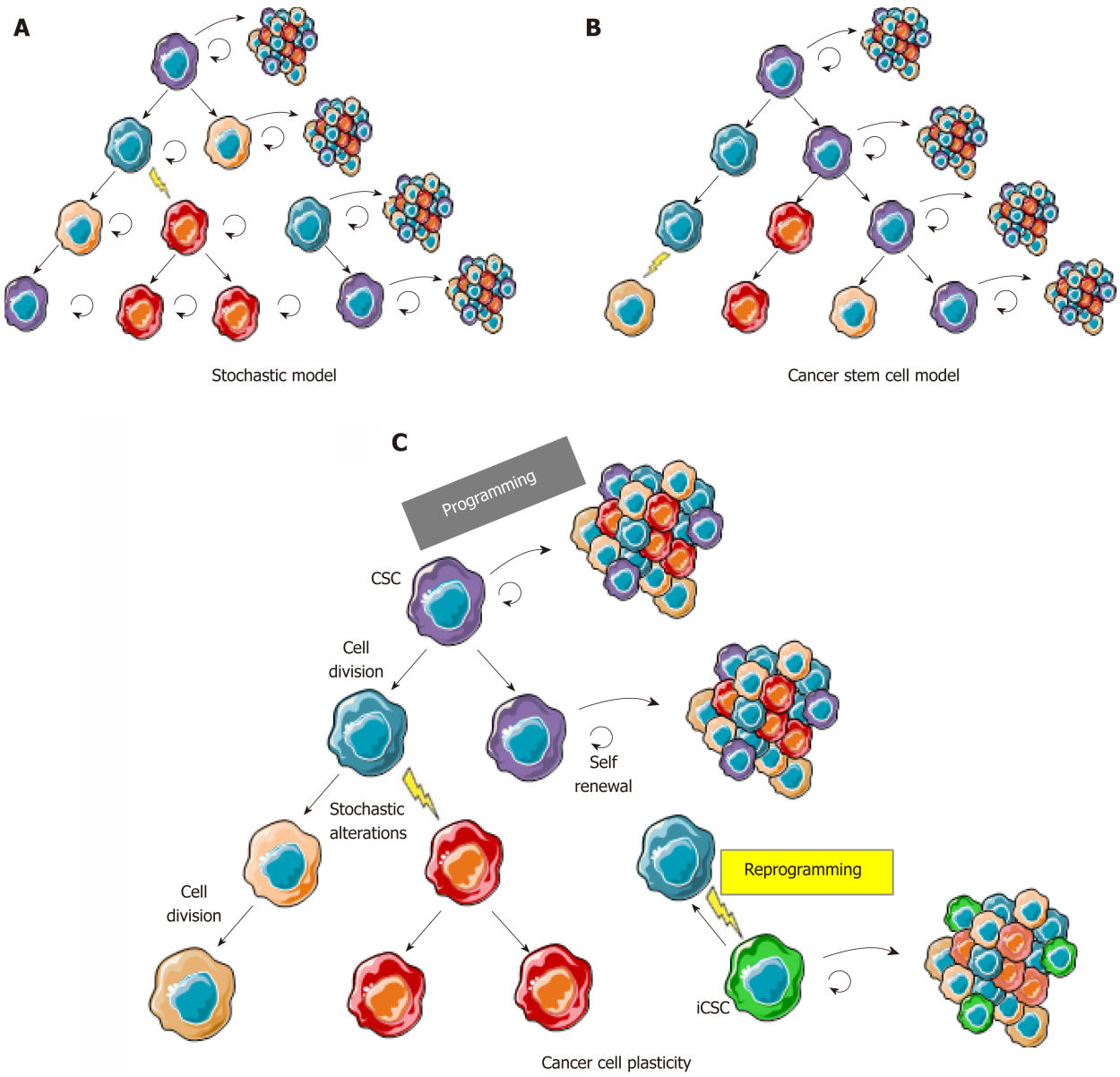

Figure 1 The cancer cell plasticity model reconciles cancer stem cell and stochastic models. A: In the stochastic model, cancer cells are heterogeneous because of accumulation of genetic and epigenetic alterations acquired through excessive proliferation, but most cells are able to proliferate and initiate new tumors; B: In the cancer stem cell model, cancer cells are organized in a hierarchy comparable to normal tissues where CSCs (in purple) are the only cells able to regenerate a tumor with its whole heterogeneity; C: In the cancer plasticity model, cancer cells are able to rapidly switch back and forth between a stem and a non-stem state. CSCs change to non-stem cell most likely occurs through epigenetic programming and silencing of cancer stem cell/pluripotency markers. Reprogramming, leading to induced CSCs (in green) from non-stem cancer cells, can either occur through reversible epigenetic modifications or genetic alterations, hence leading to a new clonal population of cancer cells in the tumor. CSC: Cancer stem cell; iCSC: Induced CSC.

to shape cellular identity and functions. However, numerous genetic alterations may render cancer cell reprogramming more complicated to target. Understanding this flexibility is crucial for the development of new anticancer drugs. Therefore, new therapeutic strategies will have to combine the targeting of the bulk of the tumor and of the CSCs, whether they are pre-existing or induced. Hence, if these different types of CSCs share the same reversible reprogramming mechanisms, epigenetic therapies would represent an interesting strategy (Figure 2).

UNRAVELING THE EPIGENETIC SIGNATURE OF CSCs: A KEY TO UNDERSTANDING CANCER CELL PLASTICITY AND REPROGRAMMING

Current research on induced pluripotent stem cells teaches us that erasing epigenetic marks of the differentiated cell of origin greatly improves reprogramming[15,16].

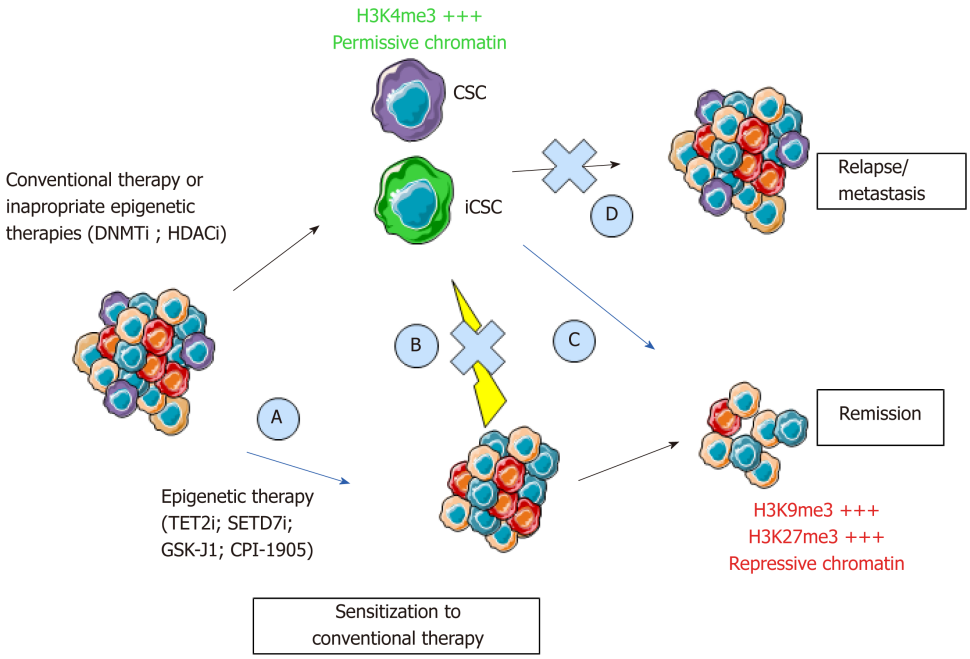

Figure 2 Epigenetic programming and reprogramming of cancer cells and consequences for therapeutic strategies. New therapeutics will have to combine the targeting of the bulk of the tumor, pre-existing CSCs, and iCSCs through inhibition of cancer cell reprogramming. Epigenetic therapies could inhibit CSCs to sensitize cancer cells to conventional therapies (A, C), inhibit cancer cells reprogramming (B), and inhibit relapse through inhibition of self-renewal (D). CSC: Cancer stem cell; iCSC: Induced CSCs; DNMTi: DNA methyltransferase inhibitor; HDACi: Histone deacetylase inhibitor; TET2i: Ten-eleven-translocation 2 inhibitor; SETD7i: SET domain containing 7 inhibitor; H3K4me3: Trimethylation of lysine 4 on histone 3; H3K9me3: Trimethylation of lysine 9 on Histone 3; H3K27me3: Trimethylation of lysine 27 on histone 3.

Mapping stemness-associated chromatin modifications would surely facilitate the development of therapeutic strategies evoking differentiation of CSCs. Indeed, the "differentiating strategy" has proven its efficiency in certain types of hematologic tumors years ago[17]. On the other hand, these strategies have failed to prove their systematic efficacy in solid tumors, where CSCs may come from multiple origins, including normal differentiated cells[8,18], or stochastic genetic events altering cancer cells along tumor evolution.

Molecular mechanisms involved in the shaping of the cancer epigenetic landscapes, and especially in CSCs, are complex. Genetic alterations leading to loss or gain of epienzyme functions have been described[19], but only rare studies focus exclusively on CSCs. Furthermore, overexpression of epienzymes may not reflect an oncogenic role. The histone methyltransferase enhancer of zeste 2 (EZH2) is the perfect example of this paradox, while its overactivation in certain types of cancers is the sole sign of a compensation mechanisms in cells where histone H3 K27 trimethylation is diluted over excessive proliferation[20-22].

Because of the rareness and diversity of CSCs and the fact that no consensus has been found for markers that would allow their proper isolation, few studies have been able to define clearly the cancer stemness-associated epigenetic profiles. It has been shown, however, that mammary and hepatic CSCs harbor more permissive chromatin profiles, more prone to gene activation, than non-stem cancer cells[12]. They also harbor decreased DNA methylation and trimethylation of lysine 27 on histone H3 at tumor suppressor genes[12]. Similarly, trimethylation of lysine 4 on histone H3 is found preferentially at pluripotency genes such as BMI1, NOTCH1, and WNT1 in CSCs from acute myeloid leukemia patients[13]. CSCs from head and neck carcinomas harbor an epigenetic signature with only 22 differentially methylated genes between cluster of differentiation (CD)-44+ CSCs and CD44 non-stem cancer cell populations[14], pointing out subtle and specific differences between stem and non-stem cancer cells. The same type of signature has been identified in breast tumors[23], but still needs to be defined for CSCs from the different colon cancer molecular subtypes.

The common findings from studies on CSC epigenetic profiles are that CSC markers are either regulated by epigenetic mechanisms in normal and/or cancer cells or harbor different epigenetic profiles between stem and non-stem cancer cells[24]. Alternatively, CSC markers can themselves be directly or indirectly responsible for chromatin modifications through their presence in Polycomb Repressive Complexes (BMI1) or through histone demethylation (JARID1B).

Among CSC markers, CD133 and CD44 have been extensively utilized to isolate

cancer cells with tumorigenic characteristics in numerous types of cancers, including colon cancers in which CD133 predicts low survival. In combination with CD166, these two markers better stratify low, intermediate, and high-risk cases of colorectal cancer[25] (CRC) than the three markers alone. We have shown that combined expression of these three markers is associated with stemness and resistance to 5-fluorouracil (5-FU) in colon cancer cells[26,27]. Interestingly, expression and splicing of these three markers are epigenetically regulated in cancer cells.

Epigenetic regulation of PROM1, encoding the CSC marker CD133

CD133 is a 120 kDa transmembrane glycoprotein that was initially identified in hematopoietic stem cells[28] and is involved in cell-cell interactions and membrane organization, through its binding to phospholipids[29]. CD133 is now used as a stem cell marker in most solid tumors including colorectal cancers[29]. More importantly, CD133 is directly involved in stemness properties as its inhibition alters self-renewal and tumorigenic capacities[30]. CD133 is also associated with metastasis and invasiveness through the decrease of metalloprotease 2 expression. Interestingly, its expression is positively correlated with the expression of ATP-binding cassette (ABC) transporters ABCG1 and ABCG2, hence associating CSC properties to chemo-resistance through the presence of multidrug efflux pumps[28]. CD133 is correlated to poor prognosis in numerous cancers including CRC.

The human PROM1 gene, which encodes CD133 (prominin-1), consists of 28 exons and is localized on chromosome 4p15. The regulation of PROM1 transcription includes five alternative promoters (P1-5) involved in embryonic phase development. PROM1 harbors seven alternative spliced variants, of which the most documented are CD133s1 and CD133s2 (lacking exon 3)[31,32]. Of those only CD133s1 is mainly associated with normal tissue in brain, bone marrow, and blood[31]. CD133s2 expression is widely observed in human fetal tissue and adult tissues and in several cancers, including breast, colon, lung, and pancreatic carcinomas. CD133s2 is also associated with the human stem cell niche[33].

PROM1 expression is inversely correlated with methylation of CpG islands in its promoter in numerous cancer cell lines[34,35]. For example, in glioma tissues, an inverse correlation has been shown between the CpG methylation status of promoter P1 and P2 and expression levels of PROM1 transcripts. Epigenetic regulation of PROM1 also includes histone modifications, since synergistic effects are observed when using histone deacetylase (HDAC) inhibitors in combination with DNA methyltransferase (DNMT) inhibitors to re-express the cell surface marker CD133 in ovarian cancer cells[24].

Epigenetic regulation of CD44

CD44 is a transmembrane glycoprotein interacting with components of the extracellular matrix including hyaluronic acid, collagens, fibronectins, integrins, and laminin[36]. These interactions induce cytoskeleton modifications and activation of signaling pathways involved in cell adhesion and migration. CD44 expression has been associated with tumor progression, epithelial-to-mesenchymal transition[37], and poor survival in colon cancers[38]. Mutations have been described in solid tumors, suggesting its implication in carcinogenesis[37]. Most importantly, CD44-variant-6 (v6) is a well-recognized marker of colon and gastric CSCs[39,40].

The human CD44 gene consists of 20 exons and is located on chromosome 11p13. Exons 1-5 and 15-19 encode homologous N-ter (extracellular) and C-ter (extracellular, transmembrane and intracellular) domains respectively forming the standard isoform CD44s. Alternative splicing of exons 5a-14 result in different variants/isoforms of CD44 (CD44v). CD44 variants are overexpressed in numerous types of solid tumors including pancreatic (CD44v2-6), breast (CD44v6/v8-10), prostate (CD44v2/v6), head and neck (CD44v3), and colon (CD44v6/v10) cancers[37]. In contrast with CD44s variant that is absent from mouse normal intestinal stem cells[37], CD44 variants (CD44v4-10) have been associated with normal and cancer stemness. For instance, CD44v6 and CD44v4 are largely overexpressed in stem cells compared to their progeny (transit-amplifying cells). CD44 variants, and not CD44s, are involved in adenoma formation in mouse models of familial polycystic adenomas[41]. Similarly, expression of CD44v6 is restricted to colon CSCs and is associated with worse survival in patients with CRC[39]. In most studies, CD44v4-10 variants are associated with aggressiveness, resistance, metastasis, and poor prognosis in solid tumors including colon cancers.

Epigenetic regulation of the CD44 gene has recently been described. DNA methylation at CpG islands located in the promoter and histone H3 acetylation regulate its silencing or expression[37], respectively. DNMT inhibition induced DNA methylation and histone modification changes at the CD44 gene promoter, increasing CD44 mRNA levels in cancer cell lines[37,42]. More importantly, alternative splicing of

CD44 and, hence, the expression of CSC specific variants is epigenetically regulated. Indeed, accumulation of histone H3 lysine 9 trimethylation and HP1 stabilizes pre-mRNA binding to the chromatin and therefore facilitates exon inclusion[43].

Epigenetic regulation of ALCAM encoding the CSC marker CD166

CD166 is a member of the immunoglobulin superfamily and is engaged in homophilic or heterophilic interactions with the cell surface receptor CD6. CD166, which is expressed on antigen-presenting cells, is involved in maturation of CD6-expressing resting T-cells and is also expressed in mesenchymal stem cells, neural cells, osteoblasts, and stromal cells of the bone marrow. It is involved in hematopoiesis, development of central and peripheral nervous system, sense organs, and differentiation of endothelial as well as epithelial lineages[44]. CD166 has proven its relevance as a CSC marker alone or in combination with CD44 in several studies including studies on colon cancer cell lines[45,46].

The human gene ALCAM, encoding CD166, is located on chromosome 3q.13 and consists of 16 exons. A soluble isoform, produced through alternative splicing, has been described, but its role remains unknown[47].

The ALCAM promoter harbors several CpG islands regulated by DNA methylation. It has been shown that the DNMT inhibitor 5-Aza-2′-deoxycytidine increased its expression in breast cancer cells[48], hence raising questions about the use of these inhibitors in breast cancer patients.

Interestingly, the three discussed CSC and survival markers (CD44, CD166, and CD133, Figure 3) are not only epigenetically regulated in cancer cells, but our transcriptomic analyses of public CRC data also revealed that the combined expression of these markers in colon cancer is correlated with a specific panel of epienzyme expression (both positive and negative correlations are listed in Tables 1-6).

EPIENZYME CORRELATION WITH COLON CSC MARKERS: A HINT FOR SUCCESS IN EPIGENETIC THERAPEUTIC STRATEGIES?

Current epigenetic strategies

Most solid tumors, including CRC, acquire chemoresistance over time. In addition to expected chemo-induced genetic alterations, the molecular mechanisms involved include transcriptional plasticity that is regulated epigenetically, for example by multiple DNA methylation changes at CpG islands[49]. Contrary to genetic alterations, epigenetic modifications are potentially reversible, paving the way for novel cancer therapies.

This past decade has seen the emergence of many epigenetic therapies, especially DNA hypomethylating drugs (DNA methyltransferase inhibitors) and HDAC inhibitors (HDACi), as well as lysine-specific histone demethylase-1, EZH2 inhibitors, and many others[50].

Epigenetic drugs have shown beneficial effects for the treatment of hematological malignancies and led to the approval of epidrugs like 5-azacitidine, decitabine, vorinostat, romidepsin, belinostat, and panobinostat for patient treatment[50]. In contrast, clinical trials assessing the efficacy of these epigenetic drugs in monotherapies for CRC and other solid tumors failed to improve clinical outcomes with, in some cases, no response at all[51], never passing the phase III trial necessary for approval (clinical trials for CRC listed in Tables 1-6).

Several hypotheses could be raised regarding this apparent lack of efficacy of epidrugs for solid tumors. First, compared to hematologic malignancies, solid tumors harbor a weaker penetrance of mutations in genes encoding chromatin modifying enzymes[19]. Second, the pleiotropic effect of current epidrugs leads to the combined inhibition of many members of a given family of epienzymes that have a broad spectrum of action and opposing roles in cancer cells. Third, and most importantly, cancer cell plasticity, and the switch between stem and non-stem state, is orchestrated by complex mechanisms, including epigenetic silencing of CSC markers and pluripotency genes. Despite genetic heterogeneity among cancer cells[52] (due to stochastic or chemo-/radio-induced mutations along tumor evolution/treatment), DNA methylation and histone deacetylation seem to represent typical mechanisms involved in repressing stemness markers in non-stem cancer cells, as previously demonstrated for CD44, CD133, and CD166. Therefore, inhibiting DNMT and HDAC may result in increased expression of CSC markers[37,42,48] along with an increased stemness potential. Last, patients included in these clinical trials often present metastatic or advanced disease and are recruited regardless of the molecular subtype

Table 1 Negative correlation between combined expression of cancer stem cell markers CD133, CD44 and CD166 and epigenetic writers

Family/gene symbol	Epidrug/chemical probe	Clinical trials for CRC	Results/status	Z-score	*P* value
DNA methyltransferases	5-azacytidine (Vidaza)[1]	Early Phase I to phase II[63,64]	No OR[63,65]		
	5-aza-2′-désoxycytidine (Decitabine)[1]	Phase I to phase II[66-68]	No OR[66]; beneficial with Panitumumab[68]		
	EGCG (Green tea extract)	Preclinical spheroid-derived cancer stem cell xenograft models[69]	Sensitization to chemotherapy		
	Zebularine	Preclinical xenografts[70]	Anticancer activity		
	RG108, Procainamide[2]				
DNMT3A, DNMT3B, DNMT3L				-2.788/-4.848/-4.321	< 0.005
Activating Lysine methyltransferases					
SETD6	vp22-RelA302-316[3][71]			-4.641	3.47E-06
SETD1A				-4.375	1.212E-05
Repressive Lysine methyltransferases					
SMYD5	-			-4.514	6.371E-06
EHMT2	UNC0224[3], UNC0642[3], BIX-01294[3]			-4.322	1.545E-05
SETDB2	-			-3.6	0.0003176
PRDM13	-			-3.442	< 0.005
SUV39H1, SUV39H2	Chaetocin[3]			-3.422/-2.934	0.0006216
PRDM12	-			-3.089	0.00201
EZH1	UNC1999[3]			-2.787	0.005314
EZH2	CPI-1205[2,4], EPZ-6438 (Tazemetostat)[2], DZNep[2], UNC1999[3]			-2.495	0.01259
Arginine methyltransferases					
CARM1	MS049[3], SGC2085[3], TP-064[3][72]			-3.812	0.0001381
PRMT1	MS023[3][72]			-3.659	0.0002534
PRMT6	MS023[3], MS049[c], EPZ020411[3][72], 6′-methyleneamine sinefungin[3][73]			-3.521	0.0004301
Histone acetylation					
KAT2A	CPTH2[3][74], γ-butyrolactone[3] (MB-3)[75]			-4.683	2.823E-06
NAA10, NAA16, NAA20, NAA38, NAA40	-			-4.335/-3.255/-3.786/-3.801/-2.665	< 0.01
NAT8, NAT9	-			-2.573/-3.995	< 0.01
NCOA5, NCOA6	-			-3.238/-3.112	< 0.002
Histone phosphorylation					
BAZ1B	-			-2.374	0.01758
Histone glycosylation					
OGT	-			-3.172	0.001512

[1]Approved for the treatment of other diseases;
[2]Used in clinical trials for other diseases;
[3]Not yet used in clinical trials;
[4]Activator. CRC: Colorectal cancer; OR: Objective response.

of cancer. As aberrant DNA methylation is an early step of carcinogenesis, advanced disease may not be the relevant stage for treatments with DNMTi and HDACi.

To refine these treatment strategies, tumor grade, heterogeneity, and subtypes of cancers will have to be considered. Indeed, determining which tumors will benefit from epigenetic differentiation strategies[53] and which tumors would acquire stemness capacities after epigenetic resetting is mandatory. Hence, modulating epigenetic alterations to sensitize cancer cells to other conventional therapies[54] or to lower their aggressiveness seems to be a reasonable goal when it comes to epigenetic strategies for advanced disease, as shown by numerous studies on cancer cell lines[53]. HDAC and DNMT inhibitors, used alone or in combination, are able to sensitize resistant cancer cells and their use after conventional or targeted therapies have

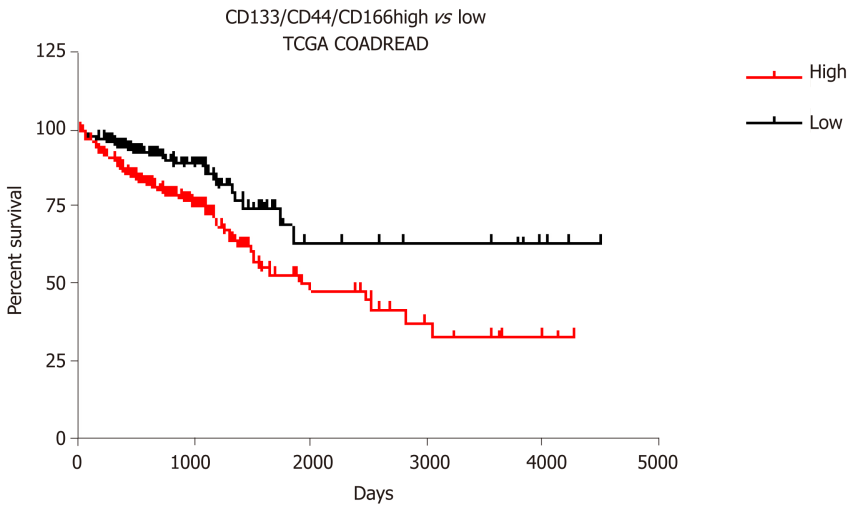

Figure 3 Survival analysis for CD133/CD44/CD166 expression profiles in colorectal cancer. The association of CD133/CD44/CD166 transcript expression with cancer survival in the COADREAD Cancer Genome Atlas dataset was analyzed using the SurvExpress portal[62]. Kaplan-Meier plot and Cox survival statistics were established with maximized risk group assessment (466 patients with 255 in low *vs* 211 in high risk profile). The log rank for equal curves indicated a significant difference (*P* value = 0.0007) with a hazard ratio of 2.12 (95%CI: 1.35-3.31, *P* value = 0.0009).

proven their efficacy in clinical trials[55]. For instance, treatment with 5-azacitidine or 5-Aza-2′-deoxycitidine increases sensitivity of colon cancer cells to irinotecan and 5-FU[56]. Irinotecan sensitivity with DNMTi was confirmed in *in vivo* CRC models showing tumor regression and increased survival in contrast with monotherapies. The same results were observed with the combination of 5-azacitidine and a BRAF inhibitor in CRC xenograft models[57]. Synergetic therapies were also observed with HDACi in combination with 5-FU. Indeed, trichostatine A in combination with 5-FU suppresses colon cancer cell viability[58]. However, initiating re-differentiation in CSCs remains a challenge dependent on the characteristics of each tumor type and with their specific genetic alterations.

Molecular subtypes of CRC or chemoresistance also predict how and whether or not patients will benefit from existing epidrug treatments. For instance, it has been shown that treatment with 5-azacitidine can restore chemosensitivity to irinotecan in microsatellite stable CRC cell lines but not in microsatellite instable CRC cell lines[56]. Moreover, microsatellite instability CRC status is associated with the hypermethylation of glutathione peroxidase 3, a gene encoding an antioxidant selenoprotein involved in drug metabolism. In this case, treatment with 5-azacitidine induced an increase of glutathione peroxidase 3 expression and a decrease of chemosensitivity to oxaliplatin in microsatellite instability CRC cell lines[59]. These findings emphasize the need for personalized therapies that consider CRC interindividual heterogeneity and classification.

Exploring new avenues for colon cancer treatment

In order to better anticipate how colon CSCs will respond to the different existing therapies, we analyzed TCGA_COADREAD data of 379 colon cancer patients using LinkedOmics[60]. With this meta-analysis we assessed the correlation Z-score estimate (Stouffer method-based) and a *P* value between the combined expression of the three colon CSC markers CD133, CD44, and CD166 and an exhaustive list of known chromatin modifying enzymes (epigenetic writers and erasers) and chromatin binding proteins (epigenetic readers). The observed negative and positive correlation of expression between the three CSC markers and a significant number of epienzymes are highlighted in Tables 1 to 6.

Strikingly, DNMT3A, DNMT3B, and DNMT3L, the DNA methyltransferases that are responsible for *de novo* DNA methylation, showed a negative correlation score with the combined expression of the three CSC markers studied (Table 1), while the expression of DNMT1, responsible for DNA methylation maintenance, was not significantly correlated with the combination of these markers (-2 < score < 2). Similarly, three class I and II HDAC as well as two sirtuins were found negatively correlated to the combination of markers (Table 2). None of the known HDAC were found positively correlated with the expression of the three CSC markers. This strongly suggests that inhibiting DNMT or HDAC activity would have no effect in colon cancers overexpressing CSC markers (and potentially harbor high stemness

properties) but may have adverse effect in low-expressing and maybe less aggressive colon cancers. These data are in accordance with disappointing clinical trials that have been conducted so far with these inhibitors in colon cancer patients. Interestingly, our analyses suggest that another strategy to regulate DNA methylation in colon CSCs may be the inhibition of the methylcytosine dioxygenase TET2, known to trigger DNA demethylation and found correlated to CSC marker expression in our analyses (Table 3).

The correlation scores we obtained for other chromatin writers, readers, and erasers seem more specific to the enzyme itself than to their role in the shaping of epigenetic landscapes (Tables 1-6).

We found a negative correlation between the expression of the three markers and several histone lysine methyltransferases associated with the establishment of constitutive or facultative heterochromatin, including EZH2 that has recently emerged as one of the new favorite targets for epigenetic therapies[20] (Table 1). These estimated scores in colon cancer expressing CD133, CD44, and CD166 suggest that an activator of EZH2, such as CPI-1205, may have better efficacy than known inhibitors in clinical trials to influence cancer stemness and are in accordance with a protective role of EZH2 in cell differentiation. Similarly, expression of EHMT2 (also known as G9A and KMT1C), encoding another lysine methyltransferase that also recently raised interests in the epidrug field, was inversely correlated with the three CSC markers expression (Table 1).

Only few lysine methyltransferases associated with gene activation were found correlated or inversely correlated with the combined expression of the three markers. Among them, SETD7 (Table 4), but not SETD6 (Table 1), may be a good candidate to inhibit stemness in colon cancer cells.

Recently, small molecules that can target specific bromodomains have been extensively developed[61]. Bromodomains are part of a family of epigenetic readers that play pivotal roles in transcriptional regulation through the binding of acetylated histones and the recruitment of other epienzymes in epigenetic complexes at specific sites. We found only a few bromodomain-containing proteins whose expression was positively (BPTF, BAZ2B, Table 5) or negatively (BRD7, Table 6) correlated to the combined expression of the three CSC markers.

Among epigenetic readers, methylated DNA binding proteins have probably been overlooked as epidrug targets since expression of both MBD1 and MBD2 is positively correlated with CSC markers (Table 5).

Targeting members of the lysine-specific histone demethylase family of histone demethylases using inhibitors such as GSK-J1 may also be a good option since only a few of them are inversely correlated with the three CSC markers while KDM3B, KDM4B/C, KDM5B, KDM6A (UTX), and KDM6B (JMJD3) are positively correlated to their expression (Table 3).

Finally, JAK1/2 kinases, which possess a histone phosphorylation activity and are the targets of numerous inhibitors already tested in the clinic, mainly for other diseases, should probably be reconsidered for colon cancer patients with high expression of CD133, CD44, and CD166 or after conventional therapies. Indeed, our meta-analyses suggest that their expression is positively correlated to the expression of the three CSC markers (Table 4).

As mentioned above, CD44, CD133, and CD166 are potent markers of CSCs from multiple tissues including digestive (gastric, pancreatic) and non-digestive cancers in which they are epigenetically regulated. Therefore, these considerations could be largely applicable to other types of cancers, in which correlation studies between epienzymes and CSC markers may be of great interest.

PERSPECTIVES

Although epigenetic therapies are conceptually very promising, several pitfalls will have to be overcome in order to take a step forward in clinical trials for solid tumors. First, while intra-tumor and inter-individual heterogeneity of CRC is now evident, epigenetic landscapes and epienzyme activity will have to be studied in all types of tumor cells. Single cell approaches will be very useful to circumvent the difficulty of exploring rare CSCs from different CRC consensus molecular subtypes. Second, studies to prove causal correlations between epienzyme expression and the control of stemness will be mandatory in order to clear up confusion relative to the oncogenic or tumor suppressive roles of chromatin modifiers. Finally, the major difficulty for the design of new epidrugs is to target efficiently a single member of entire families of epienzymes that have homologous domains but different roles in stemness. To circumvent this difficulty, increasing specificity by targeting epigenetic complexes

Table 2 Negative correlation between combined expression of cancer stem cell markers CD133, CD44 and CD166 and epigenetic erasers

Family/gene symbol	Epidrug/chemical probe	Clinical trials for CRC	Results	*Z*-score	*P* value
Histone deacetylation (Zinc-dependent)					
	Acide valproïque[1]	I to II	In combination: OR in 64% patients or SD[76,77]		
	Belinostat[2], Apicidin[3]				
	Entinostat	I to I/II	No OR[78] or SD[79]		
	Panobinostat	I	PR and SD in combination with Bevacizumab[80]		
	Vorinostat (SAHA)	I to II	No OR[81,82]; SD and PR with Bortezomib or 5FU and leucovorin or Doxorubicin[83-85]		
	Trichostatine A[2]				
	Mocetinostat[2]				
	Sodium phenylbutyrate[2]	I	In combination with 5-FU: SD[86]		
Class I	Romidepsin (Istodax)[1]	II	Ineffective[86]		
	CI-994	I	PR in combination with carboplatin and placlitaxel[87]		
HDAC8	TM-2-51[4], CUDC-101[2], Pracinostat[2], Ricolinostat[2], Citarinostat[2], Abexinostat[2], Quisinostat[3], PCI-34051[3]			-2.527	0.0115
Class IIa (1 catalytic site, mainly cytoplasmic)					
HDAC5	CUDC-101[2], Pracinostat[2], Domatinostat[2], Quisinostat[3], LMK-235[3], TMP195[3], TMP269[3]			-4.133	3.581E-05
Class IIb (2 catalytic sites, mainly cytoplasmic)					
HDAC10	CUDC-101[2], CUDC-907[2], Pracinostat[2], Domatinostat[2], Abexinostat[2], Tucidinostat[2], Quisinostat[3]			-3.17	0.001525
Histone deacetylation NAD+ dependent (Class III)					
	Resveratrol[4]	I	Reduced cell proliferation[88]		
	Salermide[3][89]				
SIRT6	OSS_128167[3]			-3.467	0.0005257
SIRT7				-2.582	0.009835
Histone demethylation					
LSD family of demethylases					
	ORY-1001[3], (±)-tranylcypromine[3]				
KDM2B	-			-3.54	0.0004003
KDM4D	-			-2.704	0.006848
JmjC containing lysine demethylases					
	JIB-04[3]				
JMJD6	IOX1[3]			-2.59	0.00961
JMJD5	IOX1[3]			-2.588	0.009654

[1]Approved for the treatment of other diseases;
[2]Used in clinical trials for other diseases;
[3]Not yet used in clinical trials;
[4]Activator. CRC: Colorectal cancer; OR: Objective response; SD: Stable disease; PR: Partial response.

Table 3 Positive correlation between combined expression of cancer stem cell markers CD133, CD44 and CD166 and epigenetic erasers

Family/gene symbol	Putative epidrug/chemical probe	*Z*-score	*P* value
DNA demethylation			
TET2	-	5.968	2.40E-09
Histone demethylation			
LSD family of demethylases			
	ORY-1001[3], (±)-tranylcypromine[3]		
KDM3B		5.636	1.74E-08
KDM4B	CP2[3][90]	5.212	1.87E-07
KDM4C	CP2[3][90]	3.895	9.81E-05
KDM5B	CPI-455[3], AS-8351[3], 59[3] (KDOAMA-25[3])[90]	9.092	9.72E-20
KDM6A	GSK-J1[3]	2.84	0.00451
KDM6B	GSK-J1[3]	4.014	5.98E-05

[1]Approved for the treatment of other diseases; [2]Used in clinical trials for other diseases; [3]Not yet used in clinical trials.

and therefore epienzyme-epienzyme interactions may be a better option for new designs. Based on these considerations, epigenetic personalized medicine will be truly envisioned.

Table 4 Positive correlation between combined expression of cancer stem cell markers CD133, CD44 and CD166 and epigenetic writers

Family/gene symbol	Epidrug/chemical probe	Clinical trials for CRC	Results/status	Z-score	*P* value
Histone acetyltransferases					
EP300	Curcumin	Early phase I to III	Low bioavailability[91]	2.513	0.01198
	Garcinol[3], C646[3]				
NCOA1	Bufalin[2][92]			5.45	5.04E-08
NCOA4	-			4.183	2.88E-05
NCOA7	-			5.788	7.14E-09
KAT2B	Ischemin[3][93]			6.514	7.31E-11
Activating Lysine methyltransferases					
ASH1L	-			2.591	0.009565
SMYD1	-			2.739	0.00616
SETD7	PFI-2[3]			5.11	3.23E-07
Repressing Lysine methyltransferases					
PRDM8	-			3.411	0.0006465
Putative Lysine methyltransferase					
PRDM10	-			2.448	0.01438
Arginine methyltransferases					
PRDM1	-			2.874	0.004056
PRMT2	-			2.901	0.003726
Histone ubiquitination					
UBE2B	-			2.748	0.005991
UBE2H	-			5.809	6.30E-09
Histone phosphorylation					
JAK1	Ruxolitinib	Phase I and II	No benefit over Regorafenib alone[94]	7.739	1.01E-14
	Baricitinib[2], Momelotinib[2], Filgotinib[2], Decernotinib[2], Cerdulatinib[2], Solcitinib[2], Oclacitinib maleate[2]				
JAK2	Ruxolitinib	Phase I and II	No benefit over Regorafenib alone[94]	6.7	2.09E-11
	Gandotinib[2], AZD1480[2], BMS-911543[2], AT9283[2], XL019[2], Baricitinib[2], Momelotinib[2], Filgotinib[2], Decernotinib[2], Cerdulatinib[2], JAK2/HDAC Dual Inhibitors[3][95]				
Histone biotinylation					
BTD	Biotinyl-methyl 4-(amidomethyl)benzoate[3][96]			4.379	1.19E-05

[1]Approved for the treatment of other diseases;
[2]Used in clinical trials for other diseases;
[3]Not yet used in clinical trials. CRC: Colorectal cancer.

Table 5 Positive correlation between combined expression of cancer stem cell markers CD133, CD44 and CD166 and epigenetic readers

Family/gene symbol	Epidrug/chemical probe	Z-score	*P* value
Methylated DNA binding			
MBD1	-	2.593	0.009517
MBD2	-	3.477	0.0005076
ZBTB4	-	5.496	3.89E-08
Methylated histone binders			
Zinc finger, PHD-type			
DPF3		3.503	0.0004602
Bromodomain	Apabetalone[2], Bromosporine[3]		

BPTF		2.621	0.008773
BAZ2B	GSK2801[3]	4.791	1.66E-06
Tudor domain			
TDRD1	-	2.459	0.01394
TP53BP1	-	2.965	0.003029
Other cofactors of epigenetic complexes			
RBBP5	-	2.966	0.003014
TADA2B	-	3.382	0.0007189
ELP2	PLX-4720[3]	3.277	0.00105
ELP3	-	2.622	0.00875
TAB2	-	2.551	0.01074
NCOR1	-	3.62	0.0002949
Chromodomain (Chromatin Organization Modifier Domain)			
CHD1, CHD3, CHD9	-	3.007/4.099/4.367	< 0.003

[1]Approved for the treatment of other diseases;
[2]Used in clinical trials for other diseases;
[3]Not yet used in clinical trials.

Table 6 Negative correlation between combined expression of cancer stem cell markers CD133, CD44 and CD166 and epigenetic readers

Family/gene symbol	Putative epidrug/chemical probe	Z-score	*P* value
Methylated DNA binding			
MBD3	-	-3.601	0.0003174
ZBTB38 (Kaiso family)	-	-2.557	0.01055
Histone binders			
Bromodomains			
BRD7	BI7273[3], BI-9564[3], TP-472[3][97]	-4.906	9.301E-07
Zinc finger, Plant Homeodomain (PHD)-type			
ING1, ING5	-	-2.544/-4.255	< 0.05
PHF20	-	-3.094	0.001973
PHF14	-	-2.934	0.003344
PHF5A	-	-2.521	0.01171
DPF1	-	-2.78	0.00543
Tudor domain			
TDRKH	-	-2.755	0.005875
WD40 motif			
EED	A-395[3][98]	-4.307	1.652E-05
Other cofactors of epigenetic complexes			
DPY30	-	-3.549	0.0003863
WDR5	OICR-9429[3]	-3.31	0.0009321
TADA2A		-2.473	0.01341

[1]Approved for the treatment of other diseases; [2]Used in clinical trials for other diseases;
[3]Not yet used in clinical trials.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Samuel Malone for his careful and critical reading of the manuscript, for the helpful comments and for English editing.

REFERENCES

1 **D'Andrea V**, Guarino S, Di Matteo FM, Maugeri Saccà M, De Maria R. Cancer stem cells in surgery. *G Chir* 2014; **35**: 257-259 [PMID: 25644725]

2 **Southam CM**, Brunschwig A. Quantitative studies of autotransplantation of human cancer. *Cancer* 1961; **14**: 971-978 [DOI: 10.1002/1097-0142(196109/10)14:5<971::AID-CNCR2820140510>3.0.CO;2-O]
3 **Huntly BJ**, Gilliland DG. Leukaemia stem cells and the evolution of cancer-stem-cell research. *Nat Rev Cancer* 2005; **5**: 311-321 [PMID: 15803157 DOI: 10.1038/nrc1592]
4 **Reya T**, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and CSCs. *Nature* 2001; **414**: 105-111 [DOI: 10.1038/35102167]
5 **Shackleton M**, Quintana E, Fearon ER, Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell* 2009; **138**: 822-829 [PMID: 19737509 DOI: 10.1016/j.cell.2009.08.017]
6 **Basicević V**, Kleut-Jelić R, Orovcanec M, Vuković D. [Frequency and significance of lambliasis in the clinical material]. *Med Pregl* 1972; **25**: 323-325 [PMID: 4636056 DOI: 10.1126/scitranslmed.aaa1408]
7 **Turajlic S**, McGranahan N, Swanton C. Inferring mutational timing and reconstructing tumour evolutionary histories. *Biochim Biophys Acta* 2015; **1855**: 264-275 [PMID: 25827356 DOI: 10.1016/j.bbcan.2015.03.005]
8 **Frohman LA**, Downs TR, Williams TC, Heimer EP, Pan YC, Felix AM. Rapid enzymatic degradation of growth hormone-releasing hormone by plasma in vitro and in vivo to a biologically inactive product cleaved at the NH2 terminus. *J Clin Invest* 1986; **78**: 906-913 [PMID: 3093533 DOI: 10.1073/pnas.1102454108]
9 **Bardají JL**, Villarroel MT, Vázquez de Prada JA, Ruano J, Olalla JJ, Martín Durán R, Martín Lorente JL, López Morante A. [Acute pericarditis and cardiac tamponade as the initial manifestation of ulcerative colitis]. *Rev Esp Cardiol* 1988; **41**: 257-260 [PMID: 3413333 DOI: 10.1002/stem.1058]
10 **Freitas DP**, Teixeira CA, Santos-Silva F, Vasconcelos MH, Almeida GM. Therapy-induced enrichment of putative lung cancer stem-like cells. *Int J Cancer* 2014; **134**: 1270-1278 [PMID: 24105655 DOI: 10.1002/ijc.28478]
11 **Sugiura T**, Iwasaka T, Takayama Y, Takahashi N, Matsutani M, Inada M. The factors associated with fascicular block in acute anteroseptal infarction. *Arch Intern Med* 1988; **148**: 529-533 [PMID: 3341854 DOI: 10.1038/bjc.2012.126]
12 **Yasuda H**, Soejima K, Watanabe H, Kawada I, Nakachi I, Yoda S, Nakayama S, Satomi R, Ikemura S, Terai H, Sato T, Suzuki S, Matsuzaki Y, Naoki K, Ishizaka A. Distinct epigenetic regulation of tumor suppressor genes in putative cancer stem cells of solid tumors. *Int J Oncol* 2010; **37**: 1537-1546 [PMID: 21042723 DOI: 10.3892/ijo_00000807]
13 **Yamazaki J**, Estecio MR, Jelinek J, Graber D, Lu Y, Ramagli L, Liang S, Kornblau SM, Issa JP. Genome-wide epigenetic analysis of CSCs in acute myeloid leukemia. ASH Annual Meeting; 2010. American Society of Hematology. 2010
14 **Furusawa J**, Zhang H, Vural E, Stone A, Fukuda S, Oridate N, Fang H, Ye Y, Suen JY, Fan CY. Distinct epigenetic profiling in head and neck squamous cell carcinoma stem cells. *Otolaryngol Head Neck Surg* 2011; **144**: 900-909 [PMID: 21493336 DOI: 10.1177/0194599811398786]
15 **Bar-Nur O**, Russ HA, Efrat S, Benvenisty N. Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. *Cell Stem Cell* 2011; **9**: 17-23 [PMID: 21726830 DOI: 10.1016/j.stem.2011.06.007]
16 **el-Fiky SM**, Kolkaila AM, Dawd DS, Wahab RM. Histochemical aspects of hydatidiform mole and choriocarcinoma. *Acta Histochem* 1973; **47**: 115-123 [PMID: 4134946 DOI: 10.1016/j.jacc.2014.04.056]
17 **Cimino G**, Lo-Coco F, Fenu S, Travaglini L, Finolezzi E, Mancini M, Nanni M, Careddu A, Fazi F, Padula F, Fiorini R, Spiriti MA, Petti MC, Venditti A, Amadori S, Mandelli F, Pelicci PG, Nervi C. Sequential valproic acid/all-trans retinoic acid treatment reprograms differentiation in refractory and high-risk acute myeloid leukemia. *Cancer Res* 2006; **66**: 8903-8911 [PMID: 16951208 DOI: 10.1158/0008-5472.CAN-05-2726]
18 **Vincent A**, Van Seuningen I. On the epigenetic origin of cancer stem cells. *Biochim Biophys Acta* 2012; **1826**: 83-88 [PMID: 22495062 DOI: 10.1016/j.bbcan.2012.03.009]
19 **Timp W**, Feinberg AP. Cancer as a dysregulated epigenome allowing cellular growth advantage at the expense of the host. *Nat Rev Cancer* 2013; **13**: 497-510 [PMID: 23760024 DOI: 10.1038/nrc3486]
20 **Kim KH**, Roberts CW. Targeting ezh2 in cancer. *Nat Med* 2016; **22**: 128-134 [DOI: 10.1038/nm.4036]
21 **van Vlerken LE**, Kiefer CM, Morehouse C, Li Y, Groves C, Wilson SD, Yao Y, Hollingsworth RE, Hurt EM. EZH2 is required for breast and pancreatic cancer stem cell maintenance and can be used as a functional cancer stem cell reporter. *Stem Cells Transl Med* 2013; **2**: 43-52 [PMID: 23283488 DOI: 10.5966/sctm.2012-0036]
22 **Tan J**, Yang X, Jiang X, Zhou J, Li Z, Lee PL, Li B, Robson P, Yu Q. Integrative epigenome analysis identifies a Polycomb-targeted differentiation program as a tumor-suppressor event epigenetically inactivated in colorectal cancer. *Cell Death Dis* 2014; **5**: e1324 [PMID: 25032847 DOI: 10.1038/cddis.2014.283]
23 **Sun JG**, Liao RX, Qiu J, Jin JY, Wang XX, Duan YZ, Chen FL, Hao P, Xie QC, Wang ZX, Li DZ, Chen ZT, Zhang SX. Microarray-based analysis of microRNA expression in breast cancer stem cells. *J Exp Clin Cancer Res* 2010; **29**: 174 [PMID: 21192833 DOI: 10.1186/1756-9966-29-174]
24 **Baba T**, Convery PA, Matsumura N, Whitaker RS, Kondoh E, Perry T, Huang Z, Bentley RC, Mori S, Fujii S, Marks JR, Berchuck A, Murphy SK. Epigenetic regulation of CD133 and tumorigenicity of CD133+ ovarian cancer cells. *Oncogene* 2009; **28**: 209-218 [PMID: 18836486 DOI: 10.1038/onc.2008.374]
25 **Horst D**, Kriegl L, Engel J, Kirchner T, Jung A. Prognostic significance of the cancer stem cell markers CD133, CD44, and CD166 in colorectal cancer. *Cancer Invest* 2009; **27**: 844-850 [PMID: 19626493 DOI: 10.1080/07357900902744502]
26 **Corvaisier M**, Bauzone M, Corfiotti F, Renaud F, El Amrani M, Monté D, Truant S, Leteurtre E, Formstecher P, Van Seuningen I, Gespach C, Huet G. Regulation of cellular quiescence by YAP/TAZ and Cyclin E1 in colon cancer cells: Implication in chemoresistance and cancer relapse. *Oncotarget* 2016; **7**: 56699-56712 [PMID: 27527859 DOI: 10.18632/oncotarget.11057]
27 **Touil Y**, Igoudjil W, Corvaisier M, Dessein AF, Vandomme J, Monté D, Stechly L, Skrypek N, Langlois C, Grard G, Millet G, Leteurtre E, Dumont P, Truant S, Pruvot FR, Hebbar M, Fan F, Ellis LM, Formstecher P, Van Seuningen I, Gespach C, Polakowska R, Huet G. Colon cancer cells escape 5FU chemotherapy-induced cell death by entering stemness and quiescence associated with the c-Yes/YAP axis. *Clin Cancer Res* 2014; **20**: 837-846 [PMID: 24323901 DOI: 10.1158/1078-0432.CCR-13-1854]
28 **Jang JW**, Song Y, Kim SH, Kim J, Seo HR. Potential mechanisms of CD133 in cancer stem cells. *Life Sci* 2017; **184**: 25-29 [PMID: 28697984 DOI: 10.1016/j.lfs.2017.07.008]
29 **Ren F**, Sheng WQ, Du X. CD133: a cancer stem cells marker, is used in colorectal cancers. *World J*

Gastroenterol 2013; **19**: 2603-2611 [PMID: 23674867 DOI: 10.3748/wjg.v19.i17.2603]
30 **Li Z**. CD133: a stem cell biomarker and beyond. *Exp Hematol Oncol* 2013; **2**: 17 [PMID: 23815814 DOI: 10.1186/2162-3619-2-17]
31 **Tabu K**, Sasai K, Kimura T, Wang L, Aoyanagi E, Kohsaka S, Tanino M, Nishihara H, Tanaka S. Promoter hypomethylation regulates CD133 expression in human gliomas. *Cell Res* 2008; **18**: 1037-1046 [PMID: 18679414 DOI: 10.1038/cr.2008.270]
32 **Fargeas CA**, Huttner WB, Corbeil D. Nomenclature of prominin-1 (CD133) splice variants - an update. *Tissue Antigens* 2007; **69**: 602-606 [PMID: 17498271 DOI: 10.1111/j.1399-0039.2007.00825.x]
33 **Yu Y**, Flint A, Dvorin EL, Bischoff J. AC133-2, a novel isoform of human AC133 stem cell antigen. *J Biol Chem* 2002; **277**: 20711-20716 [PMID: 12042327 DOI: 10.1074/jbc.M202349200]
34 **Irollo E**, Pirozzi G. CD133: to be or not to be, is this the real question? *Am J Transl Res* 2013; **5**: 563-581 [PMID: 24093054]
35 **Friel AM**, Zhang L, Curley MD, Therrien VA, Sergent PA, Belden SE, Borger DR, Mohapatra G, Zukerberg LR, Foster R, Rueda BR. Epigenetic regulation of CD133 and tumorigenicity of CD133 positive and negative endometrial cancer cells. *Reprod Biol Endocrinol* 2010; **8**: 147 [PMID: 21122138 DOI: 10.1186/1477-7827-8-147]
36 **Yan Y**, Zuo X, Wei D. Concise Review: Emerging Role of CD44 in Cancer Stem Cells: A Promising Biomarker and Therapeutic Target. *Stem Cells Transl Med* 2015; **4**: 1033-1043 [PMID: 26136504 DOI: 10.5966/sctm.2015-0048]
37 **Chen C**, Zhao S, Karnad A, Freeman JW. The biology and role of CD44 in cancer progression: therapeutic implications. *J Hematol Oncol* 2018; **11**: 64 [PMID: 29747682 DOI: 10.1186/s13045-018-0605-5]
38 **Xia P**, Xu XY. Prognostic significance of CD44 in human colon cancer and gastric cancer: Evidence from bioinformatic analyses. *Oncotarget* 2016; **7**: 45538-45546 [PMID: 27323782 DOI: 10.18632/oncotarget.9998]
39 **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]
40 **Eom DW**, Hong SM, Kim G, Bae YK, Jang KT, Yu E. Prognostic Significance of CD44v6, CD133, CD166, and ALDH1 Expression in Small Intestinal Adenocarcinoma. *Appl Immunohistochem Mol Morphol* 2015; **23**: 682-688 [PMID: 25710579 DOI: 10.1097/PAI.0000000000000140]
41 **Zeilstra J**, Joosten SP, van Andel H, Tolg C, Berns A, Snoek M, van de Wetering M, Spaargaren M, Clevers H, Pals ST. Stem cell CD44v isoforms promote intestinal cancer formation in Apc(min) mice downstream of Wnt signaling. *Oncogene* 2014; **33**: 665-670 [PMID: 23318432 DOI: 10.1038/onc.2012.611]
42 **Müller I**, Wischnewski F, Pantel K, Schwarzenbach H. Promoter- and cell-specific epigenetic regulation of CD44, Cyclin D2, GLIPR1 and PTEN by methyl-CpG binding proteins and histone modifications. *BMC Cancer* 2010; **10**: 297 [PMID: 20565761 DOI: 10.1186/1471-2407-10-297]
43 **Saint-André V**, Batsché E, Rachez C, Muchardt C. Histone H3 lysine 9 trimethylation and HP1γ favor inclusion of alternative exons. *Nat Struct Mol Biol* 2011; **18**: 337-344 [PMID: 21358630 DOI: 10.1038/nsmb.1995]
44 **Weidle UH**, Eggle D, Klostermann S, Swart GW. ALCAM/CD166: cancer-related issues. *Cancer Genomics Proteomics* 2010; **7**: 231-243 [PMID: 20952758]
45 **Dalerba P**, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, Shelton AA, Parmiani G, Castelli C, Clarke MF. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci USA* 2007; **104**: 10158-10163 [PMID: 17548814 DOI: 10.1073/pnas.0703478104]
46 **Kemper K**, Grandela C, Medema JP. Molecular identification and targeting of colorectal cancer stem cells. *Oncotarget* 2010; **1**: 387-395 [PMID: 21311095 DOI: 10.18632/oncotarget.101003]
47 **Ikeda K**, Quertermous T. Molecular isolation and characterization of a soluble isoform of activated leukocyte cell adhesion molecule that modulates endothelial cell function. *J Biol Chem* 2004; **279**: 55315-55323 [PMID: 15496415 DOI: 10.1074/jbc.M407776200]
48 **King JA**, Tan F, Mbeunkui F, Chambers Z, Cantrell S, Chen H, Alvarez D, Shevde LA, Ofori-Acquah SF. Mechanisms of transcriptional regulation and prognostic significance of activated leukocyte cell adhesion molecule in cancer. *Mol Cancer* 2010; **9**: 266 [PMID: 20929568 DOI: 10.1186/1476-4598-9-266]
49 **Wei SH**, Brown R, Huang TH. Aberrant DNA methylation in ovarian cancer: is there an epigenetic predisposition to drug response? *Ann N Y Acad Sci* 2003; **983**: 243-250 [PMID: 12724229 DOI: 10.1111/j.1749-6632.2003.tb05979.x]
50 **Baretti M**, Azad NS. The role of epigenetic therapies in colorectal cancer. *Curr Probl Cancer* 2018; **42**: 530-547 [PMID: 29625794 DOI: 10.1016/j.currproblcancer.2018.03.001]
51 **Azad N**, Zahnow CA, Rudin CM, Baylin SB. The future of epigenetic therapy in solid tumours--lessons from the past. *Nat Rev Clin Oncol* 2013; **10**: 256-266 [PMID: 23546521 DOI: 10.1038/nrclinonc.2013.42]
52 **McGranahan N**, Swanton C. Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. *Cancer Cell* 2015; **27**: 15-26 [PMID: 25584892 DOI: 10.1016/j.ccell.2014.12.001]
53 **S Franco S**, Szczesna K, Iliou MS, Al-Qahtani M, Mobasheri A, Kobolák J, Dinnyés A. In vitro models of cancer stem cells and clinical applications. *BMC Cancer* 2016; **16**: 738 [PMID: 27766946 DOI: 10.1186/s12885-016-2774-3]
54 **Ahuja N**, Easwaran H, Baylin SB. Harnessing the potential of epigenetic therapy to target solid tumors. *J Clin Invest* 2014; **124**: 56-63 [PMID: 24382390 DOI: 10.1172/JCI69736]
55 **Brown R**, Curry E, Magnani L, Wilhelm-Benartzi CS, Borley J. Poised epigenetic states and acquired drug resistance in cancer. *Nat Rev Cancer* 2014; **14**: 747-753 [PMID: 25253389 DOI: 10.1038/nrc3819]
56 **Sharma A**, Vatapalli R, Abdelfatah E, Wyatt McMahon K, Kerner Z, A Guzzetta A, Singh J, Zahnow C, B Baylin S, Yerram S, Hu Y, Azad N, Ahuja N. Hypomethylating agents synergize with irinotecan to improve response to chemotherapy in colorectal cancer cells. *PLoS One* 2017; **12**: e0176139 [PMID: 28445481 DOI: 10.1371/journal.pone.0176139]
57 **Mao M**, Tian F, Mariadason JM, Tsao CC, Lemos R, Dayyani F, Gopal YN, Jiang ZQ, Wistuba II, Tang XM, Bornman WG, Bollag G, Mills GB, Powis G, Desai J, Gallick GE, Davies MA, Kopetz S. Resistance to BRAF inhibition in BRAF-mutant colon cancer can be overcome with PI3K inhibition or demethylating agents. *Clin Cancer Res* 2013; **19**: 657-667 [PMID: 23251002 DOI: 10.1158/1078-0432.CCR-11-1446]
58 **Huang TH**, Wu SY, Huang YJ, Wei PL, Wu AT, Chao TY. The identification and validation of Trichosstatin A as a potential inhibitor of colon tumorigenesis and colon cancer stem-like cells. *Am J*

Cancer Res 2017; **7**: 1227-1237 [PMID: 28560069]
59 **Pelosof L**, Yerram S, Armstrong T, Chu N, Danilova L, Yanagisawa B, Hidalgo M, Azad N, Herman JG. GPX3 promoter methylation predicts platinum sensitivity in colorectal cancer. *Epigenetics* 2017; **12**: 540-550 [PMID: 27918237 DOI: 10.1080/15592294.2016.1265711]
60 **Vasaikar SV**, Straub P, Wang J, Zhang B. LinkedOmics: analyzing multi-omics data within and across 32 cancer types. *Nucleic Acids Res* 2018; **46**: D956-D963 [PMID: 29136207 DOI: 10.1093/nar/gkx1090]
61 **Pérez-Salvia M**, Esteller M. Bromodomain inhibitors and cancer therapy: From structures to applications. *Epigenetics* 2017; **12**: 323-339 [PMID: 27911230 DOI: 10.1080/15592294.2016.1265710]
62 **Aguirre-Gamboa R**, Gomez-Rueda H, Martínez-Ledesma E, Martínez-Torteya A, Chacolla-Huaringa R, Rodriguez-Barrientos A, Tamez-Peña JG, Treviño V. SurvExpress: an online biomarker validation tool and database for cancer gene expression data using survival analysis. *PLoS One* 2013; **8**: e74250 [PMID: 24066126 DOI: 10.1371/journal.pone.0074250]
63 **Azad NS**, El-Khoueiry A, Yin J, Oberg AL, Flynn P, Adkins D, Sharma A, Weisenberger DJ, Brown T, Medvari P, Jones PA, Easwaran H, Kamel I, Bahary N, Kim G, Picus J, Pitot HC, Erlichman C, Donehower R, Shen H, Laird PW, Piekarz R, Baylin S, Ahuja N. Combination epigenetic therapy in metastatic colorectal cancer (mCRC) with subcutaneous 5-azacitidine and entinostat: a phase 2 consortium/stand up 2 cancer study. *Oncotarget* 2017; **8**: 35326-35338 [PMID: 28186961 DOI: 10.18632/oncotarget.15108]
64 **Bauman J**, Verschraegen C, Belinsky S, Muller C, Rutledge T, Fekrazad M, Ravindranathan M, Lee SJ, Jones D. A phase I study of 5-azacytidine and erlotinib in advanced solid tumor malignancies. *Cancer Chemother Pharmacol* 2012; **69**: 547-554 [PMID: 21901396 DOI: 10.1007/s00280-011-1729-2]
65 **Overman MJ**, Morris V, Moinova H, Manyam G, Ensor J, Lee MS, Eng C, Kee B, Fogelman D, Shroff RT, LaFramboise T, Mazard T, Feng T, Hamilton S, Broom B, Lutterbaugh J, Issa JP, Markowitz SD, Kopetz S. Phase I/II study of azacitidine and capecitabine/oxaliplatin (CAPOX) in refractory CIMP-high metastatic colorectal cancer: evaluation of circulating methylated vimentin. *Oncotarget* 2016; **7**: 67495-67506 [PMID: 27542211 DOI: 10.18632/oncotarget.11317]
66 **Schwartsmann G**, Schunemann H, Gorini CN, Filho AF, Garbino C, Sabini G, Muse I, DiLeone L, Mans DR. A phase I trial of cisplatin plus decitabine, a new DNA-hypomethylating agent, in patients with advanced solid tumors and a follow-up early phase II evaluation in patients with inoperable non-small cell lung cancer. *Invest New Drugs* 2000; **18**: 83-91 [PMID: 10830142]
67 **Appleton K**, Mackay HJ, Judson I, Plumb JA, McCormick C, Strathdee G, Lee C, Barrett S, Reade S, Jadayel D, Tang A, Bellenger K, Mackay L, Setanoians A, Schätzlein A, Twelves C, Kaye SB, Brown R. Phase I and pharmacodynamic trial of the DNA methyltransferase inhibitor decitabine and carboplatin in solid tumors. *J Clin Oncol* 2007; **25**: 4603-4609 [PMID: 17925555 DOI: 10.1200/JCO.2007.10.8688]
68 **Garrido-Laguna I**, McGregor KA, Wade M, Weis J, Gilcrease W, Burr L, Soldi R, Jakubowski L, Davidson C, Morrell G, Olpin JD, Boucher K, Jones D, Sharma S. A phase I/II study of decitabine in combination with panitumumab in patients with wild-type (wt) KRAS metastatic colorectal cancer. *Invest New Drugs* 2013; **31**: 1257-1264 [PMID: 23504398 DOI: 10.1007/s10637-013-9947-6]
69 **Toden S**, Tran HM, Tovar-Camargo OA, Okugawa Y, Goel A. Epigallocatechin-3-gallate targets cancer stem-like cells and enhances 5-fluorouracil chemosensitivity in colorectal cancer. *Oncotarget* 2016; **7**: 16158-16171 [PMID: 26930714 DOI: 10.18632/oncotarget.7567]
70 **Yang PM**, Lin YT, Shun CT, Lin SH, Wei TT, Chuang SH, Wu MS, Chen CC. Zebularine inhibits tumorigenesis and stemness of colorectal cancer *via* p53-dependent endoplasmic reticulum stress. *Sci Rep* 2013; **3**: 3219 [PMID: 24225777 DOI: 10.1038/srep03219]
71 **Feldman M**, Levy D. Peptide inhibition of the SETD6 methyltransferase catalytic activity. *Oncotarget* 2017; **9**: 4875-4885 [PMID: 29435148 DOI: 10.18632/oncotarget.23591]
72 **Kaniskan HÜ**, Eram MS, Zhao K, Szewczyk MM, Yang X, Schmidt K, Luo X, Xiao S, Dai M, He F, Zang I, Lin Y, Li F, Dobrovetsky E, Smil D, Min SJ, Lin-Jones J, Schapira M, Atadja P, Li E, Barsyte-Lovejoy D, Arrowsmith CH, Brown PJ, Liu F, Yu Z, Vedadi M, Jin J. Discovery of Potent and Selective Allosteric Inhibitors of Protein Arginine Methyltransferase 3 (PRMT3). *J Med Chem* 2018; **61**: 1204-1217 [PMID: 29244490 DOI: 10.1021/acs.jmedchem.7b01674]
73 **Wu H**, Zheng W, Eram MS, Vhuiyan M, Dong A, Zeng H, He H, Brown P, Frankel A, Vedadi M, Luo M, Min J. Structural basis of arginine asymmetrical dimethylation by PRMT6. *Biochem J* 2016; **473**: 3049-3063 [PMID: 27480107 DOI: 10.1042/BCJ20160537]
74 **Chimenti F**, Bizzarri B, Maccioni E, Secci D, Bolasco A, Chimenti P, Fioravanti R, Granese A, Carradori S, Tosi F, Ballario P, Vernarecci S, Filetici P. A novel histone acetyltransferase inhibitor modulating Gcn5 network: cyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl)hydrazone. *J Med Chem* 2009; **52**: 530-536 [PMID: 19099397 DOI: 10.1021/jm800885d]
75 **Aquea F**, Timmermann T, Herrera-Vásquez A. Chemical inhibition of the histone acetyltransferase activity in Arabidopsis thaliana. *Biochem Biophys Res Commun* 2017; **483**: 664-668 [PMID: 27993678 DOI: 10.1016/j.bbrc.2016.12.086]
76 **Wheler JJ**, Janku F, Falchook GS, Jackson TL, Fu S, Naing A, Tsimberidou AM, Moulder SL, Hong DS, Yang H, Piha-Paul SA, Atkins JT, Garcia-Manero G, Kurzrock R. Phase I study of anti-VEGF monoclonal antibody bevacizumab and histone deacetylase inhibitor valproic acid in patients with advanced cancers. *Cancer Chemother Pharmacol* 2014; **73**: 495-501 [PMID: 24435060 DOI: 10.1007/s00280-014-2384-1]
77 **Münster P**, Marchion D, Bicaku E, Schmitt M, Lee JH, DeConti R, Simon G, Fishman M, Minton S, Garrett C, Chiappori A, Lush R, Sullivan D, Daud A. Phase I trial of histone deacetylase inhibition by valproic acid followed by the topoisomerase II inhibitor epirubicin in advanced solid tumors: a clinical and translational study. *J Clin Oncol* 2007; **25**: 1979-1985 [PMID: 17513804]
78 **Pili R**, Salumbides B, Zhao M, Altiok S, Qian D, Zwiebel J, Carducci MA, Rudek MA. Phase I study of the histone deacetylase inhibitor entinostat in combination with 13-cis retinoic acid in patients with solid tumours. *Br J Cancer* 2012; **106**: 77-84 [PMID: 22134508 DOI: 10.1038/bjc.2011.527]
79 **Ngamphaiboon N**, Dy GK, Ma WW, Zhao Y, Reungwetwattana T, DePaolo D, Ding Y, Brady W, Fetterly G, Adjei AA. A phase I study of the histone deacetylase (HDAC) inhibitor entinostat, in combination with sorafenib in patients with advanced solid tumors. *Invest New Drugs* 2015; **33**: 225-232 [PMID: 25371323 DOI: 10.1007/s10637-014-0174-6]
80 **Strickler JH**, Starodub AN, Jia J, Meadows KL, Nixon AB, Dellinger A, Morse MA, Uronis HE, Marcom PK, Zafar SY, Haley ST, Hurwitz HI. Phase I study of bevacizumab, everolimus, and panobinostat (LBH-589) in advanced solid tumors. *Cancer Chemother Pharmacol* 2012; **70**: 251-258 [PMID: 22744359 DOI: 10.1007/s00280-012-1911-1]
81 **Ree AH**, Dueland S, Folkvord S, Hole KH, Seierstad T, Johansen M, Abrahamsen TW, Flatmark K.

Vorinostat, a histone deacetylase inhibitor, combined with pelvic palliative radiotherapy for gastrointestinal carcinoma: the Pelvic Radiation and Vorinostat (PRAVO) phase 1 study. *Lancet Oncol* 2010; **11**: 459-464 [PMID: 20378407 DOI: 10.1016/S1470-2045(10)70058-9]

82 **Wilson PM**, El-Khoueiry A, Iqbal S, Fazzone W, LaBonte MJ, Groshen S, Yang D, Danenberg KD, Cole S, Kornacki M, Ladner RD, Lenz HJ. A phase I/II trial of vorinostat in combination with 5-fluorouracil in patients with metastatic colorectal cancer who previously failed 5-FU-based chemotherapy. *Cancer Chemother Pharmacol* 2010; **65**: 979-988 [PMID: 20062993 DOI: 10.1007/s00280-009-1236-x]

83 **Fakih MG**, Groman A, McMahon J, Wilding G, Muindi JR. A randomized phase II study of two doses of vorinostat in combination with 5-FU/LV in patients with refractory colorectal cancer. *Cancer Chemother Pharmacol* 2012; **69**: 743-751 [PMID: 22020318 DOI: 10.1007/s00280-011-1762-1]

84 **Munster PN**, Marchion D, Thomas S, Egorin M, Minton S, Springett G, Lee JH, Simon G, Chiappori A, Sullivan D, Daud A. Phase I trial of vorinostat and doxorubicin in solid tumours: histone deacetylase 2 expression as a predictive marker. *Br J Cancer* 2009; **101**: 1044-1050 [PMID: 19738609 DOI: 10.1038/sj.bjc.6605293]

85 **Deming DA**, Ninan J, Bailey HH, Kolesar JM, Eickhoff J, Reid JM, Ames MM, McGovern RM, Alberti D, Marnocha R, Espinoza-Delgado I, Wright J, Wilding G, Schelman WR. A Phase I study of intermittently dosed vorinostat in combination with bortezomib in patients with advanced solid tumors. *Invest New Drugs* 2014; **32**: 323-329 [PMID: 24114123 DOI: 10.1007/s10637-013-0035-8]

86 **Whitehead RP**, Rankin C, Hoff PM, Gold PJ, Billingsley KG, Chapman RA, Wong L, Ward JH, Abbruzzese JL, Blanke CD. Phase II trial of romidepsin (NSC-630176) in previously treated colorectal cancer patients with advanced disease: a Southwest Oncology Group study (S0336). *Invest New Drugs* 2009; **27**: 469-475 [PMID: 18941712 DOI: 10.1007/s10637-008-9190-8]

87 **Pauer LR**, Olivares J, Cunningham C, Williams A, Grove W, Kraker A, Olson S, Nemunaitis J. Phase I study of oral CI-994 in combination with carboplatin and paclitaxel in the treatment of patients with advanced solid tumors. *Cancer Invest* 2004; **22**: 886-896 [PMID: 15641487]

88 **Patel KR**, Scott E, Brown VA, Gescher AJ, Steward WP, Brown K. Clinical trials of resveratrol. *Ann N Y Acad Sci* 2011; **1215**: 161-169 [PMID: 21261655 DOI: 10.1111/j.1749-6632.2010.05853.x]

89 **Rotili D**, Tarantino D, Nebbioso A, Paolini C, Huidobro C, Lara E, Mellini P, Lenoci A, Pezzi R, Botta G, Lahtela-Kakkonen M, Poso A, Steinkühler C, Gallinari P, De Maria R, Fraga M, Esteller M, Altucci L, Mai A. Discovery of salermide-related sirtuin inhibitors: binding mode studies and antiproliferative effects in cancer cells including cancer stem cells. *J Med Chem* 2012; **55**: 10937-10947 [PMID: 23189967 DOI: 10.1021/jm3011614]

90 **Lin H**, Li Q, Li Q, Zhu J, Gu K, Jiang X, Hu Q, Feng F, Qu W, Chen Y, Sun H. Small molecule KDM4s inhibitors as anti-cancer agents. *J Enzyme Inhib Med Chem* 2018; **33**: 777-793 [PMID: 29651880 DOI: 10.1080/14756366.2018.1455676]

91 **Bar-Sela G**, Epelbaum R, Schaffer M. Curcumin as an anti-cancer agent: review of the gap between basic and clinical applications. *Curr Med Chem* 2010; **17**: 190-197 [PMID: 20214562 DOI: 10.2174/092986710790149738]

92 **Wang J**, Chen C, Wang S, Zhang Y, Yin P, Gao Z, Xu J, Feng D, Zuo Q, Zhao R, Chen T. Bufalin Inhibits HCT116 Colon Cancer Cells and Its Orthotopic Xenograft Tumor in Mice Model through Genes Related to Apoptotic and PTEN/AKT Pathways. *Gastroenterol Res Pract* 2015; **2015**: 457193 [PMID: 26770191 DOI: 10.1155/2015/457193]

93 **Wapenaar H**, Dekker FJ. Histone acetyltransferases: challenges in targeting bi-substrate enzymes. *Clin Epigenetics* 2016; **8**: 59 [PMID: 27231488 DOI: 10.1186/s13148-016-0225-2]

94 **Fogelman D**, Cubillo A, García-Alfonso P, Mirón MLL, Nemunaitis J, Flora D, Borg C, Mineur L, Vieitez JM, Cohn A, Saylors G, Assad A, Switzky J, Zhou L, Bendell J. Randomized, double-blind, phase two study of ruxolitinib plus regorafenib in patients with relapsed/refractory metastatic colorectal cancer. *Cancer Med* 2018; **7**: 5382-5393 [PMID: 30123970 DOI: 10.1002/cam4.1703]

95 **Chu-Farseeva YY**, Mustafa N, Poulsen A, Tan EC, Yen JJY, Chng WJ, Dymock BW. Design and synthesis of potent dual inhibitors of JAK2 and HDAC based on fusing the pharmacophores of XL019 and vorinostat. *Eur J Med Chem* 2018; **158**: 593-619 [PMID: 30243158 DOI: 10.1016/j.ejmech.2018.09.024]

96 **Kobza KA**, Chaiseeda K, Sarath G, Takacs JM, Zempleni J. Biotinyl-methyl 4-(amidomethyl)benzoate is a competitive inhibitor of human biotinidase. *J Nutr Biochem* 2008; **19**: 826-832 [PMID: 18479898 DOI: 10.1016/j.jnutbio.2007.11.002]

97 **Moustakim M**, Clark PG, Trulli L, Fuentes de Arriba AL, Ehebauer MT, Chaikuad A, Murphy EJ, Mendez-Johnson J, Daniels D, Hou CD, Lin YH, Walker JR, Hui R, Yang H, Dorrell L, Rogers CM, Monteiro OP, Fedorov O, Huber KV, Knapp S, Heer J, Dixon DJ, Brennan PE. Discovery of a PCAF Bromodomain Chemical Probe. *Angew Chem Int Ed Engl* 2017; **56**: 827-831 [PMID: 27966810 DOI: 10.1002/anie.201610816]

98 **He Y**, Selvaraju S, Curtin ML, Jakob CG, Zhu H, Comess KM, Shaw B, The J, Lima-Fernandes E, Szewczyk MM, Cheng D, Klinge KL, Li HQ, Pliushchev M, Algire MA, Maag D, Guo J, Dietrich J, Panchal SC, Petros AM, Sweis RF, Torrent M, Bigelow LJ, Senisterra G, Li F, Kennedy S, Wu Q, Osterling DJ, Lindley DJ, Gao W, Galasinski S, Barsyte-Lovejoy D, Vedadi M, Buchanan FG, Arrowsmith CH, Chiang GG, Sun C, Pappano WN. The EED protein-protein interaction inhibitor A-395 inactivates the PRC2 complex. *Nat Chem Biol* 2017; **13**: 389-395 [PMID: 28135237 DOI: 10.1038/nchembio.2306]

World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2019 November 26; 11(11): 937-956

DOI: 10.4252/wjsc.v11.i11.937

ISSN 1948-0210 (online)

REVIEW

CRISPR/Cas system: An emerging technology in stem cell research

Maria Teresa Valenti, Michela Serena, Luca Dalle Carbonare, Donato Zipeto

ORCID number: Maria Teresa Valenti (0000-0003-1166-8033); Michela Serena (0000-0002-4697-2003); Luca Dalle Carbonare (0000-0003-3263-6671); Donato Zipeto (0000-0002-2168-4144).

Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting, critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

Manuscript source: Invited manuscript

Received: March 23, 2019
Peer-review started: March 23, 2019
First decision: August 1, 2019
Revised: August 12, 2019
Accepted: September 11, 2019
Article in press: September 11, 2019
Published online: November 26, 2019

P-Reviewer: Li SC ,Tanabe S

Maria Teresa Valenti, Luca Dalle Carbonare, Department of Medicine, Section of Internal Medicine D, University of Verona, Verona 37134, Italy

Michela Serena, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

Donato Zipeto, Department of Neurosciences, Biomedicine and Movement Sciences, Laboratory of Molecular Biology, Verona 37134, Italy

Corresponding author: Maria Teresa Valenti, BSc, PhD, Research Fellow, Department of Medicine, University of Verona, Ple Scuro 10, Verona 37100, Italy.
mariateresa.valenti@univr.it
Telephone: +39-45-8128450
Fax: +39-45-8027403

Abstract

The identification of new and even more precise technologies for modifying and manipulating the genome has been a challenge since the discovery of the DNA double helix. The ability to modify selectively specific genes provides a powerful tool for characterizing gene functions, performing gene therapy, correcting specific genetic mutations, eradicating diseases, engineering cells and organisms to achieve new and different functions and obtaining transgenic animals as models for studying specific diseases. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology has recently revolutionized genome engineering. The application of this new technology to stem cell research allows disease models to be developed to explore new therapeutic tools. The possibility of translating new systems of molecular knowledge to clinical research is particularly appealing for addressing degenerative diseases. In this review, we describe several applications of CRISPR/Cas9 to stem cells related to degenerative diseases. In addition, we address the challenges and future perspectives regarding the use of CRISPR/Cas9 as an important technology in the medical sciences.

Key words: Gene editing; CRISPR/Cas9; Stem cells; Degenerative diseases

Core tip: The possibility of translating new molecular knowledge systems to clinical research is particularly appealing for counteracting degenerative diseases as well as infective pathologies and cancer. A novel gene-editing technique, CRISPR/Cas9, has recently emerged for inducing targeted genetic modifications. Therefore, in this review, we describe recent applications of CRISPR/Cas9 to stem cells for counteracting

S-Editor: Zhang L
L-Editor: Filipodia
E-Editor: Zhou BX

degenerative diseases.

Citation: Valenti MT, Serena M, Carbonare LD, Zipeto D. CRISPR/Cas system: An emerging technology in stem cell research. *World J Stem Cells* 2019; 11(11): 937-956
URL: https://www.wjgnet.com/1948-0210/full/v11/i11/937.htm
DOI: https://dx.doi.org/10.4252/wjsc.v11.i11.937

INTRODUCTION

Gene editing

The development of gene targeting by homologous recombination (HR) was one of the fundamental steps forward in the field of genome editing, allowing site-directed specific mutation of a desired locus by exploiting homology arms to facilitate recombination at the donor site[1]. HR-mediated gene targeting led to the generation of both knock-in and knock-out cell lines as well as many transgenic animal models. However, one of the weaknesses of this technology is that the frequency of recombination events is low (one in 10^6-10^9 cells)[1], thus limiting its application for large-scale experiments. A subsequent fundamental discovery was the observation that targeted DNA double-strand breaks (DSBs) could directly induce homology-directed repair (HDR)[2,3]. It was also shown that in the presence of a DSB without any homology repair template, the error-prone nonhomologous end-joining (NHEJ) repair pathway induces insertion or deletion mutations (indels) at the break site. These observations led to the development of programmable nuclease-based genome editing strategies based on the design of molecular machines composed of a specific DNA-binding domain and an effector domain to induce a DSB, thus increasing the rate of gene editing at the desired locus.

In particular, the zinc-finger nucleases (ZFNs; based on eukaryotic transcription factors)[4] and the transcription activator-like nucleases (TALENs) from *Xanthomonas* bacteria[5], which consist of individual modules targeting three or one nucleotides of DNA, respectively, can be assembled in different combinations and attached to the FokI nuclease domain to direct DSBs at a specific desired genomic site. Both types of proteins can be easily engineered due to the possibility of customizing the DNA-binding domain to recognize any sequence in the genome. A ZF consists of approximately 30 amino acids and can recognize 3 bp in the major groove of DNA. The possibility of developing synthetic arrays containing more than three zinc-finger domains allows the targeting of 9-18-bp-long DNA sequences, thus conferring enough targeting specificity within the human genome[6]. A TALEN consists of a DNA-binding domain composed of a series of 33-35-amino acid modular repeats (each recognizing a single base pair) that are linked together to recognize contiguous DNA sequences. TALEN specificity is based on the exploitation of two hypervariable amino acids, known as repeat-variable di-residues[7]. Compared to zinc-finger proteins, TALEN array engineering requires more technical work due to the extensive identical repeat sequences involved, but many strategies have been developed to overcome this issue.

ZFNs and TALENs applications

Both ZFNs and TALENs have been used to edit a number of genes and to introduce genome modifications. ZFN engineering has been applied to correct X-linked severe combined immune deficiency[4], haemophilia B[8] and sickle cell disease[9,10]. ZFNs have also been applied for disease eradication *via* DSB-induced NHEJ, particularly in the field of acquired immune deficiency syndrome (AIDS). They were exploited to disable the human immunodeficiency virus 1 (HIV-1) co-receptor C-C chemokine receptor type 5 (CCR5), thus conferring virus resistance in T cells[11] and haematopoietic stem cells[12]; both approaches are currently in clinical trials. Another approach consists of the targeted integration of anti-HIV-1 restriction factors into the CCR5 locus to obtain T cells that are resistant to both CCR5-tropic (R5-tropic) and CXCR4-tropic HIV-1[13]. The CCR5 deletion has twice been proven to be a powerful and effective way to eradicate HIV-1 from the human body. The first case dates back to a decade ago[14]: the so-called "Berlin patient", who was receiving treatment with highly active antiretroviral therapy (HAART) after the diagnosis of HIV-1 infection, underwent two allogeneic haematopoietic stem cell transplantations from a donor with a homozygous mutation in the HIV-1 co-receptor CCR5 (CCR5Δ32/Δ32) to treat acute myeloid

leukaemia. The newly implanted cells no longer supported R5-tropic HIV-1 replication, and even after interruption of HAART, no active HIV-1 has since been detected in this patient. The second case, the so-called "London patient", was actually very recent[15]: An HIV-1-infected adult underwent allogeneic haematopoietic stem cell transplantation to treat Hodgkin's lymphoma, again from a CCR5Δ32/Δ32 donor, but *via* a less aggressive and toxic approach, avoiding total body irradiation. At present, HIV-1 remission has been maintained in this patient. These two cases suggest that CCR5Δ32 bone marrow stem cell transplantation represents a possible strategy for achieving HIV-1 remission and should be deeply investigated in the future.

Similar to ZFNs, TALENs have been used to perform homologous recombination-based gene correction in induced pluripotent stem cells (iPSCs) from patients with β-thalassemia[16]. TALENs were also exploited to induce point mutations in the *Oryza sativa* genome to obtain a new rice variety with enhanced resistance to herbicides[17]. The first clinical application of TALENs consisted of a cell therapy approach based on the generation of universal chimeric antigen receptor 19 (CAR19) T cells by depletion of both TCR and CD52 molecules to eliminate the risk of graft-versus-host disease[18].

However, the engineering of site-specific nucleases such as ZFNs and TALENs requires a great deal of effort, since the nucleases need to be *de novo* reengineered through a very labour-intensive and time-consuming procedure.

The CRISPR/Cas9 technology

A novel gene-editing technique, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, has recently emerged as an efficient alternative to ZFNs and TALENs for inducing targeted genetic modifications. The revolutionary feature of this technology is that Cas9 is an RNA-guided nuclease containing an HNH nuclease domain that cleaves the target strand of DNA and a RuvC-like nuclease domain that cleaves the non-target strand. Target sequence specificity arises from Watson–Crick base pairing between the guide RNA and the target DNA site[19]. As a consequence, unlike previous strategies based on DNA-binding proteins, the CRISPR/Cas9 system can be easily programmed to target new sites by merely changing its guide RNA sequence, thus making it a suitable tool for high-throughput gene editing in many cell types and organisms.

The discovery of the CRISPR/Cas system originates in 1987, from mysterious 29-nt repetitive elements identified downstream of the *iap* gene in *E. coli*. Interestingly, these repeats were interspaced with five intervening 32-nt nonrepetitive sequences[20]. During the following 10 years, the same pattern of repeated elements was reported in the genomes of different bacterial and archaeal strains, and in 2002 the acronym CRISPR was introduced to specify microbial genomic loci consisting of an interspaced repeat array[21,22]. In parallel, a series of CRISPR-associated (*Cas*) genes adjacent to these repeat elements were identified[22]. It was subsequently shown that CRISPR loci are actually transcribed[23], and that bacteriophages are unable to infect archaeal cells carrying spacers corresponding to their own genomes[24]. The first evidence that the CRISPR system serves as a microbial molecular immune memory and defence mechanism against viruses came from the Danisco company, where researchers were working to improve the lifespan of bacterial cultures for manufacturing yogurt and ice cream[21]. Thus far, at least six types (I–VI, with types I-III the most characterized) of CRISPR/Cas systems have been identified in many Bacteria and in the majority of characterized Archaea; these systems consist of a cluster of CRISPR-associated (*Cas*) genes, noncoding RNAs and a distinct array of repetitive elements.

In general, a CRISPR system functions *via* three steps that are necessary to achieve a full immune response against foreign DNA[25]. In the first stage, the invading DNA is fragmented into short sequences that are incorporated into the host crRNA array as spacers between the CRISPR RNA (crRNA) repeats. This stage is mediated by a complex of the Cas1 and Cas2 proteins, which are shared by all known CRISPR/Cas systems. In the second stage, the CRISPR array is transcribed into pre-crRNA, which is then cleaved and processed into mature crRNAs by Cas proteins and host factors[26]. This crRNA acts as a guide containing the spacer sequence necessary to target specifically the Cas proteins to the invading genome upon recognition of the crRNA by the Cas proteins themselves. In particular, in type II CRISPR systems, the presence of a noncoding transactivating crRNA (tracrRNA) that hybridizes with the pre-crRNA is necessary for crRNA processing, Cas binding and target cleavage[27]. crRNA maturation is mediated by either a Cas6-related ribonuclease (in type I and III systems) or housekeeping RNaseIII (type II system) that specifically cleaves double-stranded RNA hybrids of pre-crRNA and tracrRNA. In the third stage, the Cas proteins recognize the target DNA and induce cleavage of the invading genome, thus protecting the host cells from infection.

In the most recent classification, the various CRISPR/Cas systems are divided into two simple classes: class 1 CRISPR systems (types I, III, IV) utilize several Cas proteins

and crRNAs to form an effector complex, whereas class 2 CRISPR systems (types II, V, VI) exploit a large single-component Cas protein in conjunction with crRNAs to mediate interference[28]. The type II CRISPR system is currently one of the best characterized, consisting of the Cas9 nuclease, a crRNA array that encodes guide RNAs and the required auxiliary tracrRNA, which helps to process the crRNA array into discrete units containing a 20-nt guide sequence and a partial direct repeat[27]. Within the DNA target, each spacer is always associated with a protospacer-adjacent motif (PAM), which can vary depending on the specific CRISPR system[29,30].

To simplify the system and make it utilizable for genome editing, the crRNA-tracrRNA duplex can be fused into a chimeric single guide RNA (sgRNA) and expressed in a plasmid under the control of the human U6 polymerase III promoter, whose only requirement for transcription initiation is the presence of a G nucleotide, which can eventually be added at the 5′ end of the guide[27,31]. A human codon-optimized version of Cas9 fused to the C-terminal SV40 nuclear localization signal has also been generated for the mammalian expression system[31]. As a consequence, the Cas9-sgRNA complex can specifically target the DNA sequence that base pairs with the sgRNA and is adjacent to the PAM sequence and induce a DSB. Cas9 can therefore be targeted to any genomic locus only by customizing an approximately 20-nucleotide sequence complementary to the target DNA, making it an easily programmable platform for high-throughput gene targeting[32].

Indeed, the CRISPR/Cas9 system has been used for both NHEJ- and HDR-induced gene editing in eukaryotic cells[31,33-35]. Direct embryonic injection of sgRNA and Cas9 mRNA allowed transgenic mice with multiple modified alleles to be obtained[36]. To improve the specificity of CRISPR/Cas9-mediated HDR, a nickase version of Cas9 (Cas9n) was generated by aspartate-to-alanine mutation in the RuvC catalytic domain to nick rather than cleave DNA, leading to a single-strand break[19,27,37]. It has been reported that the combination of Cas9n together with a pair of offset sgRNAs complementary to opposite strands of the target DNA induces a double nick (one per DNA strand), leading to a DSB and NHEJ-based indels[32]. Due to the combination of two sgRNAs, Cas9n shows fewer off-target effects than does Cas9, since possible individual single-stranded nicks are repaired by the high-fidelity base excision repair mechanism. Recently, the type V CRISPR/Cas system was discovered[38], based on the Cpf1 ribonucleoprotein (CRISPR from *Prevotella* and *Francisella 1*), containing only the RuvC-like domain and not the HNH domain. In contrast to Cas9, Cpf1-mediated DNA cleavage is guided by only a crRNA and does not require a tracrRNA. Additionally, Cpf1 requires a short T-rich PAM preceding the target sequence, unlike the G-rich PAM downstream of the target sequence required for Cas9, and the seed region is within approximately the first five nucleotides at the 5′ end of the target sequence.

Within the past few years, the RNA-targeting type VI CRISPR/Cas system was also discovered and characterized. This system is based on the Cas13 protein, which forms a crRNA-guided RNA-targeting effector complex when assembled with crRNA. The type VI CRISPR/Cas system can be divided into four subtypes (A–D) based on the phylogeny of the effector complexes[39-41]. However, all type VI systems are based on Cas13, which exhibits two enzymatically distinct ribonuclease activities: One responsible for pre-crRNA processing and one provided by two Hhigher Eeukaryotes and Pprokaryotes Nnucleotide-binding (HEPN) domains, which are required for the degradation of the target RNA[42-44]. These properties of Cas13 led to the rapid development of a new generation of RNA-targeting tools for many applications. In particular, Cas13 has been tested for human RNA knockdown, showing high specificity and fewer off-targets compared to RNAi[45,46,51].

Source of human stem cells for genome editing

The possibility of combining the potential of human pluripotent stem cells (hPSCs) with this new genome-editing technique makes important applications in biomedical research possible. hPSCs can be generated either from human embryonic stem cells (hESCs), arising directly from embryos[47], or from iPSCs. iPSCs are generated from fibroblasts or other somatic cells by the transfection of "reprogramming genes"[48,49]. In addition, by transferring a nucleus from differentiated cells to a de-nucleated ovum, the third type of stem cell (SCNT stem cells) can be obtained[50]. hPSCs generally share several characteristic features, such as the possibility of being maintained in culture for many passages with the same karyotype without genomic loss. hPSCs are pluripotent cells and can differentiate into different somatic cell types based on the protocol used[51]. The ability of hPSCs to self-renew indefinitely and differentiate into different types of somatic cells represents an important tool for regenerative medicine. With this tool, mutations can be introduced in cell lines to generate disease models, and genetic defects can be corrected to rescue pathological conditions.

iPSC technology has provided appealing tools to the field of degenerative disease

research. iPSCs can be produced by injecting several key transcription factors into somatic cells. Initially, Takahashi *et al*[52] were able to reprogram murine fibroblast cells by injecting several transcription factors, such as octamer-binding transcription factor 4 (Oct4), sex-determining region Y-box 2 (Sox2), Krüppel-like factor 4 and cMyc. In particular, Oct4 prevents the expression of genes involved in the differentiation of ESCs and can reprogram somatic cells[53,54]. In 2007, Yu *et al*[55] applied this technique to human somatic cells. Thus, human somatic cells can be reprogrammed to iPSCs by combining factors such as Oct4, Sox2, NANOG and LIN28[52,55].

Moreover, this technology has been improved by using newly defined factors as well as different delivery systems. It has been demonstrated that in the absence of the Oct4 and Sox2 factors, genes involved in mesendodermal *(i.e. GATA3*, *GATA6*, and *SOX7*) and ectodermal commitment (*i.e. SOX1*, *SOX3*, and *GMNN*) can induce cell reprogramming[56,57]. Additionally, the use of miRNAs such as miR-291-3p, miR-294, miR-295 and the miR-302/367 cluster, has been suggested for enhancing the reprogramming cells[58,59]. In addition to fibroblasts, other kinds of cells can be induced to undergo reprogramming; the cells include B lymphocytes; neural progenitors; keratinocytes; cells arising from amniotic fluid, the liver, the stomach, or the pancreas; or cells harvested from blood or urine[60].

Importantly, as iPSCs originate from the somatic cells of patients, they represent a specific source for transplantation therapy that prevents immunologic reactions. iPSCs have the same background as the patients from whom they are harvested. Because they carry the same genetic mutations as the patient, these cells provide a perfect disease model, which is important for understanding pathological conditions or identifying personalized therapeutic tools.

CRISPR/CAS9 APPLICATIONS IN DEGENERATIVE DISEASES

Haematological disorders

By improving the development of experimental models, CRISPR/Cas9 technology has contributed to a deep understanding of haematological disorders. The first haematological disorder to which CRISPR/Cas 9 was applied was sickle cell disease (SCD). SCD is caused by a single-nucleotide polymorphism in one of the haemoglobin genes and induces severe organ complications[61,62]. Dewitt *et al*[61] corrected the mutation in CD34+ haematopoietic stem/progenitor cells (HSPCs). In particular, they delivered a ribonucleoprotein complex containing the Cas9 protein, an unmodified single guide RNA and a single-stranded DNA oligonucleotide donor to replace the mutation in HSPCs[61]. When these cells were differentiated to erythroblasts, they produced low mRNA and protein levels of sickle haemoglobin and increased levels of wild-type haemoglobin. Also, when transplanted into mice, the cells maintained the edited gene for 16 wk and showed improved clinical characteristics[61].

Recently, an *in vivo* model for studying myeloid malignancies by using CRISPR/Cas9 technology was proposed[63]. Patients affected by these malignancies harbour three or five mutations contributing to a poor diagnosis. By using CRISPR/Cas9, researchers inactivated eight different alleles in a single HSPC; cells arising from this HPSC were able to induce leukaemia after transplantation in mice[64]. Bejar *et al*[65] also used engineered CRISPR/Cas9 HSPCs carrying specific mutations to demonstrate that these cells are sensitive to azacitidine.

CRISPR and HIV

HIV-1 infection is currently treated with HAART, involving a combination of antiretroviral drugs that help to control viral load, thus delaying or preventing progression towards AIDS. This therapy does not eradicate the virus from the body, and it has to be continued throughout a patient's life. One of the main problems in achieving an effective HIV-1 cure is the persistence of latent viral reservoirs that cannot be cleared by current treatments. The establishment of these reservoirs is due to the integration of HIV-1 DNA into the cellular genome[66], and the only way to eradicate them would be to delete directly or deactivate proviral DNA. To this end, it has been reported that the CRISPR/Cas9 system can be exploited to target and inactivate HIV-1 integrated DNA in Jurkat cells, resulting in no difference between active and inactive HIV-1 DNA transcription, suggesting a promising strategy for addressing latently infected cells[67]. Other studies have demonstrated that it is possible to apply the CRISPR/Cas9 system to remove entirely the HIV-1 genome by using specific gRNAs directed at the long terminal repeats of the integrated HIV-1 genome in latently infected cells[68-70]. The efficacy of adeno-associated virus (AAV)

vectors in the delivery of the CRISPR/Cas9 system into transgenic HIV-1-infected mice and rats through tail-vein injection to excise proviral DNA has also been shown[69,71]. Additionally, the CRISPR/Cas9 system has been applied to reactivate the latent HIV-1 reservoir by using catalytically deficient Cas9-synergistic activation mediator technology[72]. Zhang *et al*[73] showed that reactivation of the HIV-1 provirus was achieved in latently HIV-1-infected TZM-bl, Jurkat and CHME5 microglial cells, indicating the potential application of CRISPR/deficient Cas9-synergistic activation mediator as a "shock and kill" strategy to reactivate and induce cell death of latently HIV-1-infected cells.

Neurodegenerative diseases

Neurodegenerative diseases are severe pathological conditions with critical social outcomes. Unfortunately, the available therapeutic approaches are not able to treat effectively these degenerative disorders. In fact, the molecular and cellular defects causing neurodegeneration are not entirely understood, and specific therapeutic targets are lacking. Therefore, to identify the cellular and molecular pathways involved in neurodegenerative diseases, genetic screening performed by applying CRISPR technology has been proposed. Different targets involved in neurodegenerative diseases have been identified using CRISPR technology applied to human neurons obtained from iPSCs. Based on this strategy, Nakamoto *et al*[74] investigated the role of coenzyme Q10 in patients with multiple-system atrophy, a neurodegenerative disorder characterized by various combinations of neuronal dysfunction[74]. Their findings demonstrated that a reduction in coenzyme Q10 levels, particularly in patients with COQ2 variants, contributes to neuronal apoptosis in patients affected by multiple-system atrophy, suggesting an effective therapy[74]. The benefit of using CRISPR technology in studies related to Alzheimer's disease (AD) is under debate because most AD cases are sporadic and have different causes. Mutations in the gene encoding amyloid precursor protein are found in a small percentage of patients (> 0.1%) even when overexpression of beta-amyloid peptide is detected in all AD patients[75]. However, CRISPR technology can be useful for correcting autosomal-dominant mutations in presenilin 1 and presenilin 2 (PSEN2) that are found in the early onset AD[76]. In fact, CRISPR/Cas9 has been employed to correct PSEN2 in iPSC neurons from a patient with a PSEN2N141I mutation[77].

The APOE4 isoform is involved in the development of late-onset AD[78]. In contrast, the APOE2 isoform seems to reduce the risk of developing AD by up to 40%. Therefore, the application of CRISPR/Cas9 to replace APOE4 with APOE2 may be considered a useful tool for treating patients carrying the APOE4 variant[79].

Huntington's disease (HD) is characterized by muscular, psychiatric and cognitive disorders due to heterozygous expanded (CAG)n trinucleotide repeats in the gene that encodes huntingtin (HTT). This disorder causes alteration of the medium spiny neurons. Cellular strategies have been suggested for the generation of an HD disease model and the identification of therapeutic tools for treating HD. Therefore, therapies based on stem cell transplantation have been indicated as promising therapeutic tools[80]. In addition, iPSC lines originating from patients with juvenile HD have been generated[81]. In this context, the application of the CRISPR technique to target the HTT locus in iPSCs has given rise to new perspectives for the treatment of HD[82]

Bone and musculoskeletal disorders

The application of CRISPR technology to iPSCs originating from patients with skeletal disorders has been suggested to explore bone diseases. This approach has been applied to investigate cleidocranial dysplasia (CCD), a skeletal disease caused by a mutation in the transcription factor RUNX2. In particular, the CRISPR/Cas9 system has been applied to two iPSC lines generated from CCD patients with different RUNX2 mutations to restore the normal phenotype[83]. The CRISPR-edited cells were then evaluated *in vitro* and in a rat model, and correct osteo-induction was observed, thus indicating the molecular mechanism involved and suggesting a novel therapeutic approach for treating CCD[83]. The most abundant non-collagenous protein found in bone is osteocalcin, and an *in vivo* osteocalcin deficiency model shows impaired skeletal structure[84]. To understand better the role of osteocalcin in skeletal disorders, a rat model was generated by Lambert *et al*[85]. Specifically, these researchers injected CRISPR/Cas9 to knock out osteocalcin in the pronuclei of Sprague-Dawley embryos. With the development of this system, the authors provided a model of the disease that can be used in the field of osteoporosis and osteoarthritis research.

Duchenne muscular dystrophy (DMD) is a severe disease that affects skeletal and cardiac muscles in childhood. The absence of the dystrophin protein, encoded by the dystrophin gene (Dmd), prevents the muscular sarcolemma from being protected from injuries due to contractions, causing DMD[86]. Mutations in the Dmd gene are frequent, among which frameshift mutations are the most common, although in-

frame and out-of frame mutations may also occur; frameshift mutations generally result in a premature stop codon by altering the reading frame[87]. Therefore, as DMD is a genetic disorder, the possibility of identifying a therapeutic approach for DMD based on the application of CRISPR/Cas9 technology to stem cells appears intriguing.

To repair damaged muscle by correcting the dystrophin gene, CRISPR/Cas9 has been applied in mdx mice, a model of DMD[86]. By using this technique, the researchers obtained genetically mosaic animals with heterogeneous percentages of DMD gene correction (from 2% to 100%). These different percentages of gene correction allowed comparison of the percentage of correction with the level of muscular rescue[86]. Interestingly, the dystrophin protein has also been restored in iPSCs obtained from patients affected by DMD by using CRISPR/Cas9 technology[88]. However, five off-target sites were affected by the procedure in this model[88].

Musculoskeletal disorders also occur in lysosomal storage diseases (LSDs). LSDs include different genetic diseases characterized by deleterious mutations causing the disruption of lysosomal enzymes. Therapeutic approaches for counteracting LSDs include enzyme replacement therapy, pharmacological chaperone therapy and haematopoietic stem cell transplantation. However, all of these treatments cause secondary side effects[89]. To identify new therapeutic approaches, experimental models using CRISPR/Cas9 and iPSCs have been adopted. Pompe disease is an LSD caused by mutations in the gene that encodes the lysosomal hydrolase acid-alpha glucosidase and is characterized by a severe myopathy[90] . Possible therapy for these patients is provided by the enzyme replacement therapy Myozyme®; unfortunately, this therapy is expensive. Therefore, a useful therapeutic approach involving targeting the mutation in hematopoietic stem cells (HSCs) derived from the same patient *via* the CRISPR/Cas9 system has been suggested[91]. Other LSDs can certainly also be considered prospective targets for this therapy based on the CRISPR/Cas9- and iPSC system.

Cardiovascular diseases

CRISPR/Cas9 editing has emerged as a useful technology in the cardiovascular field. Cardiovascular disorders affect a large number of patients, and the incidence of these pathologies has increased considerably in recent decades. Therefore, an important challenge is to understand the molecular mechanisms that affect vascular and cardiac systems and determine cardiovascular mortality[92]. Among the pathological conditions affecting the cardiovascular system, cardiomyopathies, arrhythmias, rheumatic heart disease, stroke and congenital cardiac defects have been reported. Molecular tests and bioinformatics analyses allow the identification of individuals predisposed to cardiac disorders. However, there are some limitations to a complete understanding of the molecular signalling causing these pathologies because mechanistic studies aimed at understanding the causes of the diseases are limited by the complexity of culturing human cardiomyocytes[93]. However, CRISPR/Cas9 technology has allowed cardiac disease models to be generated, and it is possible to study cardiovascular diseases by injecting the CRISPR/Cas9 system components into the embryos of rats, rabbits and primates[94,95]. In addition, the coupling of iPSC technology with the application of the CRISPR/Cas9 system has provided useful cell models for better understanding the molecular mechanisms involved in cardiac pathologies and for recovering specific mutations causing cardiovascular diseases. The combination of iPSCs and CRISPR/Cas9 technologies has allowed the generation of a cellular model characterized by mitochondrial dysfunction originating from patients affected by Barth syndrome. By introducing a mutation in the *tafazzin* gene with the CRISPR/Cas9 system, the authors demonstrated that this mutation caused the mitochondrial phenotype and that normal mitochondrial function could be recovered by the administration of specific antioxidants[96].

CRISPR/Cas9 technology has also allowed the analysis of titin gene mutations in cardiomyopathy. By introducing either missense or frameshift mutations in the titin gene, researchers were able to generate contractile deficits in iPSCs that differentiated into cardiomyocytes (iPSC-CM)[97]. Similarly, iPSC-CMs have been obtained from patients affected by Jervell and Lange-Nielsen syndrome, a severe cardiac arrhythmia[98], and iPSCs carrying a mutation in the *CALM2* gene reproducing long QT syndrome have been generated with the same technique[99]. As CRISPR/Cas9 may introduce changes in noncoding regions, Beaudoin *et al*[100] were able to delete a sequence in an intronic region in the *PHACTR1* gene (associated with premature myocardial infarction) in iPSCs to generate a cell model of the pathology.

Hypertrophic cardiomyopathy is a severe cardiovascular disease with different clinical aspects characterized by cardiac arrhythmias. To identify therapeutic strategies for rescuing arrhythmias, hPSCs-MC have been engineered by using CRISPR/Cas9. In particular, Mosqueira *et al*[101] generated in three hPSC lines carrying 11 variants of the c.C9123T-MYH7 mutation, which affects the myosin heavy chain to

cause hypertrophic cardiomyopathy. By using this disease model, the authors demonstrated the possibility of correcting arrhythmias by pharmacological treatment and identified the ratio between MHY7: MYH6 and mutant: wild-type MYH7 isoforms as a diagnostic tool[101].

Diabetes

Stem cell therapy has been proposed for the treatment of diabetes, a metabolic disorder characterized by the disruption of insulin production. Two different types of diabetes are known: type 1 diabetes (T1D), which is an autoimmune disease, and type 2 diabetes (T2D), which is the most common and heterogeneous form of diabetes[102]. Both T1D and T2D are characterized by the disruption of pancreatic β-cell function[102].

The generation of pancreatic cells followed by their transplantation in patients with T1DM has been proposed. In this context, the use of iPSCs and the concurrent application of CRISPR/Cas9 technology can improve the generation of pancreatic organs[103]. In addition, this system avoids the controversial use of hESCs. Despite the advantages of using hESCs, such as the ease of differentiating these cells into β cells *in vivo*, the reduction in viral transgene incorporation and the greater efficiency of these cells in producing insulin compared to iPSCs, ethical concerns due to the induction process restrict their use[103].

T2D pathophysiology is complex because various factors, such as genetic, epigenetic and lifestyle factors, can contribute to the development of this disease. iPSC lines generated from T2D patients have allowed the detection of several mutations in transcription factors involved in pancreas development (*HNF1B*, *HNF4A* and *HNF1A*), genes encoding enzymes related to insulin secretion and proteins devoted to exocrine pancreas function[104]. Interestingly, genome-wide association studies (GWAS) revealed a robust statistical association between T2D and genetic variants located in noncoding regions. Therefore, in association with GWAS, CRISPR/Cas9 has been suggested to be a useful tool for improved understanding of the molecular factors involved in the pathogenesis of T2D[105]. A form of diabetes caused by mutations in the gene encoding insulin can appear during neonatal life (neonatal diabetes)[106]. Recently, Balboa *et al*[106] demonstrated that insulin mutations cause abnormal β-cell differentiation in a neonatal diabetes model. In particular, the researchers obtained iPSCs from affected patients. Then, by applying the CRISPR/Cas9 system, they corrected a missense mutation in the insulin gene and compared these corrected iPSCs to mutant iPSCs. Interestingly, by single-cell RNA sequencing, these authors observed increased endoplasmic reticulum stress and reduced proliferation[106] in mutant cells compared to corrected cells.

Cancer

iPSCs can be generated from cancer cells. Therefore, this technology will allow the molecular bases of malignant transformation to be identified. In addition, this approach can result in the screening of therapeutic formulations and the identification of useful biomarkers. The generation of iPSCs *via* the application of CRISPR/Cas9 methodology is particularly important to identify genetic disruptions that induce cellular transformation and have not yet been found, *e.g.*, in the case of glioblastoma (GBM). GBMs belong to the gliomas, a heterogeneous type of cancer, and originate from cells showing neural stem and progenitor cell characteristics[107]. Even though GWAS have allowed the identification of many genetic and epigenetic targets, other key molecular targets still need to be identified. For example, the *PKMYT1* gene has been identified as a candidate target for therapy in GBM patients by the application of CRISPR/Cas9 libraries to stem cell-like cells originating from GBM patients[108].

T cell-based immunotherapy represents a useful tool for the treatment of malignant cells. These cells show a reduced proliferative ability, but the possibility of using iPSCs from antigen-specific T cells overcomes this limit. Unfortunately, the rearrangement of the T cell receptor chain gene during reprogramming causes loss of their antigen specificity. However, Minagawa *et al*[109] were able to prevent this additional rearrangement by obtaining functional iPSCs from antigen-specific T cells *via* the application of CRISPR.

In the context of precision oncology, the application of CRISPR/Cas9 combined with iPSC technology offers effective tools for identifying appropriate therapies. Recently, this system allowed the investigation of the individual roles of two co-recurrent genetic lesions involved in myeloid malignancy: A mutation in the SRSF2 factor and a chromosome 7q deletion[110]. The authors found that the SRSF2 mutation induces dysplasia, whereas the chromosome 7 deletion prevents differentiation and is associated with disease progression[110].

The use of CRISPR/Cas9 technology associated with iPSC generation has been applied to the study of RET mutations in multiple endocrine neoplasm type 2 (MEN2). MEN2 is a rare syndrome that affects organs originating from neural crest

and endoderm and causes medullary thyroid cancer, pheochromocytoma, cutaneous lichen amyloidosis and primary hyperparathyroidism. In addition, it can cause Hirschprung disease[111]. iPSCs from a MEN2 patient with the most frequent mutation in RET (RET^{C634Y}) have been used to better understand the molecular mechanism by which the RET mutation causes MEN2[112]. These researchers generated CRISPR-corrected isogenic counterparts of these cells and, by performing transcriptomic analyses, identified early growth response 1 as a key molecular target in MEN2A[112].

In addition to the work described above, many other studies related to the application of the CRISPR/Cas9 System in stem cell research have been recently performed (Table 1).

CHALLENGES

As previously described, CRISPR/Cas9 has become a powerful technology that allows the manipulation of almost any biological organism. The relative simplicity of the technique has made it possible to develop new models for studying the effect of mutations in genetic diseases and for revealing previously unknown gene functions, among many other applications.

Despite the enormous therapeutic potential of the technique, it will be necessary to address various challenges before it can be safely used in the field of gene therapy and in clinical applications.

Off-target

The specificity of CRISPR/Cas9 is fundamental for its clinical application. Off-target mutations can impair the fitness and/or the functionality of edited cells and, even more problematically, can generate potential oncogenic cell clones[113].

Initial reports of the whole-genome sequencing of edited cells indicate a low rate of off-target mutations, supporting the good specificity of the system[114-116]. A study published in 2017 raised concerns about the extent of unexpected mutations introduced by Cas9[117], but the study was retracted in 2018 due to insufficient data to support the claim[118].

Subsequent studies based on whole-genome sequencing addressed concerns about potential off-target effects, reporting no unexpected off-target activity of CRISPR/Cas9[119,120]. Another study indicated that by appropriately designing gRNAs, it is possible to achieve efficient *in vivo* editing with no detectable off-target mutations[121].

A recent study revealed that sgRNAs are very sensitive to chromatin state, suggesting that off-target effects are inhibited by chromatin, thus favouring specificity[122].

Overall, CRISPR/Cas9 appears to be a very specific tool for genome editing, and the initial discordant reports might have been more closely related to the appropriate choice of sgRNAs, rather than to Cas9 activity[113]. Similar to PCR protocols, it is possible to envision that in the future, when sufficient data are available, a database of optimal sgRNAs can be generated to be used in different cellular models, paired with improved computational analysis, for gene editing.

Despite these reassuring data, new methods are being developed to detect potential off-target CRISPR mutations, as well as new systems and protocols to reduce further the risk. These approaches include the development of better *in silico* computational prediction tools, the use of more-specific nucleases, such as Cpf1, and the development of cell-free genomic DNA assays to detect double-stranded breaks based on sequencing, such as Digenome-seq[123] (in which Cas9 cleavage is followed by next-generation sequencing) and newer, more-sensitive methods such as CIRCLE-Seq and SITE-Seq. Additional methods are being developed using cell-based assays and are aimed at identifying potential off-target sites in specific cell types; these methods include GUIDE-Seq[124] and LAM-HTGTS[125], the latter of which is aimed at identifying genomic rearrangements following DSBs[126].

It has been reported that high concentrations of Cas9 nucleases may increase the rate of off-target mutations[127,128]. To address this issue, new strategies such as the double nickase system[32] or the use of high-fidelity recombinant Cas9 variants have been developed[128-131]. In addition, the discovery and characterization of Cas9 orthologues from other prokaryotic organisms may help to identify Cas variants with higher specificity[132,133].

Recently, protocols based on the transfection of Cas9-coding mRNA and gRNA as well as gRNA-Cas9 complexes have been proposed as systems to reduce further off-target effects[134]. The delivery of CRISPR/Cas9 components as RNA ands gRNA-Cas9 complexes may present an additional advantage, since circular plasmid DNA may

Table 1 Recent studies related to the gene-editing technology applied to stem cells research on degenerative diseases

Authors and year	Disorder
Zhou *et al*[163], 2018	Spinal muscular atrophy
Calvo-Garrido *et al*[164], 2019	Neuronal
Dong *et al*[165], 2019	Hereditary hearing loss
Zhao *et al*[166], 2019	Breast cancer
Yanagihara *et al*[167], 2019	Skeletal diseases
Vrugt *et al*[168], 2019	Fanconi anemia
Blanas *et al*[169], 2019	Colorectal cancer
Sun *et al*[170], 2019	Glioblastoma
Jelinkova *et al*[171], 2019	Duchenne muscular dystrophy
Hurtado *et al*[172], 2018	Renal
Tang *et al*[173], 2019	Cardiac hypertrophy
Tian *et al*[174], 2019	Pediatric biliary atresia
Wang *et al*[175], 2018	Werner syndrome
Barnes *et al*[176], 2018	Neuronal
Frasier *et al*[177], 2018	Cardiac arrhythmia
Sasaki-Honda *et al*[178], 2018	facioscapulohumeral muscular dystrophy
Wang *et al*[179], 2018	Hepatoma
Moghaddas *et al*[180], 2018	Autoinflammatory
Liu *et al*[181], 2018	Colon cancer
Jiao *et al*[182], 2018	Cardiac disorders
Lyu *et al*[183], 2018	Haemophilia
Deng *et al*[184], 2018	Retinitis pigmentosa
Wattanapanitch *et al*[185], 2018	Thalassemia
Suda *et al*[186], 2018	Parkinson's disease

(presumably only rarely) be randomly integrated into the host genome[135].

Cellular challenges

The editing of a specific gene sequence relies on HDR rather than NHEJ. HDR is selectively expressed during mitosis and is downregulated after cell division[136]. For this reason, gene editing may be very difficult to achieve in non-dividing cells, such as neurons. Different strategies are currently under study to address this issue[137].

In vivo delivery challenges

Some genetic diseases may be treated by collecting, modifying and reinfusing stem cells, but others will require the correction of many cells in formed tissues in the patient's body.

An *ex vivo* strategy based on the collection of stem cells from a patient (usually from bone marrow), followed by their modification and reimplantation, presents almost the same general risks previously described for genome editing in cell cultures. Additional challenges clearly remain concerning the *in vivo* delivery of the CRISPR/Cas9 system. Lentiviral vectors have been widely and successfully used in different applications.

However, permanent integration of lentiviral vectors in the host cell genome will most likely cause permanent expression of the Cas9 nuclease, increasing the potential for off-target effects *in vivo* and the related oncogenic risk, which adds to the intrinsic risk of random insertion of these vectors in the cell genome.

Unlike lentiviral vectors, adenoviral (AV) vectors do not integrate into the host cell genome, thus avoiding permanent expression and reducing the risk of off-target effects. AV vectors also allow the insertion of larger DNA fragments, making it possible to include additional sgRNA sequences or reporter genes. On the other hand, AV vectors present risks of immunotoxicity due to cellular immune responses, and studies are consequently needed to define the immunogenicity of Cas9 for *in vivo* applications[138].

AAV vectors have been proposed as more suitable and less risky viral vectors, and these vectors have been approved for use in clinical trials[139]. Problems due to the

small genome size of AAV vectors have been addressed by using a smaller Cas9 variant from *Streptococcus aureus*, *Streptococcus thermophilus*[37] or *Neisseria meningitidis*[37], rather than the commonly used *Streptococcus pyrogenes* Cas9 (SpCas9)[140].

Even if AAV vectors are used, the problem of the persistent Cas9 expression remains, as do the potential risks of a lower editing efficiency due to previous immunity against AAV.

Delivery to embryos to generate knock-out or other mutants is possible through direct microinjection, which is a costly and technically challenging procedure, although it is useful in generating permanent germline modifications. This approach is the most common tool used by researchers to generate new animal models.

Recently, different approaches based on the development of non-viral vectors have been developed. Such delivery alternatives involve the use of lipid-based vectors, polymeric cationic vectors and chitosan[141].

These methods are characterized by lower immunogenicity and higher safety, reducing the risk of short and long-term adverse effects. However, a low delivery efficiency remains the principal problem[141]. Studies in the field of nanotechnology will most likely result in new, optimized synthetic delivery systems based on nanoparticles that will facilitate the delivery of CRISPR/Cas9 components *in vivo*.

Immunity against Cas9

Other issues that will need to be addressed include the risk of an immune response against Cas9, a prokaryotic protein, when used in gene therapy applications and how this may impact the application of the technique in a clinical context[142].

A recent study[143] of human donors documented a high frequency of antibodies and anti-Cas9 cytotoxic T-lymphocytes (CTLs) against SaCas9 and SpCas9; these Cas9 orthologues are the most widely used and are derived from *Staphylococcus aureus* and *Streptococcus pyogenes*, respectively. Since these are two common bacterial species infecting humans, the study raises concerns about the impact of pre-existing humoral and cellular immune responses to Cas9 in future clinical trials. A possible solution may be to use Cas9 orthologues derived from bacterial species that do not commonly infect humans, to avoid the destruction of cells "treated" using CRISPR/Cas9 due to pre-existing anti-Cas9 cellular immunity.

HIV resistance

In the field of AIDS, the advantage of CRISPR/Cas9 engineering consists of conferring permanent protection against HIV-1, which is not achieved with antiviral drugs, but an important unanswered question is whether and how HIV-1 might escape from this genome editing system. HIV-1 evolution experiments have been performed in CD4+ T cells expressing both Cas9 and sgRNAs targeting different regions of the HIV-1 genome[144,145], showing that although there was apparent initial virus inhibition, viral replication re-bounded over time, resulting in high levels of HIV-1 production. In particular, rapid escape was observed when non-conserved HIV-1 sequences were deleted, while a longer time was needed to escape in the case of more conserved sequences. When the targeted viral DNA was sequenced, mutations were specifically identified in the sgRNA complementarity region, suggesting that HIV-1 can adapt its genome to escape CRISPR/Cas9-mediated editing. In particular, most of the identified resistance mutations were indels matching the specific site at which Cas9 was expected to cleave viral DNA, suggesting that a variety of mutations at the cleavage site might actually be induced by NHEJ: some of these mutations would not be selected because of abolishing viral replication, while other mutations would be selected because they are not deleterious to the virus, thus generating CRISPR/Cas9-resistant viral particles[145].

To overcome this unique viral escape mechanism, one solution may be to exploit multiple sgRNAs to target conserved proviral regions. It has been shown that multiplexed targeting of HIV-1 DNA leads to much stronger suppression of HIV-1 infection, although possible viral escape cannot be excluded[146]. Another approach might involve modified versions of Cas9 that can cleave the DNA outside of the target sequence, so that any mutation generated by NHEJ will not prevent the CRISPR/Cas9 machinery from rebinding and cleaving proviral DNA again. The newly discovered Cpf1 that cleaves DNA in the more distal region of the target sequence[38] may provide a possible strategy for addressing this issue. Another solution could be to suppress the NHEJ machinery enzymes through the use of specific anticancer drugs[147].

Other possible strategies for the suppression of viral infections are based on targeting host cell factors necessary for HIV-1 replication, such as inactivation of the co-receptor genes CXCR4 and CCR5. Several studies have already demonstrated the feasible application of CRISPR/Cas9 to inactivate both receptors[148-150], thus generating HIV-1 resistant cells. CXCR4 or CCR5 knock-out T cells have also been produced by

direct electroporation of the CRISPR/Cas9 ribonucleoproteins[151], which is a particularly useful strategy for cells that are difficult to transfect, such as primary cells. The immediate activity of the proteins is observed following transfection, and this approach may limit off-target effects, since the protein complex is quickly degraded within the cell.

Despite the promising efficacy of CRISPR/Cas9 for genome editing, the procedure is still too unsafe to be applied in human embryos because unwanted germline mutations might be passed to future generations, with unpredictable effects.

The first trial was carried out by Chinese researchers who used the CRISPR/Cas9 system to modify genetically the human β-globulin gene, whose mutation causes β-thalassemia, in human embryos[152]. Unfortunately, a higher frequency of mutations was detected in the CRISPR/Cas9-treated human embryos compared to the results observed in modified adult mouse or human cells. This result confirmed that the fidelity and specificity of the CRISPR/Cas9 system still require further investigation, which will be a prerequisite for any clinical applications of genome editing. Despite these ethical concerns, the first genetically modified babies were recently reported to have been generated in China, giving rise to strong international criticism[153]. He Jiankui, a genome-editing researcher at the Southern University of Science and Technology of China in Shenzhen, injected the CRISPR/Cas9 machinery into human embryos to disable the *CCR5* gene, thus generating R5-tropic HIV-1-resistant human babies. When the embryos were 3-5 five days old, a few cells were removed and checked for editing. Sixteen of 22 embryos were actually found to have been edited, and 11 of them were used in six implantation attempts before a twin pregnancy was achieved. Genetic tests suggest that both CCR5 alleles had been correctly modified in one twin, while the other twin is heterozygous for the modification. At present, this type of gene editing is prohibited in most countries, as the CRISPR/Cas9 technology is still experimental. The rate of off-target mutations is still too high, which might lead to long-term unexpected side effects, including the development of cancers that may be passed to future generations. Furthermore, CCR5 depletion provides higher susceptibility to other viral infections, such as West Nile and influenza viruses, and if a working vaccine against HIV-1 is found in the future, harbouring the CCR5 deletion will provide no benefits[154].

P53 mutations

hPSCs are very difficult to treat using CRISPR/Cas9 and exhibit a very low efficiency of genome editing compared to laboratory tumour cell lines[155]. These characteristics are due to the toxicity of DSBs induced by Cas9 in hPSCs, which appear to be p53 dependent[155]. Since stem cells may acquire p53 mutations[156], clonal expansion of stem cells that are more tolerant to DNA damage poses severe risks of cancer development. A careful genetic analysis of hPSC-treated cells, therefore, needs to be carried out before clinical use.

Nature spread

Homing gene drives based on CRISPR/Cas9 may be used to design mutations that will spread within a target population or species, for instance, to confer resistance to a parasite[157]. Such drives have been studied as a potential tool for the eradication of mosquitos to prevent diseases such as malaria[158,159] or other vector-borne diseases. This possibility, though fascinating, raises many concerns, since it may potentially cause the genetic modification of an entire species if modified organisms are accidentally released in the environment. Safeguarding strategies are under development to avoid the risk of premature release in the wild[157].

Ethical concerns

The technique needs to be used carefully and responsibly. Where does a cure end and improvement start?

A committee of the National Academy of Science addressed clinical, social, ethical and legal issues linked to genome editing, releasing a report entitled "Human Genome Editing: Science, Ethics, and Governance" in 2017[160]. Permanently editing germlines raises many concerns[161,162]. While there is no doubt that the correction of a genetic defect may help to eradicate, or at least significantly reduce, the burden of severe genetic diseases in the general population, the technical shortcomings of the technique will necessitate the discarding of embryos or even recurrent selective abortion when the editing procedure does not succeed, raising ethical, religious and practical concerns when applied to humans.

In addition, it may be difficult to distinguish between the correction of a detrimental mutation and genetic enhancement. For this reason, the use of genome editing technologies in human embryos may result in unexpected, unpredictable and potentially harmful consequences for future generations, since it may result in

reduced human genetic variability and cross the borders of eugenics, baby design and the removal of certain characteristics, to be substituted with others that are more desirable[161,162].

Thus, the boundaries between a cure and eugenics applications are becoming very thin. It may be fundamental to promote general, worldwide-accepted protocols, which will require close interaction between the regulatory agencies, scientific communities and governments of different countries. It is for this reason that a global moratorium on the use of genome editing technologies for human germline modification has recently been called for[154], to allow time to discuss the relevant scientific and ethical issues.

In conclusion, the potential of CRISPR/Cas9 is enormous, but researchers need to proceed with caution. It is very likely that new discoveries, data and protocols will help to address the many obstacles involved, and CRISPR will lead to a new revolution in the field of molecular biology, similar to polymerase chain reaction in the 1980s.

REFERENCES

1 **Capecchi MR**. Altering the genome by homologous recombination. *Science* 1989; **244**: 1288-1292 [PMID: 2660260 DOI: 10.1126/science.2660260]

2 **Rouet P**, Smih F, Jasin M. Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol Cell Biol* 1994; **14**: 8096-8106 [PMID: 7969147 DOI: 10.1128/mcb.14.12.8096]

3 **Choulika A**, Perrin A, Dujon B, Nicolas JF. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of Saccharomyces cerevisiae. *Mol Cell Biol* 1995; **15**: 1968-1973 [PMID: 7891691 DOI: 10.1128/mcb.15.4.1968]

4 **Urnov FD**, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 2005; **435**: 646-651 [PMID: 15806097 DOI: 10.1038/nature03556]

5 **Christian M**, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Bogdanove AJ, Voytas DF. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 2010; **186**: 757-761 [PMID: 20660643 DOI: 10.1534/genetics.110.120717]

6 **Gaj T**, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 2013; **31**: 397-405 [PMID: 23664777 DOI: 10.1016/j.tibtech.2013.04.004]

7 **Deng D**, Yan C, Pan X, Mahfouz M, Wang J, Zhu JK, Shi Y, Yan N. Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* 2012; **335**: 720-723 [PMID: 22223738 DOI: 10.1126/science.1215670]

8 **Li H**, Haurigot V, Doyon Y, Li T, Wong SY, Bhagwat AS, Malani N, Anguela XM, Sharma R, Ivanciu L, Murphy SL, Finn JD, Khazi FR, Zhou S, Paschon DE, Rebar EJ, Bushman FD, Gregory PD, Holmes MC, High KA. In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature* 2011; **475**: 217-221 [PMID: 21706032 DOI: 10.1038/nature10177]

9 **Sebastiano V**, Maeder ML, Angstman JF, Haddad B, Khayter C, Yeo DT, Goodwin MJ, Hawkins JS, Ramirez CL, Batista LF, Artandi SE, Wernig M, Joung JK. In situ genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. *Stem Cells* 2011; **29**: 1717-1726 [PMID: 21898685 DOI: 10.1002/stem.718]

10 **Zou J**, Mali P, Huang X, Dowey SN, Cheng L. Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. *Blood* 2011; **118**: 4599-4608 [PMID: 21881051 DOI: 10.1182/blood-2011-02-335554]

11 **Perez EE**, Wang J, Miller JC, Jouvenot Y, Kim KA, Liu O, Wang N, Lee G, Bartsevich VV, Lee YL, Guschin DY, Rupniewski I, Waite AJ, Carpenito C, Carroll RG, Orange JS, Urnov FD, Rebar EJ, Ando D, Gregory PD, Riley JL, Holmes MC, June CH. Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nat Biotechnol* 2008; **26**: 808-816 [PMID: 18587387 DOI: 10.1038/nbt1410]

12 **Holt N**, Wang J, Kim K, Friedman G, Wang X, Taupin V, Crooks GM, Kohn DB, Gregory PD, Holmes MC, Cannon PM. Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. *Nat Biotechnol* 2010; **28**: 839-847 [PMID: 20601939 DOI: 10.1038/nbt.1663]

13 **Voit RA**, McMahon MA, Sawyer SL, Porteus MH. Generation of an HIV resistant T-cell line by targeted "stacking" of restriction factors. *Mol Ther* 2013; **21**: 786-795 [PMID: 23358186 DOI: 10.1038/mt.2012.284]

14 **Hütter G**, Nowak D, Mossner M, Ganepola S, Müssig A, Allers K, Schneider T, Hofmann J, Kücherer C, Blau O, Blau IW, Hofmann WK, Thiel E. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med* 2009; **360**: 692-698 [PMID: 19213682 DOI: 10.1056/NEJMoa0802905]

15 **Gupta RK**, Abdul-Jawad S, McCoy LE, Mok HP, Peppa D, Salgado M, Martinez-Picado J, Nijhuis M, Wensing AMJ, Lee H, Grant P, Nastouli E, Lambert J, Pace M, Salasc F, Monit C, Innes AJ, Muir L, Waters L, Frater J, Lever AML, Edwards SG, Gabriel IH, Olavarria E. HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. *Nature* 2019; **568**: 244-248 [PMID: 30836379 DOI: 10.1038/s41586-019-1027-4]

16 **Ma N**, Liao B, Zhang H, Wang L, Shan Y, Xue Y, Huang K, Chen S, Zhou X, Chen Y, Pei D, Pan G. Transcription activator-like effector nuclease (TALEN)-mediated gene correction in integration-free β-thalassemia induced pluripotent stem cells. *J Biol Chem* 2013; **288**: 34671-34679 [PMID: 24155235 DOI: 10.1074/jbc.M113.496174]

17 **Li T**, Liu B, Chen CY, Yang B. TALEN-Mediated Homologous Recombination Produces Site-Directed DNA Base Change and Herbicide-Resistant Rice. *J Genet Genomics* 2016; **43**: 297-305 [PMID: 27180265 DOI: 10.1016/j.jgg.2016.03.005]

18 **Qasim W**, Zhan H, Samarasinghe S, Adams S, Amrolia P, Stafford S, Butler K, Rivat C, Wright G,

Somana K, Ghorashian S, Pinner D, Ahsan G, Gilmour K, Lucchini G, Inglott S, Mifsud W, Chiesa R, Peggs KS, Chan L, Farzeneh F, Thrasher AJ, Vora A, Pule M, Veys P. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. *Sci Transl Med* 2017; 9 [PMID: 28123068 DOI: 10.1126/scitranslmed.aaj2013]

19 **Gasiunas G**, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci U S A* 2012; **109**: E2579-E2586 [PMID: 22949671 DOI: 10.1073/pnas.1208507109]

20 **Ishino Y**, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J Bacteriol* 1987; **169**: 5429-5433 [PMID: 3316184 DOI: 10.1128/jb.169.12.5429-5433.1987]

21 **Barrangou R**, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 2007; **315**: 1709-1712 [PMID: 17379808 DOI: 10.1126/science.1138140]

22 **Jansen R**, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. *Mol Microbiol* 2002; **43**: 1565-1575 [PMID: 11952905 DOI: 10.1046/j.1365-2958.2002.02839.x]

23 **Tang TH**, Bachellerie JP, Rozhdestvensky T, Bortolin ML, Huber H, Drungowski M, Elge T, Brosius J, Hüttenhofer A. Identification of 86 candidates for small non-messenger RNAs from the archaeon Archaeoglobus fulgidus. *Proc Natl Acad Sci U S A* 2002; **99**: 7536-7541 [PMID: 12032318 DOI: 10.1073/pnas.112047299]

24 **Mojica FJ**, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* 2005; **60**: 174-182 [PMID: 15791728 DOI: 10.1007/s00239-004-0046-3]

25 **Rath D**, Amlinger L, Rath A, Lundgren M. The CRISPR-Cas immune system: biology, mechanisms and applications. *Biochimie* 2015; **117**: 119-128 [PMID: 25868999 DOI: 10.1016/j.biochi.2015.03.025]

26 **Deltcheva E**, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 2011; **471**: 602-607 [PMID: 21455174 DOI: 10.1038/nature09886]

27 **Jinek M**, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012; **337**: 816-821 [PMID: 22745249 DOI: 10.1126/science.1225829]

28 **Makarova KS**, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Barrangou R, Brouns SJ, Charpentier E, Haft DH, Horvath P, Moineau S, Mojica FJ, Terns RM, Terns MP, White MF, Yakunin AF, Garrett RA, van der Oost J, Backofen R, Koonin EV. An updated evolutionary classification of CRISPR-Cas systems. *Nat Rev Microbiol* 2015; **13**: 722-736 [PMID: 26411297 DOI: 10.1038/nrmicro3569]

29 **Bolotin A**, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* 2005; **151**: 2551-2561 [PMID: 16079334 DOI: 10.1099/mic.0.28048-0]

30 **Mojica FJ**, Díez-Villaseñor C, García-Martínez J, Almendros C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* 2009; **155**: 733-740 [PMID: 19246744 DOI: 10.1099/mic.0.023960-0]

31 **Hwang WY**, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 2013; **31**: 227-229 [PMID: 23360964 DOI: 10.1038/nbt.2501]

32 **Ran FA**, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 2013; **154**: 1380-1389 [PMID: 23992846 DOI: 10.1016/j.cell.2013.08.021]

33 **Cho SW**, Kim S, Kim JM, Kim JS. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat Biotechnol* 2013; **31**: 230-232 [PMID: 23360966 DOI: 10.1038/nbt.2507]

34 **Jinek M**, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. *Elife* 2013; **2**: e00471 [PMID: 23386978 DOI: 10.7554/eLife.00471]

35 **Mali P**, Esvelt KM, Church GM. Cas9 as a versatile tool for engineering biology. *Nat Methods* 2013; **10**: 957-963 [PMID: 24076990 DOI: 10.1038/nmeth.2649]

36 **Shen B**, Zhang J, Wu H, Wang J, Ma K, Li Z, Zhang X, Zhang P, Huang X. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res* 2013; **23**: 720-723 [PMID: 23545779 DOI: 10.1038/cr.2013.46]

37 **Cong L**, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013; **339**: 819-823 [PMID: 23287718 DOI: 10.1126/science.1231143]

38 **Zetsche B**, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV, Zhang F. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 2015; **163**: 759-771 [PMID: 26422227 DOI: 10.1016/j.cell.2015.09.038]

39 **Shmakov S**, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI, Severinov K, Zhang F, Koonin EV. Diversity and evolution of class 2 CRISPR-Cas systems. *Nat Rev Microbiol* 2017; **15**: 169-182 [PMID: 28111461 DOI: 10.1038/nrmicro.2016.184]

40 **Yan WX**, Chong S, Zhang H, Makarova KS, Koonin EV, Cheng DR, Scott DA. Cas13d Is a Compact RNA-Targeting Type VI CRISPR Effector Positively Modulated by a WYL-Domain-Containing Accessory Protein. *Mol Cell* 2018; **70**: 327-339.e5 [PMID: 29551514 DOI: 10.1016/j.molcel.2018.02.028]

41 **Konermann S**, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD. Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. *Cell* 2018; **173**: 665-676.e14 [PMID: 29551272 DOI: 10.1016/j.cell.2018.02.033]

42 **East-Seletsky A**, O'Connell MR, Knight SC, Burstein D, Cate JH, Tjian R, Doudna JA. Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. *Nature* 2016; **538**: 270-273 [PMID: 27669025 DOI: 10.1038/nature19802]

43 **Smargon AA**, Cox DBT, Pyzocha NK, Zheng K, Slaymaker IM, Gootenberg JS, Abudayyeh OA, Essletzbichler P, Shmakov S, Makarova KS, Koonin EV, Zhang F. Cas13b Is a Type VI-B CRISPR-Associated RNA-Guided RNase Differentially Regulated by Accessory Proteins Csx27 and Csx28. *Mol Cell* 2017; **65**: 618-630.e7 [PMID: 28065598 DOI: 10.1016/j.molcel.2016.12.023]

44 **O'Connell MR**. Molecular Mechanisms of RNA Targeting by Cas13-containing Type VI CRISPR-Cas Systems. *J Mol Biol* 2019; **431**: 66-87 [PMID: 29940185 DOI: 10.1016/j.jmb.2018.06.029]

45 **Abudayyeh OO**, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DBT,

Kellner MJ, Regev A, Lander ES, Voytas DF, Ting AY, Zhang F. RNA targeting with CRISPR-Cas13. *Nature* 2017; **550**: 280-284 [PMID: 28976959 DOI: 10.1038/nature24049]

46 **Cox DBT**, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J, Zhang F. RNA editing with CRISPR-Cas13. *Science* 2017; **358**: 1019-1027 [PMID: 29070703 DOI: 10.1126/science.aaq0180]

47 **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]

48 **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]

49 **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]

50 **Tachibana M**, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, Kang E, Fulati A, Lee HS, Sritanaudomchai H, Masterson K, Larson J, Eaton D, Sadler-Fredd K, Battaglia D, Lee D, Wu D, Jensen J, Patton P, Gokhale S, Stouffer RL, Wolf D, Mitalipov S. Human embryonic stem cells derived by somatic cell nuclear transfer. *Cell* 2013; **153**: 1228-1238 [PMID: 23683578 DOI: 10.1016/j.cell.2013.05.006]

51 **Lian X**, Zhang J, Azarin SM, Zhu K, Hazeltine LB, Bao X, Hsiao C, Kamp TJ, Palecek SP. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nat Protoc* 2013; **8**: 162-175 [PMID: 23257984 DOI: 10.1038/nprot.2012.150]

52 **Takahashi K**, Okita K, Nakagawa M, Yamanaka S. Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* 2007; **2**: 3081-3089 [PMID: 18079707 DOI: 10.1038/nprot.2007.418]

53 **Chen SM**, Lee MS, Chang CY, Lin SZ, Cheng EH, Liu YH, Pan HC, Lee HC, Su HL. Prerequisite OCT4 Maintenance Potentiates the Neural Induction of Differentiating Human Embryonic Stem Cells and Induced Pluripotent Stem Cells. *Cell Transplant* 2015; **24**: 829-844 [PMID: 24256943 DOI: 10.3727/096368913X675179]

54 **Krohne TU**, Westenskow PD, Kurihara T, Friedlander DF, Lehmann M, Dorsey AL, Li W, Zhu S, Schultz A, Wang J, Siuzdak G, Ding S, Friedlander M. Generation of retinal pigment epithelial cells from small molecules and OCT4 reprogrammed human induced pluripotent stem cells. *Stem Cells Transl Med* 2012; **1**: 96-109 [PMID: 22532929 DOI: 10.5966/sctm.2011-0057]

55 **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]

56 **Buganim Y**, Jaenisch R. Transdifferentiation by defined factors as a powerful research tool to address basic biological questions. *Cell Cycle* 2012; **11**: 4485-4486 [PMID: 23165203 DOI: 10.4161/cc.22665]

57 **Moon JH**, Heo JS, Kwon S, Kim J, Hwang J, Kang PJ, Kim A, Kim HO, Whang KY, Yoon BS, You S. Two-step generation of induced pluripotent stem cells from mouse fibroblasts using Id3 and Oct4. *J Mol Cell Biol* 2012; **4**: 59-62 [PMID: 22131360 DOI: 10.1093/jmcb/mjr038]

58 **Judson RL**, Babiarz JE, Venere M, Blelloch R. Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat Biotechnol* 2009; **27**: 459-461 [PMID: 19363475 DOI: 10.1038/nbt.1535]

59 **Anokye-Danso F**, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y, Zhang Y, Yang W, Gruber PJ, Epstein JA, Morrisey EE. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* 2011; **8**: 376-388 [PMID: 21474102 DOI: 10.1016/j.stem.2011.03.001]

60 **Yang J**, Li S, He XB, Cheng C, Le W. Induced pluripotent stem cells in Alzheimer's disease: applications for disease modeling and cell-replacement therapy. *Mol Neurodegener* 2016; **11**: 39 [PMID: 27184028 DOI: 10.1186/s13024-016-0106-3]

61 **DeWitt MA**, Magis W, Bray NL, Wang T, Berman JR, Urbinati F, Heo SJ, Mitros T, Muñoz DP, Boffelli D, Kohn DB, Walters MC, Carroll D, Martin DI, Corn JE. Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci Transl Med* 2016; **8**: 360ra134 [PMID: 27733558 DOI: 10.1126/scitranslmed.aaf9336]

62 **Dalle Carbonare L**, Matte A, Valenti MT, Siciliano A, Cristellon A, De Franceschi L. Zoledronic Acid Reverses Acute Bone Impairment in a Mouse Model for Sickle Cell Disease. *Blood* 2014; **124**: 122

63 **Lucas D**, O'Leary HA, Ebert BL, Cowan CA, Tremblay CS. Utility of CRISPR/Cas9 systems in hematology research. *Exp Hematol* 2017; **54**: 1-3 [PMID: 28668351 DOI: 10.1016/j.exphem.2017.06.006]

64 **Heckl D**, Kowalczyk MS, Yudovich D, Belizaire R, Puram RV, McConkey ME, Thielke A, Aster JC, Regev A, Ebert BL. Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. *Nat Biotechnol* 2014; **32**: 941-946 [PMID: 24952903 DOI: 10.1038/nbt.2951]

65 **Bejar R**, Lord A, Stevenson K, Bar-Natan M, Pérez-Ladaga A, Zaneveld J, Wang H, Caughey B, Stojanov P, Getz G, Garcia-Manero G, Kantarjian H, Chen R, Stone RM, Neuberg D, Steensma DP, Ebert BL. TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. *Blood* 2014; **124**: 2705-2712 [PMID: 25224413 DOI: 10.1182/blood-2014-06-582809]

66 **Donahue DA**, Wainberg MA. Cellular and molecular mechanisms involved in the establishment of HIV-1 latency. *Retrovirology* 2013; **10**: 11 [PMID: 23375003 DOI: 10.1186/1742-4690-10-11]

67 **Zhu W**, Lei R, Le Duff Y, Li J, Guo F, Wainberg MA, Liang C. The CRISPR/Cas9 system inactivates latent HIV-1 proviral DNA. *Retrovirology* 2015; **12**: 22 [PMID: 25808449 DOI: 10.1186/s12977-015-0150-z]

68 **Ebina H**, Misawa N, Kanemura Y, Koyanagi Y. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. *Sci Rep* 2013; **3**: 2510 [PMID: 23974631 DOI: 10.1038/srep02510]

69 **Kaminski R**, Bella R, Yin C, Otte J, Ferrante P, Gendelman HE, Li H, Booze R, Gordon J, Hu W, Khalili K. Excision of HIV-1 DNA by gene editing: a proof-of-concept in vivo study. *Gene Ther* 2016; **23**: 696 [PMID: 27488023 DOI: 10.1038/gt.2016.45]

70 **Hu W**, Kaminski R, Yang F, Zhang Y, Cosentino L, Li F, Luo B, Alvarez-Carbonell D, Garcia-Mesa Y, Karn J, Mo X, Khalili K. RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. *Proc Natl Acad Sci U S A* 2014; **111**: 11461-11466 [PMID: 25049410 DOI: 10.1073/pnas.1405186111]

71 **Yin C**, Zhang T, Qu X, Zhang Y, Putatunda R, Xiao X, Li F, Xiao W, Zhao H, Dai S, Qin X, Mo X, Young WB, Khalili K, Hu W. In Vivo Excision of HIV-1 Provirus by saCas9 and Multiplex Single-Guide RNAs in Animal Models. *Mol Ther* 2017; **25**: 1168-1186 [PMID: 28366764 DOI: 10.1016/j.ymthe.2017.03.012]

72 **Konermann S**, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O, Zhang F. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 2015; **517**: 583-588 [PMID: 25494202 DOI: 10.1038/nature14136]

73 **Zhang Y**, Yin C, Zhang T, Li F, Yang W, Kaminski R, Fagan PR, Putatunda R, Young WB, Khalili K, Hu W. CRISPR/gRNA-directed synergistic activation mediator (SAM) induces specific, persistent and robust reactivation of the HIV-1 latent reservoirs. *Sci Rep* 2015; **5**: 16277 [PMID: 26538064 DOI: 10.1038/srep16277]

74 **Nakamoto FK**, Okamoto S, Mitsui J, Sone T, Ishikawa M, Yamamoto Y, Kanegae Y, Nakatake Y, Imaizumi K, Ishiura H, Tsuji S, Okano H. The pathogenesis linked to coenzyme Q10 insufficiency in iPSC-derived neurons from patients with multiple-system atrophy. *Sci Rep* 2018; **8**: 14215 [PMID: 30242188 DOI: 10.1038/s41598-018-32573-1]

75 **Bettens K**, Sleegers K, Van Broeckhoven C. Genetic insights in Alzheimer's disease. *Lancet Neurol* 2013; **12**: 92-104 [PMID: 23237904 DOI: 10.1016/S1474-4422(12)70259-4]

76 **Schellenberg GD**, Bird TD, Wijsman EM, Orr HT, Anderson L, Nemens E, White JA, Bonnycastle L, Weber JL, Alonso ME. Genetic linkage evidence for a familial Alzheimer's disease locus on chromosome 14. *Science* 1992; **258**: 668-671 [PMID: 1411576 DOI: 10.1126/science.1411576]

77 **Ortiz-Virumbrales M**, Moreno CL, Kruglikov I, Marazuela P, Sproul A, Jacob S, Zimmer M, Paull D, Zhang B, Schadt EE, Ehrlich ME, Tanzi RE, Arancio O, Noggle S, Gandy S. CRISPR/Cas9-Correctable mutation-related molecular and physiological phenotypes in iPSC-derived Alzheimer's PSEN2 N141I neurons. *Acta Neuropathol Commun* 2017; **5**: 77 [PMID: 29078805 DOI: 10.1186/s40478-017-0475-z]

78 **Eisenstein M**. Genetics: finding risk factors. *Nature* 2011; **475**: S20-S22 [PMID: 21760580 DOI: 10.1038/475S20a]

79 **Rohn TT**, Kim N, Isho NF, Mack JM. The Potential of CRISPR/Cas9 Gene Editing as a Treatment Strategy for Alzheimer's Disease. *J Alzheimers Dis Parkinsonism* 2018; 8 [PMID: 30090689 DOI: 10.4172/2161-0460.1000439]

80 **Golas MM**, Sander B. Use of human stem cells in Huntington disease modeling and translational research. *Exp Neurol* 2016; **278**: 76-90 [PMID: 26826449 DOI: 10.1016/j.expneurol.2016.01.021]

81 **Rosati J**, Bidollari E, Rotundo G, Ferrari D, Torres B, Bernardini L, Consoli F, De Luca A, Santimone I, Lamorte G, Squitieri F, Vescovi AL. Generation of induced pluripotent stem cell line, CSSi002-A (2851), from a patient with juvenile Huntington Disease. *Stem Cell Res* 2018; **27**: 86-89 [PMID: 29342448 DOI: 10.1016/j.scr.2018.01.011]

82 **Golas MM**. Human cellular models of medium spiny neuron development and Huntington disease. *Life Sci* 2018; **209**: 179-196 [PMID: 30031060 DOI: 10.1016/j.lfs.2018.07.030]

83 **Saito A**, Ooki A, Nakamura T, Onodera S, Hayashi K, Hasegawa D, Okudaira T, Watanabe K, Kato H, Onda T, Watanabe A, Kosaki K, Nishimura K, Ohtaka M, Nakanishi M, Sakamoto T, Yamaguchi A, Sueishi K, Azuma T. Targeted reversion of induced pluripotent stem cells from patients with human cleidocranial dysplasia improves bone regeneration in a rat calvarial bone defect model. *Stem Cell Res Ther* 2018; **9**: 12 [PMID: 29357927 DOI: 10.1186/s13287-017-0754-4]

84 **Ducy P**, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Karsenty G. Increased bone formation in osteocalcin-deficient mice. *Nature* 1996; **382**: 448-452 [PMID: 8684484 DOI: 10.1038/382448a0]

85 **Lambert LJ**, Challa AK, Niu A, Zhou L, Tucholski J, Johnson MS, Nagy TR, Eberhardt AW, Estep PN, Kesterson RA, Grams JM. Increased trabecular bone and improved biomechanics in an osteocalcin-null rat model created by CRISPR/Cas9 technology. *Dis Model Mech* 2016; **9**: 1169-1179 [PMID: 27483347 DOI: 10.1242/dmm.025247]

86 **Long C**, McAnally JR, Shelton JM, Mireault AA, Bassel-Duby R, Olson EN. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science* 2014; **345**: 1184-1188 [PMID: 25123483 DOI: 10.1126/science.1254445]

87 **Lapidos KA**, Kakkar R, McNally EM. The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. *Circ Res* 2004; **94**: 1023-1031 [PMID: 15117830 DOI: 10.1161/01.RES.0000126574.61061.25]

88 **Li HL**, Fujimoto N, Sasakawa N, Shirai S, Ohkame T, Sakuma T, Tanaka M, Amano N, Watanabe A, Sakurai H, Yamamoto T, Yamanaka S, Hotta A. Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Reports* 2015; **4**: 143-154 [PMID: 25434822 DOI: 10.1016/j.stemcr.2014.10.013]

89 **van Gelder CM**, Vollebregt AA, Plug I, van der Ploeg AT, Reuser AJ. Treatment options for lysosomal storage disorders: developing insights. *Expert Opin Pharmacother* 2012; **13**: 2281-2299 [PMID: 23009070 DOI: 10.1517/14656566.2012.729039]

90 **van der Ploeg AT**, Reuser AJ. Pompe's disease. *Lancet* 2008; **372**: 1342-1353 [PMID: 18929906 DOI: 10.1016/S0140-6736(08)61555-X]

91 **van Til NP**, Stok M, Aerts Kaya FS, de Waard MC, Farahbakhshian E, Visser TP, Kroos MA, Jacobs EH, Willart MA, van der Wegen P, Scholte BJ, Lambrecht BN, Duncker DJ, van der Ploeg AT, Reuser AJ, Verstegen MM, Wagemaker G. Lentiviral gene therapy of murine hematopoietic stem cells ameliorates the Pompe disease phenotype. *Blood* 2010; **115**: 5329-5337 [PMID: 20385789 DOI: 10.1182/blood-2009-11-252874]

92 **The European Heart Network**. European Cardiovascular Disease Statistics 2017. Available from: http://www.ehnheart.org/cvd-statistics.html

93 **Louch WE**, Sheehan KA, Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. *J Mol Cell Cardiol* 2011; **51**: 288-298 [PMID: 21723873 DOI: 10.1016/j.yjmcc.2011.06.012]

94 **Niu Y**, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W, Sha J. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* 2014; **156**: 836-843 [PMID: 24486104 DOI: 10.1016/j.cell.2014.01.027]

95 **Shao Y**, Guan Y, Wang L, Qiu Z, Liu M, Chen Y, Wu L, Li Y, Ma X, Liu M, Li D. CRISPR/Cas-mediated genome editing in the rat via direct injection of one-cell embryos. *Nat Protoc* 2014; **9**: 2493-2512 [PMID: 25255092 DOI: 10.1038/nprot.2014.171]

96 **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]
97 **Hinson JT**, Chopra A, Nafissi N, Polacheck WJ, Benson CC, Swist S, Gorham J, Yang L, Schafer S, Sheng CC, Haghighi A, Homsy J, Hubner N, Church G, Cook SA, Linke WA, Chen CS, Seidman JG, Seidman CE. HEART DISEASE. Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. *Science* 2015; **349**: 982-986 [PMID: 26315439 DOI: 10.1126/science.aaa5458]
98 **Denjoy I**, Lupoglazoff JM, Villain E, Vaksmann G, Godart F, Lucet V, Leenhardt A, Guicheney P, Schwartz P. [The Jervell and Lange-Nielsen syndrome. Natural history, molecular basis and clinical outcome]. *Arch Mal Coeur Vaiss* 2007; **100**: 359-364 [PMID: 17646758]
99 **Yamamoto Y**, Makiyama T, Harita T, Sasaki K, Wuriyanghai Y, Hayano M, Nishiuchi S, Kohjitani H, Hirose S, Chen J, Yokoi F, Ishikawa T, Ohno S, Chonabayashi K, Motomura H, Yoshida Y, Horie M, Makita N, Kimura T. Allele-specific ablation rescues electrophysiological abnormalities in a human iPS cell model of long-QT syndrome with a CALM2 mutation. *Hum Mol Genet* 2017; **26**: 1670-1677 [PMID: 28335032 DOI: 10.1093/hmg/ddx073]
100 **Beaudoin M**, Gupta RM, Won HH, Lo KS, Do R, Henderson CA, Lavoie-St-Amour C, Langlois S, Rivas D, Lehoux S, Kathiresan S, Tardif JC, Musunuru K, Lettre G. Myocardial Infarction-Associated SNP at 6p24 Interferes With MEF2 Binding and Associates With PHACTR1 Expression Levels in Human Coronary Arteries. *Arterioscler Thromb Vasc Biol* 2015; **35**: 1472-1479 [PMID: 25838425 DOI: 10.1161/ATVBAHA.115.305534]
101 **Mosqueira D**, Mannhardt I, Bhagwan JR, Lis-Slimak K, Katili P, Scott E, Hassan M, Prondzynski M, Harmer SC, Tinker A, Smith JGW, Carrier L, Williams PM, Gaffney D, Eschenhagen T, Hansen A, Denning C. CRISPR/Cas9 editing in human pluripotent stem cell-cardiomyocytes highlights arrhythmias, hypocontractility, and energy depletion as potential therapeutic targets for hypertrophic cardiomyopathy. *Eur Heart J* 2018; **39**: 3879-3892 [PMID: 29741611 DOI: 10.1093/eurheartj/ehy249]
102 **Balboa D**, Saarimäki-Vire J, Otonkoski T. Concise Review: Human Pluripotent Stem Cells for the Modeling of Pancreatic β-Cell Pathology. *Stem Cells* 2019; **37**: 33-41 [PMID: 30270471 DOI: 10.1002/stem.2913]
103 **Coombe L**, Kadri A, Martinez JF, Tatachar V, Gallicano GI. Current approaches in regenerative medicine for the treatment of diabetes: introducing CRISPR/CAS9 technology and the case for non-embryonic stem cell therapy. *Am J Stem Cells* 2018; **7**: 104-113 [PMID: 30697454]
104 **Beer NL**, Gloyn AL. Genome-edited human stem cell-derived beta cells: a powerful tool for drilling down on type 2 diabetes GWAS biology. *F1000Res* 2016; 5 [PMID: 27508066 DOI: 10.12688/f1000research.8682.1]
105 **Kyono Y**, Kitzman JO, Parker SCJ. Genomic annotation of disease-associated variants reveals shared functional contexts. *Diabetologia* 2019; **62**: 735-743 [PMID: 30756131 DOI: 10.1007/s00125-019-4823-3]
106 **Balboa D**, Saarimäki-Vire J, Borshagovski D, Survila M, Lindholm P, Galli E, Eurola S, Ustinov J, Grym H, Huopio H, Partanen J, Wartiovaara K, Otonkoski T. Insulin mutations impair beta-cell development in a patient-derived iPSC model of neonatal diabetes. *Elife* 2018; 7 [PMID: 30412052 DOI: 10.7554/eLife.38519]
107 **Lathia JD**, Mack SC, Mulkearns-Hubert EE, Valentim CL, Rich JN. Cancer stem cells in glioblastoma. *Genes Dev* 2015; **29**: 1203-1217 [PMID: 26109046 DOI: 10.1101/gad.261982.115]
108 **Toledo CM**, Ding Y, Hoellerbauer P, Davis RJ, Basom R, Girard EJ, Lee E, Corrin P, Hart T, Bolouri H, Davison J, Zhang Q, Hardcastle J, Aronow BJ, Plaisier CL, Baliga NS, Moffat J, Lin Q, Li XN, Nam DH, Lee J, Pollard SM, Zhu J, Delrow JJ, Clurman BE, Olson JM, Paddison PJ. Genome-wide CRISPR-Cas9 Screens Reveal Loss of Redundancy between PKMYT1 and WEE1 in Glioblastoma Stem-like Cells. *Cell Rep* 2015; **13**: 2425-2439 [PMID: 26673326 DOI: 10.1016/j.celrep.2015.11.021]
109 **Minagawa A**, Yoshikawa T, Yasukawa M, Hotta A, Kunitomo M, Iriguchi S, Takiguchi M, Kassai Y, Imai E, Yasui Y, Kawai Y, Zhang R, Uemura Y, Miyoshi H, Nakanishi M, Watanabe A, Hayashi A, Kawana K, Fujii T, Nakatsura T, Kaneko S. Enhancing T Cell Receptor Stability in Rejuvenated iPSC-Derived T Cells Improves Their Use in Cancer Immunotherapy. *Cell Stem Cell* 2018; **23**: 850-858.e4 [PMID: 30449714 DOI: 10.1016/j.stem.2018.10.005]
110 **Chang CJ**, Kotini AG, Olszewska M, Georgomanoli M, Teruya-Feldstein J, Sperber H, Sanchez R, DeVita R, Martins TJ, Abdel-Wahab O, Bradley RK, Papapetrou EP. Dissecting the Contributions of Cooperating Gene Mutations to Cancer Phenotypes and Drug Responses with Patient-Derived iPSCs. *Stem Cell Reports* 2018; **10**: 1610-1624 [PMID: 29681544 DOI: 10.1016/j.stemcr.2018.03.020]
111 **Wells SA**, Pacini F, Robinson BG, Santoro M. Multiple endocrine neoplasia type 2 and familial medullary thyroid carcinoma: an update. *J Clin Endocrinol Metab* 2013; **98**: 3149-3164 [PMID: 23744408 DOI: 10.1210/jc.2013-1204]
112 **Hadoux J**, Desterke C, Féraud O, Guibert M, De Rose RF, Opolon P, Divers D, Gobbo E, Griscelli F, Schlumberger M, Bennaceur-Griscelli A, Turhan AG. Transcriptional landscape of a RETC634Y-mutated iPSC and its CRISPR-corrected isogenic control reveals the putative role of EGR1 transcriptional program in the development of multiple endocrine neoplasia type 2A-associated cancers. *Stem Cell Res* 2018; **26**: 8-16 [PMID: 29197744 DOI: 10.1016/j.scr.2017.11.015]
113 **O'Geen H**, Yu AS, Segal DJ. How specific is CRISPR/Cas9 really? *Curr Opin Chem Biol* 2015; **29**: 72-78 [PMID: 26517564 DOI: 10.1016/j.cbpa.2015.10.001]
114 **Tan EP**, Li Y, Velasco-Herrera Mdel C, Yusa K, Bradley A. Off-target assessment of CRISPR-Cas9 guiding RNAs in human iPS and mouse ES cells. *Genesis* 2015; **53**: 225-236 [PMID: 25378133 DOI: 10.1002/dvg.22835]
115 **Veres A**, Gosis BS, Ding Q, Collins R, Ragavendran A, Brand H, Erdin S, Cowan CA, Talkowski ME, Musunuru K. Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. *Cell Stem Cell* 2014; **15**: 27-30 [PMID: 24996167 DOI: 10.1016/j.stem.2014.04.020]
116 **Yang L**, Grishin D, Wang G, Aach J, Zhang CZ, Chari R, Homsy J, Cai X, Zhao Y, Fan JB, Seidman C, Seidman J, Pu W, Church G. Targeted and genome-wide sequencing reveal single nucleotide variations impacting specificity of Cas9 in human stem cells. *Nat Commun* 2014; **5**: 5507 [PMID: 25425480 DOI: 10.1038/ncomms6507]
117 **Schaefer KA**, Wu WH, Colgan DF, Tsang SH, Bassuk AG, Mahajan VB. Unexpected mutations after CRISPR-Cas9 editing in vivo. *Nat Methods* 2017; **14**: 547-548 [PMID: 28557981 DOI: 10.1038/nmeth.4293]
118 CRISPR off-targets: a reassessment. *Nat Methods* 2018; **15**: 229-230 [DOI: 10.1038/Nmeth.4664]
119 **Iyer V**, Boroviak K, Thomas M, Doe B, Riva L, Ryder E, Adams DJ. No unexpected CRISPR-Cas9 off-

target activity revealed by trio sequencing of gene-edited mice. *PLoS Genet* 2018; **14**: e1007503 [PMID: 29985941 DOI: 10.1371/journal.pgen.1007503]
120 **Anderson KR**, Haeussler M, Watanabe C, Janakiraman V, Lund J, Modrusan Z, Stinson J, Bei Q, Buechler A, Yu C, Thamminana SR, Tam L, Sowick MA, Alcantar T, O'Neil N, Li J, Ta L, Lima L, Roose-Girma M, Rairdan X, Durinck S, Warming S. CRISPR off-target analysis in genetically engineered rats and mice. *Nat Methods* 2018; **15**: 512-514 [PMID: 29786090 DOI: 10.1038/s41592-018-0011-5]
121 **Akcakaya P**, Bobbin ML, Guo JA, Malagon-Lopez J, Clement K, Garcia SP, Fellows MD, Porritt MJ, Firth MA, Carreras A, Baccega T, Seeliger F, Bjursell M, Tsai SQ, Nguyen NT, Nitsch R, Mayr LM, Pinello L, Bohlooly-Y M, Aryee MJ, Maresca M, Joung JK. In vivo CRISPR editing with no detectable genome-wide off-target mutations. *Nature* 2018; **561**: 416-419 [PMID: 30209390 DOI: 10.1038/s41586-018-0500-9]
122 **Kim D**, Kim JS. DIG-seq: a genome-wide CRISPR off-target profiling method using chromatin DNA. *Genome Res* 2018; **28**: 1894-1900 [PMID: 30413470 DOI: 10.1101/gr.236620.118]
123 **Kim D**, Bae S, Park J, Kim E, Kim S, Yu HR, Hwang J, Kim JI, Kim JS. Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods* 2015; **12**: 237-243, 1 p following 243 [PMID: 25664545 DOI: 10.1038/nmeth.3284]
124 **Tsai SQ**, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, Aryee MJ, Joung JK. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol* 2015; **33**: 187-197 [PMID: 25513782 DOI: 10.1038/nbt.3117]
125 **Frock RL**, Hu J, Meyers RM, Ho YJ, Kii E, Alt FW. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol* 2015; **33**: 179-186 [PMID: 25503383 DOI: 10.1038/nbt.3101]
126 **Ravindran S**. New Methods to Detect CRISPR Off-Target Mutations. Available from: https://www.the-scientist.com/lab-tools/new-methods-to-detect-crispr-off-target-mutations-30013
127 **Fu Y**, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol* 2013; **31**: 822-826 [PMID: 23792628 DOI: 10.1038/nbt.2623]
128 **Cho SW**, Kim S, Kim Y, Kweon J, Kim HS, Bae S, Kim JS. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res* 2014; **24**: 132-141 [PMID: 24253446 DOI: 10.1101/gr.162339.113]
129 **Casini A**, Olivieri M, Petris G, Montagna C, Reginato G, Maule G, Lorenzin F, Prandi D, Romanel A, Demichelis F, Inga A, Cereseto A. A highly specific SpCas9 variant is identified by in vivo screening in yeast. *Nat Biotechnol* 2018; **36**: 265-271 [PMID: 29431739 DOI: 10.1038/nbt.4066]
130 **Kleinstiver BP**, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 2016; **529**: 490-495 [PMID: 26735016 DOI: 10.1038/nature16526]
131 **Vakulskas C**, Collingwood M, Behlke M. High Fidelity Genome Editing with a Novel Mutant HIF1 Cas9. *Mol Ther* 2018; **26**: 92-92
132 **Hou Z**, Zhang Y, Propson NE, Howden SE, Chu LF, Sontheimer EJ, Thomson JA. Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proc Natl Acad Sci U S A* 2013; **110**: 15644-15649 [PMID: 23940360 DOI: 10.1073/pnas.1313587110]
133 **Kleinstiver BP**, Tsai SQ, Prew MS, Nguyen NT, Welch MM, Lopez JM, McCaw ZR, Aryee MJ, Joung JK. Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. *Nat Biotechnol* 2016; **34**: 869-874 [PMID: 27347757 DOI: 10.1038/nbt.3620]
134 **Farboud B**, Jarvis E, Roth TL, Shin J, Corn JE, Marson A, Meyer BJ, Patel NH, Hochstrasser ML. Enhanced Genome Editing with Cas9 Ribonucleoprotein in Diverse Cells and Organisms. *J Vis Exp* 2018 [PMID: 29889198 DOI: 10.3791/57350]
135 **Yang H**, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 2013; **154**: 1370-1379 [PMID: 23992847 DOI: 10.1016/j.cell.2013.08.022]
136 **Genovese P**, Schiroli G, Escobar G, Tomaso TD, Firrito C, Calabria A, Moi D, Mazzieri R, Bonini C, Holmes MC, Gregory PD, van der Burg M, Gentner B, Montini E, Lombardo A, Naldini L. Targeted genome editing in human repopulating haematopoietic stem cells. *Nature* 2014; **510**: 235-240 [PMID: 24870228 DOI: 10.1038/nature13420]
137 **Cox DB**, Platt RJ, Zhang F. Therapeutic genome editing: prospects and challenges. *Nat Med* 2015; **21**: 121-131 [PMID: 25654603 DOI: 10.1038/nm.3793]
138 **Lino CA**, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: a review of the challenges and approaches. *Drug Deliv* 2018; **25**: 1234-1257 [PMID: 29801422 DOI: 10.1080/10717544.2018.1474964]
139 **Wirth T**, Parker N, Ylä-Herttuala S. History of gene therapy. *Gene* 2013; **525**: 162-169 [PMID: 23618815 DOI: 10.1016/j.gene.2013.03.137]
140 **Ran FA**, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F. In vivo genome editing using Staphylococcus aureus Cas9. *Nature* 2015; **520**: 186-191 [PMID: 25830891 DOI: 10.1038/nature14299]
141 **Li L**, He ZY, Wei XW, Gao GP, Wei YQ. Challenges in CRISPR/CAS9 Delivery: Potential Roles of Nonviral Vectors. *Hum Gene Ther* 2015; **26**: 452-462 [PMID: 26176432 DOI: 10.1089/hum.2015.069]
142 **Crudele JM**, Chamberlain JS. Cas9 immunity creates challenges for CRISPR gene editing therapies. *Nat Commun* 2018; **9**: 3497 [PMID: 30158648 DOI: 10.1038/s41467-018-05843-9]
143 **Charlesworth CT**, Deshpande PS, Dever DP, Camarena J, Lemgart VT, Cromer MK, Vakulskas CA, Collingwood MA, Zhang L, Bode NM, Behlke MA, Dejene B, Cieniewicz B, Romano R, Lesch BJ, Gomez-Ospina N, Mantri S, Pavel-Dinu M, Weinberg KI, Porteus MH. Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nat Med* 2019; **25**: 249-254 [PMID: 30692695 DOI: 10.1038/s41591-018-0326-x]
144 **Wang G**, Zhao N, Berkhout B, Das AT. CRISPR-Cas9 Can Inhibit HIV-1 Replication but NHEJ Repair Facilitates Virus Escape. *Mol Ther* 2016; **24**: 522-526 [PMID: 26796669 DOI: 10.1038/mt.2016.24]
145 **Wang Z**, Pan Q, Gendron P, Zhu W, Guo F, Cen S, Wainberg MA, Liang C. CRISPR/Cas9-Derived Mutations Both Inhibit HIV-1 Replication and Accelerate Viral Escape. *Cell Rep* 2016; **15**: 481-489 [PMID: 27068471 DOI: 10.1016/j.celrep.2016.03.042]
146 **Liao HK**, Gu Y, Diaz A, Marlett J, Takahashi Y, Li M, Suzuki K, Xu R, Hishida T, Chang CJ, Esteban CR, Young J, Izpisua Belmonte JC. Use of the CRISPR/Cas9 system as an intracellular defense against HIV-1 infection in human cells. *Nat Commun* 2015; **6**: 6413 [PMID: 25752527 DOI: 10.1038/ncomms7413]

147 **Vartak SV**, Raghavan SC. Inhibition of nonhomologous end joining to increase the specificity of CRISPR/Cas9 genome editing. *FEBS J* 2015; **282**: 4289-4294 [PMID: 26290158 DOI: 10.1111/febs.13416]

148 **Hou P**, Chen S, Wang S, Yu X, Chen Y, Jiang M, Zhuang K, Ho W, Hou W, Huang J, Guo D. Genome editing of CXCR4 by CRISPR/cas9 confers cells resistant to HIV-1 infection. *Sci Rep* 2015; **5**: 15577 [PMID: 26481100 DOI: 10.1038/srep15577]

149 **Wang W**, Ye C, Liu J, Zhang D, Kimata JT, Zhou P. CCR5 gene disruption via lentiviral vectors expressing Cas9 and single guided RNA renders cells resistant to HIV-1 infection. *PLoS One* 2014; **9**: e115987 [PMID: 25541967 DOI: 10.1371/journal.pone.0115987]

150 **Ye L**, Wang J, Beyer AI, Teque F, Cradick TJ, Qi Z, Chang JC, Bao G, Muench MO, Yu J, Levy JA, Kan YW. Seamless modification of wild-type induced pluripotent stem cells to the natural CCR5Δ32 mutation confers resistance to HIV infection. *Proc Natl Acad Sci U S A* 2014; **111**: 9591-9596 [PMID: 24927590 DOI: 10.1073/pnas.1407473111]

151 **Hultquist JF**, Schumann K, Woo JM, Manganaro L, McGregor MJ, Doudna J, Simon V, Krogan NJ, Marson A. A Cas9 Ribonucleoprotein Platform for Functional Genetic Studies of HIV-Host Interactions in Primary Human T Cells. *Cell Rep* 2016; **17**: 1438-1452 [PMID: 27783955 DOI: 10.1016/j.celrep.2016.09.080]

152 **Liang P**, Xu Y, Zhang X, Ding C, Huang R, Zhang Z, Lv J, Xie X, Chen Y, Li Y, Sun Y, Bai Y, Songyang Z, Ma W, Zhou C, Huang J. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein Cell* 2015; **6**: 363-372 [PMID: 25894090 DOI: 10.1007/s13238-015-0153-5]

153 **Cyranoski D**, Ledford H. Genome-edited baby claim provokes international outcry. *Nature* 2018; **563**: 607-608 [PMID: 30482929 DOI: 10.1038/d41586-018-07545-0]

154 **Lander ES**, Baylis F, Zhang F, Charpentier E, Berg P, Bourgain C, Friedrich B, Joung JK, Li J, Liu D, Naldini L, Nie JB, Qiu R, Schoene-Seifert B, Shao F, Terry S, Wei W, Winnacker EL. Adopt a moratorium on heritable genome editing. *Nature* 2019; **567**: 165-168 [PMID: 30867611 DOI: 10.1038/d41586-019-00726-5]

155 **Ihry RJ**, Worringer KA, Salick MR, Frias E, Ho D, Theriault K, Kommineni S, Chen J, Sondey M, Ye C, Randhawa R, Kulkarni T, Yang Z, McAllister G, Russ C, Reece-Hoyes J, Forrester W, Hoffman GR, Dolmetsch R, Kaykas A. p53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat Med* 2018; **24**: 939-946 [PMID: 29892062 DOI: 10.1038/s41591-018-0050-6]

156 **Merkle FT**, Ghosh S, Kamitaki N, Mitchell J, Avior Y, Mello C, Kashin S, Mekhoubad S, Ilic D, Charlton M, Saphier G, Handsaker RE, Genovese G, Bar S, Benvenisty N, McCarroll SA, Eggan K. Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations. *Nature* 2017; **545**: 229-233 [PMID: 28445466 DOI: 10.1038/nature22312]

157 **Champer J**, Chung J, Lee YL, Liu C, Yang E, Wen Z, Clark AG, Messer PW. Molecular safeguarding of CRISPR gene drive experiments. *Elife* 2019; 8 [PMID: 30666960 DOI: 10.7554/eLife.41439]

158 **Gantz VM**, Jasinskiene N, Tatarenkova O, Fazekas A, Macias VM, Bier E, James AA. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. *Proc Natl Acad Sci U S A* 2015; **112**: E6736-E6743 [PMID: 26598698 DOI: 10.1073/pnas.1521077112]

159 **Hammond A**, Galizi R, Kyrou K, Simoni A, Siniscalchi C, Katsanos D, Gribble M, Baker D, Marois E, Russell S, Burt A, Windbichler N, Crisanti A, Nolan T. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. *Nat Biotechnol* 2016; **34**: 78-83 [PMID: 26641531 DOI: 10.1038/nbt.3439]

160 **The Lancet**. Safeguarding the future of human gene editing. *Lancet* 2017; **389**: 671 [PMID: 28229861 DOI: 10.1016/S0140-6736(17)30389-6]

161 **Khurshid AT**. Safeguarding the Impacts of Human Genome Editing. *Adv Biotech and Micro* 2018; 9 [DOI: 10.19080/AIBM.2018.09.555775]

162 **Otieno MO**. CRISPR-Cas9 Human Genome Editing: Challenges, Ethical Concerns and Implications. *Journal of Clinical Research and Bioethics* 2015; 6 [DOI: 10.4172/2155-9627.1000253]

163 **Zhou M**, Hu Z, Qiu L, Zhou T, Feng M, Hu Q, Zeng B, Li Z, Sun Q, Wu Y, Liu X, Wu L, Liang D. Seamless Genetic Conversion of SMN2 to SMN1 via CRISPR/Cpf1 and Single-Stranded Oligodeoxynucleotides in Spinal Muscular Atrophy Patient-Specific Induced Pluripotent Stem Cells. *Hum Gene Ther* 2018; **29**: 1252-1263 [PMID: 29598153 DOI: 10.1089/hum.2017.255]

164 **Calvo-Garrido J**, Maffezzini C, Schober FA, Clemente P, Uhlin E, Kele M, Stranneheim H, Lesko N, Bruhn H, Svenningsson P, Falk A, Wedell A, Freyer C, Wredenberg A. SQSTM1/p62-Directed Metabolic Reprogramming Is Essential for Normal Neurodifferentiation. *Stem Cell Reports* 2019; **12**: 696-711 [PMID: 30827875 DOI: 10.1016/j.stemcr.2019.01.023]

165 **Dong Y**, Peng T, Wu W, Tan D, Liu X, Xie D. Efficient introduction of an isogenic homozygous mutation to induced pluripotent stem cells from a hereditary hearing loss family using CRISPR/Cas9 and single-stranded donor oligonucleotides. *J Int Med Res* 2019; **47**: 1717-1730 [PMID: 30819013 DOI: 10.1177/0300060519829990]

166 **Sabol RA**, Bunnell BA. Discussion: CRISPR/Cas9-Mediated BRCA1 Knockdown Adipose Stem Cells Promote Breast Cancer Progression. *Plast Reconstr Surg* 2019; **143**: 757-758 [PMID: 30817647 DOI: 10.1097/PRS.0000000000005391]

167 **Yanagihara Y**, Inoue K, Saeki N, Sawada Y, Yoshida S, Lee J, Iimura T, Imai Y. Zscan10 suppresses osteoclast differentiation by regulating expression of Haptoglobin. *Bone* 2019; **122**: 93-100 [PMID: 30771488 DOI: 10.1016/j.bone.2019.02.011]

168 **van de Vrugt HJ**, Harmsen T, Riepsaame J, Alexantya G, van Mil SE, de Vries Y, Bin Ali R, Huijbers IJ, Dorsman JC, Wolthuis RMF, Te Riele H. Effective CRISPR/Cas9-mediated correction of a Fanconi anemia defect by error-prone end joining or templated repair. *Sci Rep* 2019; **9**: 768 [PMID: 30683899 DOI: 10.1038/s41598-018-36506-w]

169 **Blanas A**, Cornelissen LAM, Kotsias M, van der Horst JC, van de Vrugt HJ, Kalay H, Spencer DIR, Kozak RP, van Vliet SJ. Transcriptional activation of fucosyltransferase (FUT) genes using the CRISPR-dCas9-VPR technology reveals potent N-glycome alterations in colorectal cancer cells. *Glycobiology* 2019; **29**: 137-150 [PMID: 30476078 DOI: 10.1093/glycob/cwy096]

170 **Sun T**, Patil R, Galstyan A, Klymyshyn D, Ding H, Chesnokova A, Cavenee WK, Furnari FB, Ljubimov VA, Shatalova ES, Wagner S, Li D, Mamelak AN, Bannykh SI, Patil CG, Rudnick JD, Hu J, Grodzinski ZB, Rekechenetskiy A, Falahatian V, Lyubimov AV, Chen YL, Leoh LS, Daniels-Wells TR, Penichet ML, Holler E, Ljubimov AV, Black KL, Ljubimova JY. Blockade of a Laminin-411-Notch Axis with CRISPR/Cas9 or a Nanobioconjugate Inhibits Glioblastoma Growth through Tumor-Microenvironment

Cross-talk. *Cancer Res* 2019; **79**: 1239-1251 [PMID: 30659021 DOI: 10.1158/0008-5472.CAN-18-2725]
171 **Jelinkova S**, Fojtik P, Kohutova A, Vilotic A, Marková L, Pesl M, Jurakova T, Kruta M, Vrbsky J, Gaillyova R, Valášková I, Frák I, Lacampagne A, Forte G, Dvorak P, Meli AC, Rotrekl V. Dystrophin Deficiency Leads to Genomic Instability in Human Pluripotent Stem Cells via NO Synthase-Induced Oxidative Stress. *Cells* 2019; 8 [PMID: 30650618 DOI: 10.3390/cells8010053]
172 **Hurtado Del Pozo C**, Garreta E, Izpisúa Belmonte JC, Montserrat N. Modeling epigenetic modifications in renal development and disease with organoids and genome editing. *Dis Model Mech* 2018; 11 [PMID: 30459215 DOI: 10.1242/dmm.035048]
173 **Tang L**, Yao F, Wang H, Wang X, Shen J, Dai B, Wu H, Zhou D, Guo F, Wang J, Li T, Wang H, Gong T, Su J, Wang L, Liang P. Inhibition of TRPC1 prevents cardiac hypertrophy via NF-κB signaling pathway in human pluripotent stem cell-derived cardiomyocytes. *J Mol Cell Cardiol* 2019; **126**: 143-154 [PMID: 30423318 DOI: 10.1016/j.yjmcc.2018.10.020]
174 **Tian L**, Ye Z, Kafka K, Stewart D, Anders R, Schwarz KB, Jang YY. Biliary Atresia Relevant Human Induced Pluripotent Stem Cells Recapitulate Key Disease Features in a Dish. *J Pediatr Gastroenterol Nutr* 2019; **68**: 56-63 [PMID: 30358741 DOI: 10.1097/MPG.0000000000002187]
175 **Wang S**, Liu Z, Ye Y, Li B, Liu T, Zhang W, Liu GH, Zhang YA, Qu J, Xu D, Chen Z. Ectopic hTERT expression facilitates reprograming of fibroblasts derived from patients with Werner syndrome as a WS cellular model. *Cell Death Dis* 2018; **9**: 923 [PMID: 30206203 DOI: 10.1038/s41419-018-0948-4]
176 **Barnes J**, Salas F, Mokhtari R, Dolstra H, Pedrosa E, Lachman HM. Modeling the neuropsychiatric manifestations of Lowe syndrome using induced pluripotent stem cells: defective F-actin polymerization and WAVE-1 expression in neuronal cells. *Mol Autism* 2018; **9**: 44 [PMID: 30147856 DOI: 10.1186/s13229-018-0227-3]
177 **Frasier CR**, Zhang H, Offord J, Dang LT, Auerbach DS, Shi H, Chen C, Goldman AM, Eckhardt LL, Bezzerides VJ, Parent JM, Isom LL. Channelopathy as a SUDEP Biomarker in Dravet Syndrome Patient-Derived Cardiac Myocytes. *Stem Cell Reports* 2018; **11**: 626-634 [PMID: 30146492 DOI: 10.1016/j.stemcr.2018.07.012]
178 **Sasaki-Honda M**, Jonouchi T, Arai M, Hotta A, Mitsuhashi S, Nishino I, Matsuda R, Sakurai H. A patient-derived iPSC model revealed oxidative stress increases facioscapulohumeral muscular dystrophy-causative DUX4. *Hum Mol Genet* 2018; **27**: 4024-4035 [PMID: 30107443 DOI: 10.1093/hmg/ddy293]
179 **Wang H**, Guo R, Du Z, Bai L, Li L, Cui J, Li W, Hoffman AR, Hu JF. Epigenetic Targeting of Granulin in Hepatoma Cells by Synthetic CRISPR dCas9 Epi-suppressors. *Mol Ther Nucleic Acids* 2018; **11**: 23-33 [PMID: 29858058 DOI: 10.1016/j.omtn.2018.01.002]
180 **Moghaddas F**, Zeng P, Zhang Y, Schützle H, Brenner S, Hofmann SR, Berner R, Zhao Y, Lu B, Chen X, Zhang L, Cheng S, Winkler S, Lehmberg K, Canna SW, Czabotar PE, Wicks IP, De Nardo D, Hedrich CM, Zeng H, Masters SL. Autoinflammatory mutation in NLRC4 reveals a leucine-rich repeat (LRR)-LRR oligomerization interface. *J Allergy Clin Immunol* 2018; **142**: 1956-1967.e6 [PMID: 29778503 DOI: 10.1016/j.jaci.2018.04.033]
181 **Liu C**, Banister CE, Weige CC, Altomare D, Richardson JH, Contreras CM, Buckhaults PJ. PRDM1 silences stem cell-related genes and inhibits proliferation of human colon tumor organoids. *Proc Natl Acad Sci U S A* 2018; **115**: E5066-E5075 [PMID: 29760071 DOI: 10.1073/pnas.1802902115]
182 **Jiao J**, Tian W, Qiu P, Norton EL, Wang MM, Chen YE, Yang B. Induced pluripotent stem cells with NOTCH1 gene mutation show impaired differentiation into smooth muscle and endothelial cells: Implications for bicuspid aortic valve-related aortopathy. *J Thorac Cardiovasc Surg* 2018; **156**: 515-522.e1 [PMID: 29653750 DOI: 10.1016/j.jtcvs.2018.02.087]
183 **Lyu C**, Shen J, Wang R, Gu H, Zhang J, Xue F, Liu X, Liu W, Fu R, Zhang L, Li H, Zhang X, Cheng T, Yang R, Zhang L. Targeted genome engineering in human induced pluripotent stem cells from patients with hemophilia B using the CRISPR-Cas9 system. *Stem Cell Res Ther* 2018; **9**: 92 [PMID: 29625575 DOI: 10.1186/s13287-018-0839-8]
184 **Deng WL**, Gao ML, Lei XL, Lv JN, Zhao H, He KW, Xia XX, Li LY, Chen YC, Li YP, Pan D, Xue T, Jin ZB. Gene Correction Reverses Ciliopathy and Photoreceptor Loss in iPSC-Derived Retinal Organoids from Retinitis Pigmentosa Patients. *Stem Cell Reports* 2018; **10**: 2005 [PMID: 29874627 DOI: 10.1016/j.stemcr.2018.05.012]
185 **Wattanapanitch M**, Damkham N, Potirat P, Trakarnsanga K, Janan M, U-Pratya Y, Kheolamai P, Klincumhom N, Issaragrisil S. One-step genetic correction of hemoglobin E/beta-thalassemia patient-derived iPSCs by the CRISPR/Cas9 system. *Stem Cell Res Ther* 2018; **9**: 46 [PMID: 29482624 DOI: 10.1186/s13287-018-0779-3]
186 **Suda Y**, Kuzumaki N, Sone T, Narita M, Tanaka K, Hamada Y, Iwasawa C, Shibasaki M, Maekawa A, Matsuo M, Akamatsu W, Hattori N, Okano H, Narita M. Down-regulation of ghrelin receptors on dopaminergic neurons in the substantia nigra contributes to Parkinson's disease-like motor dysfunction. *Mol Brain* 2018; **11**: 6 [PMID: 29458391 DOI: 10.1186/s13041-018-0349-8]

World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2019 November 26; 11(11): 957-967

DOI: 10.4252/wjsc.v11.i11.957

ISSN 1948-0210 (online)

REVIEW

Cytokine interplay among the diseased retina, inflammatory cells and mesenchymal stem cells - a clue to stem cell-based therapy

Vladimir Holan, Barbora Hermankova, Magdalena Krulova, Alena Zajicova

ORCID number: Vladimir Holan (0000-0002-2011-3390); Barbora Hermankova (0000-0002-8236-0012); Magdalena Krulova (0000-0003-3622-376X); Alena Zajicova (0000-0002-6348-2436).

Author contributions: All authors reviewed the literature, contributed to writing, and approved the manuscript.

Supported by the Grant Agency of the Czech Republic, No. 17-04800S, No. 18-04393S, No. 19-02290S; the Ministry of Education, Youth and Sports of the Czech Republic, No. SVV 244-260435, No. NPU-I: LO1508, No. NPU-I: LO1309.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Manuscript source: Unsolicited manuscript

Received: May 25, 2019
Peer-review started: May 27, 2019
First decision: July 31, 2019

Vladimir Holan, Barbora Hermankova, Magdalena Krulova, Alena Zajicova, Department of Transplantation Immunology, Institute of Experimental Medicine of the Czech Academy of Sciences, Prague 14220, Czech Republic

Vladimir Holan, Barbora Hermankova, Magdalena Krulova, Department of Cell Biology, Faculty of Science, Charles University, Prague 12843, Czech Republic

Corresponding author: Vladimir Holan, PhD, Professor, Department of Transplantation Immunology, Institute of Experimental Medicine of the Czech Academy of Sciences, Videnska 1083, Prague 14220, Czech Republic. vladimir.holan@iem.cas.cz
Telephone: +420-241063226

Abstract

Retinal degenerative disorders, such as diabetic retinopathy, retinitis pigmentosa, age-related macular degeneration or glaucoma, represent the most common causes of loss of vision and blindness. In spite of intensive research, treatment options to prevent, stop or cure these diseases are limited. Newer therapeutic approaches are offered by stem cell-based therapy. To date, various types of stem cells have been evaluated in a range of models. Among them, mesenchymal stem/stromal cells (MSCs) derived from bone marrow or adipose tissue and used as autologous cells have been proposed to have the potential to attenuate the negative manifestations of retinal diseases. MSCs delivered to the vicinity of the diseased retina can exert local anti-inflammatory and repair-promoting/regenerative effects on retinal cells. However, MSCs also produce numerous factors that could have negative impacts on retinal regeneration. The secretory activity of MSCs is strongly influenced by the cytokine environment. Therefore, the interactions among the molecules produced by the diseased retina, cytokines secreted by inflammatory cells and factors produced by MSCs will decide the development and propagation of retinal diseases. Here we discuss the interactions among cytokines and other factors in the environment of the diseased retina treated by MSCs, and we present results supporting immunoregulatory and trophic roles of molecules secreted in the vicinity of the retina during MSC-based therapy.

Key words: Retina; Degenerative diseases; Stem cell therapy; Mesenchymal stem cells; Cytokines; Growth factors

Revised: September 2, 2019
Accepted: September 13, 2019
Article in press: September 13, 2019
Published online: November 26, 2019

P-Reviewer: Bonartsev AP, Hassan AI, Cheng TH
S-Editor: Dou Y
L-Editor: Filipodia
E-Editor: Xing YX

Core tip: Cell-based therapy using autologous mesenchymal stem cells represents a perspective approach for the treatment of so far incurable degenerative retinal diseases. However, the therapeutic potential of mesenchymal stem cells strongly depends on the cytokine environment, where these cells are delivered. In this study, we discuss recent knowledge regarding the interactions and interplay among cytokines produced by cells of the diseased retina, inflammatory immune cells and therapeutically administered mesenchymal stem cells. We suggest that these interactions among cytokines and growth factors occurring in the microenvironment of the diseased retina could be critical for the outcome of the stem cell-based therapy for retinal disorders.

Citation: Holan V, Hermankova B, Krulova M, Zajicova A. Cytokine interplay among the diseased retina, inflammatory cells and mesenchymal stem cells - a clue to stem cell-based therapy. *World J Stem Cells* 2019; 11(11): 957-967
URL: https://www.wjgnet.com/1948-0210/full/v11/i11/957.htm
DOI: https://dx.doi.org/10.4252/wjsc.v11.i11.957

RETINAL DEGENERATIVE DISORDERS

The retina is a highly specialized tissue. This structure is composed of several layers of functionally different cell types that are inter-connected. Disease or a damage to any particular cell or layer has secondary effects on the surrounding cell types, and the progression of retinal damage results in retinal degenerative disorders. Inherited and age-related retinal degenerative disorders represent the most common cause of reduced vision and blindness. Among the most common retinal degenerative diseases are age-related macular degeneration, diabetic retinopathy, retinitis pigmentosa and glaucoma. These disorders have different etiology, various causes and starting mechanisms, and distinct retinal cell types are affected. However, they are all associated with chronic inflammation, immune cell infiltration and enhanced cytokine secretion.

Because all retinal degenerative diseases in the advanced stages are associated with a loss or a function damage of specialized retinal cells, their replacement or a support of the surviving cells would be the only effective approach to stop spreading of the disease or even return the visual function of the retina. Currently, a direct transplantation of healthy retinal explants is strongly limited. Therefore, the transfer of stem cells that can support survival and functioning of the remaining retinal cells, or even replace missing cells, offers a perspective approach to stop and treat retinal degenerative diseases. Recent experimental and preclinical data suggest a great potential of such cell therapies for the treatment of so far incurable ophthalmological diseases.

TYPES OF STEM CELLS FOR RETINA REGENERATION

Stem cells are characterized by their permanent growth *in vitro* and by their ability to differentiate or even transdifferentiate into other cell types. According to their origin, stem cells can be divided into embryonic stem cells and adult stem cells. The third type of stem cells is artificially prepared from any somatic cell by reprogramming its properties to a pluripotent state. For this intervention, genes associated with stemness are introduced into somatic cells, which gain some characteristics of stem cells. These cells, called induced pluripotent stem cells, have attracted a lot of attention as a possible source of autologous stem cells that could avoid immune rejection. However, these genetically modified cells have proved to be immunogenic even in an autologous host[1], and their often uncontrolled growth and the formation of teratomas limit their clinical potential. Similarly, the use of embryonic stem cells, which have a high differentiation potential and can be relatively easily differentiated into numerous different cell types, have ethical limitations associated with their origin. They are always used as allogeneic cells, and they often suffer from uncontrolled growth. In comparison with embryonic stem cells or induced pluripotent stem cells, adult stem cells have a lesser differentiation potential but can be obtained as autologous (patient's own) cells, do not form teratomas or cancers and often fulfil the demands for use in regenerative medicine. Among the numerous types of adult stem cells, the

highest potential have been proposed from mesenchymal stem/stromal cells (MSCs), which can be obtained relatively easily from the patient, propagated *in vitro*, if needed differentiated *ex vivo* and finally used as autologous therapeutic cells.

Numerous experimental studies have shown the beneficial effects of MSCs in the treatment of ophthalmological diseases. Intravitreal or subretinal transplantation of MSCs significantly delayed retinal degeneration and supported normal retinal functions[2-4]. In addition, in other types of eye diseases, such as experimental autoimmune uveitis[5,6], dry eye syndrome[7] or corneal epithelium damage[8,9], the therapeutic effects of MSCs on tissue regeneration have been demonstrated. The ability of MSCs to support interaction between retinal cells with neurons of optic nerve has been also documented. For example, Mead *et al*[10] showed that dental pulp stem cells promoted neuroprotection and axon regeneration after optic nerve injury. In other models, MSCs transplanted to the damaged area of the retina differentiated into retinal nerve cells[11] and promoted regeneration in a rat optic tract model[12].

MSCs

MSCs represent a heterogenous population of non-hematopoietic cells with multi-lineage differentiation potential. Originally, these cells were described as spindle shaped cells derived from bone marrow that adhere to plastic and form fibrocyte-like colonies[13]. For therapeutic purposes, MSCs are isolated mainly from the bone marrow or adipose tissue, but they can be obtained from nearly all tissues of the body. According to the International Society for Cellular Therapy, human MSCs are characterized by their ability to adhere to plastic in standard culture conditions, by their potential to differentiate into adipocytes, chondroblasts and osteoblasts, and by being positive for the surface markers CD105, CD73 and CD90 and negative for CD45, CD34, CD14, CD19 and CD11b[14]. MSCs from different sources (including bone marrow, adipose tissue, umbilical cord blood, *etc*) possess similar properties[15,16]. Under appropriate conditions, MSCs can be differentiated or even transdifferentiated into different cell types. It has been demonstrated that in the presence of selective chemicals or retinal cells, MSCs can differentiate into cells expressing retinal cell markers and characteristics[17-19]. We have shown that highly purified mouse bone marrow-derived cells fulfilling all the criteria proposed for MSCs and cultured in the presence of retinal cell extract and supernatant from activated T cells (to mimic the inflammatory environment of diseased retina) differentiated into cells expressing rhodopsin, S-antigen, recoverin, retinaldehyde binding protein, calbindin and retinal pigment epithelium (RPE) 65, which are the markers of specialized retinal cells[20]. The ability of MSCs to extensively proliferate, to be expanded *in vitro* and to differentiate into various cell types makes them attractive targets for regenerative and reparative medical applications. However, before clinical application these cells have to be precisely characterized and their preparation standardized, as it has been recently proposed[21].

The therapeutic effects of MSCs are mediated by multiple mechanisms, as demonstrated in Figure 1. Among them, the immunomodulatory and secretory properties appear to be the most important. It has been shown that MSCs inhibit T and B cell functions, attenuate production of cytokines, decrease activity of cytotoxic T and NK cells and suppress transplantation, anti-cancer and inflammatory reactions[22,23]. On the other hand, MSCs are potent producers of numerous cytokines and growth factors[24,25]. It has been shown that mouse MSCs spontaneously produce transforming growth factor (TGF-β) and inducible interleukin-6 (IL-6), which are the basic cytokines regulating the development of anti-inflammatory regulatory T cells (Tregs) and pro-inflammatory Th17 cells[25]. The immunomodulatory properties make MSCs a promising tool for the treatment of harmful inflammatory reactions that accompany inherited retinal degenerative diseases and injuries. However, the spectrum and concentrations of immunoregulatory molecules produced by MSCs strongly depend on the cytokine environment[26,27]. This has to be taken into account when MSCs are delivered into the inflammatory environment of the diseased retina.

PRODUCTION OF CYTOKINES BY RETINAL CELLS

The retina is composed of a few layers of functionally different cell types that ensure visual acuity and internal homeostasis. The cells of the retina produce numerous cytokines and growth factors that support, in a paracrine mode, the survival of other retinal cells and contribute to the immune privilege of the eye[28]. However, from the very beginning of retinal disease or damage, the spectrum of produced cytokines is

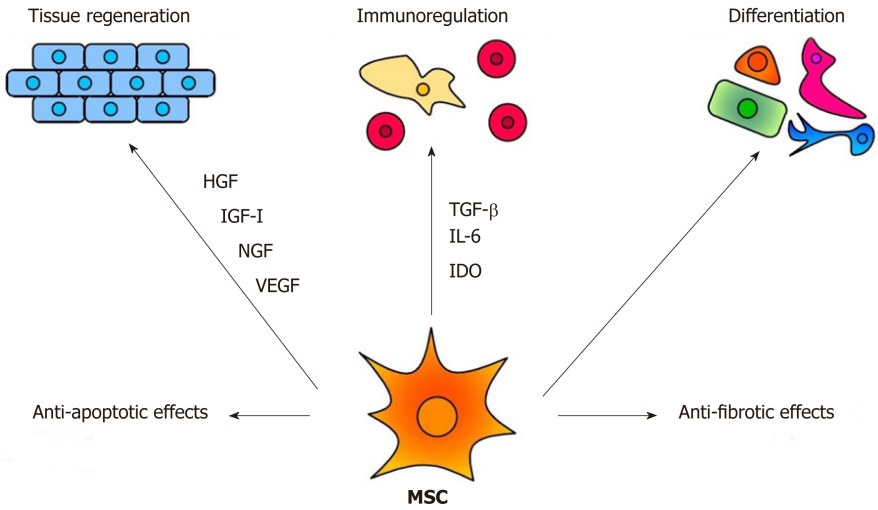

Figure 1 The main mechanisms of the therapeutic effect of MSCs. MSC: Mesenchymal stem cell; HGF: Hepatocyte growth factor; IGF-1: Insulin-like growth factor-1; NGF: Nerve growth factor; VEGF: Vascular endothelial growth factor; TGF-β: Transforming growth factor-β; IDO: Indoleamine-2,3-dioxygenase; IL-6: Interleukin-6.

significantly changed, and the retina starts to produce elevated levels of molecules that are not produced or only minimally secreted in a steady-state.

It has been shown that increased levels of pro-inflammatory molecules, such as tumor necrosis factor (TNF-α), IL-6 or inducible nitric oxide synthase (iNOS), are found in the retina or aqueous humor of patients with degenerative retinal diseases or in animal models of retinal disorders[29-32]. All of these molecules have a wider spectrum of immunoregulatory activities but generally contribute to the development of a local inflammatory reaction. On the contrary, IL-10, IL-11 or TGF-β, which are also produced by retinal cells[32,33], have anti-inflammatory effects. Therefore, the balance between the production of pro- and anti-inflammatory molecules influences the extent of the inflammation and damage in the diseased retina.

The complexity of the action of individual cytokines is supported by the observation that another pro-inflammatory cytokine, IL-6, that is produced by several retinal cell types, increases the survival of retinal ganglion cells[34]. Eastlake *et al*[35] have shown that Müller glia cells produce numerous factors, such as granulocyte-growth factor, monocyte chemoattractant protein-1, platelet-derived growth factor-BB, vascular endothelial growth factor (VEGF) or TGF-β2 and that their production is increased in the gliotic retina. Recently, IL-33 produced by Müller cells of the retina was identified as a key regulator of inflammation and photoreceptor degeneration after retinal stress injury[36]. Thus, retinal microglia and RPE cells were proposed as the main sources of the majority of factors with immunomodulatory effects that play a pivotal role in the initiation and propagation of the neurodegenerative processes[37-39]. In addition to their high secretory potential, Müller glia were recently shown to be able to directly restore vision after *de novo* induction of genesis of rod photoreceptors in mammalian retinas[40]. It has been shown that cells of individual parts of the eye, such as the cornea, ciliary body or retina are able to inhibit the intraocular immune response and to contribute to the immune privilege of the eye[41]. In this respect, we observed that the explants of the mouse retina or the supernatants from the cultures of retinal explants inhibited the production of pro-inflammatory cytokines by activated spleen cells (unpublished results).

In addition to the production of immunoregulatory cytokines, such as TGF-β or IL-6, cells of the retina produce a number of molecules that function in the paracrine mode as growth and trophic factors. Brain-derived neurotrophic factor, ciliary neurotrophic factor, glial cell line-derived neurotrophic factor, nerve growth factor, neurotrophin-3 and basic fibroblast growth factor released by microglia and other retinal cell types have been shown to protect retinal cells and support the survival of photoreceptors[42]. On the contrary, pro-angiogenic factors, such as VEGF A-E, insulin-like growth factor-I (IGF-I), platelet-derived growth factor, placental growth factor, hepatocyte growth factor (HGF) and FGF-2, which can be produced by the retina and act paracrinely, are involved in neovascularization and rather worsen retinal diseases[43,44]. In the opposite way, the RPE cells produce retinal pigment epithelium-derived factor and trombospondin-1 which have been shown to counterbalance angiogenesis and inflammation[45].

Thus, the retina is a producer of numerous cytokines and factors, which act in a

protective way, *i.e.* they inhibit the inflammatory reaction and support the survival and the growth of retinal cells. However, there are a number of cytokines and factors that attract pro-inflammatory cells, enhance inflammation, increase neovascularization and contribute to the damage of individual retinal layers. The identification of these factors and understanding of the mechanisms of the interplay among them will increase the efficacy of stem cell therapy for retinal degenerative disorders.

CYTOKINES PRODUCED BY INFLAMMATORY CELLS IN THE DISEASED RETINA

All retinal degenerative diseases are accompanied by a local cytokine disbalance, by increased production of chemokines and by infiltration with inflammatory cells. One of the first inflammatory cell populations detected in the diseased retina is macrophages and neutrophils. The accumulation of macrophages can be associated with elevated levels of vitreal granulocyte-macrophage colony-stimulating factor[46]. Macrophages are producers of numerous cytokines and factors, and their secretory profile depends on their polarization into M1 or M2 population. With the progression of the disease, increased concentrations of pro-inflammatory cytokines and chemokines, such as IL-1, IL-6, IL-8, TNF-α, interferon (IFN)-γ and monocyte chemoattractant protein-1 can be detected in vitreal liquid[47]. The deleterious role of these cytokines for the development and spreading of the disease was directly proved by the observation that intravitreal administration of IL-1β and TNF-α in mice induced vessel dilatation, bleading, retinal edema and microglia upregulation[48]. Kutty *et al*[49] showed that RPE cells exposed to IL-1, TNF-α and IFN-γ, which are secreted by lymphocytes or macrophages in the retina, decreased the expression of key genes involved in the visual cycle, epithelial morphology and phagocytosis. We observed that intravitreal administration of pro-inflammatory cytokines (such as IL-1α, TNF-α and IFN-γ) induced in the mouse retina an enhanced expression of genes for a large number of cytokines and pro-inflammatory molecules (Figure 2). With the progression of the retinal disease, the infiltration with cells of adaptive immunity can be detected[49,50]. Johnsen-Soriano *et al*[52] showed cytokine disbalance and significantly increased levels of Th1 cytokines IL-2 and IFN-γ and NO in the retina of diabetic rats. These pro-inflammatory molecules could contribute to the development of diabetic retinopathy. This concept is supported by the observation that transgenic mice expressing IFN-γ in the retina develop ocular inflammation and photoreceptor loss[53].

Thus, cytokines produced by cells infiltrating the diseased retina contribute to the development and spreading of retinal disease. They have additive and synergistic effects with cytokines produced by the diseased retina, and these interactions can further deteriorate or attenuate the disease progression.

CYTOKINES PRODUCED BY MSCS

MSC-based therapy has been proposed and tested as a prospective treatment in various models of retinal degenerative diseases[2,3,54-56]. MSCs mediate their therapeutic effect by multiple mechanisms involving immunomodulation, production of trophic factors and a possible differentiation into other cell types. The paracrine trophic effects of molecules secreted by MSCs have recently been suggested as the primary mechanisms of MSC action. Some of these immunomodulatory and trophic factors are produced by MSCs spontaneously (such as HGF or CCL2), while others are secreted only after stimulation (for example IL-6 or LIF) or are secreted in lower quantities spontaneously and in increased levels after stimulation (VEGF or IGF-I) (Figure 3).

MSCs are well known as cells with a potent immunosuppressive potential. It has been demonstrated that MSCs inhibit the secretion of cytokines and have the ability to suppress transplantation, anti-cancer or inflammatory reactions[23,57]. Mathew *et al*[58] showed that intravitreal administration of MSCs decreased the intraocular level of inflammatory molecules TNF-α, IL-1β and IL-6 and rescued the retina from ischemic damage. We have shown that MSCs transferred into the damaged ocular surface significantly decreased infiltration with T lymphocytes and attenuated a local inflammatory reaction[8,59]. Furthermore, we observed that the cultivation of retinal explants with pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ induced enhanced expression of genes for numerous cytokines and that this enhanced gene expression was attenuated in the presence of MSCs (Figure 4). The suppressive activity of MSCs could be profitable for the therapeutic inhibition of the inflammatory reaction in the diseased retina. Although different mechanisms can be responsible for the

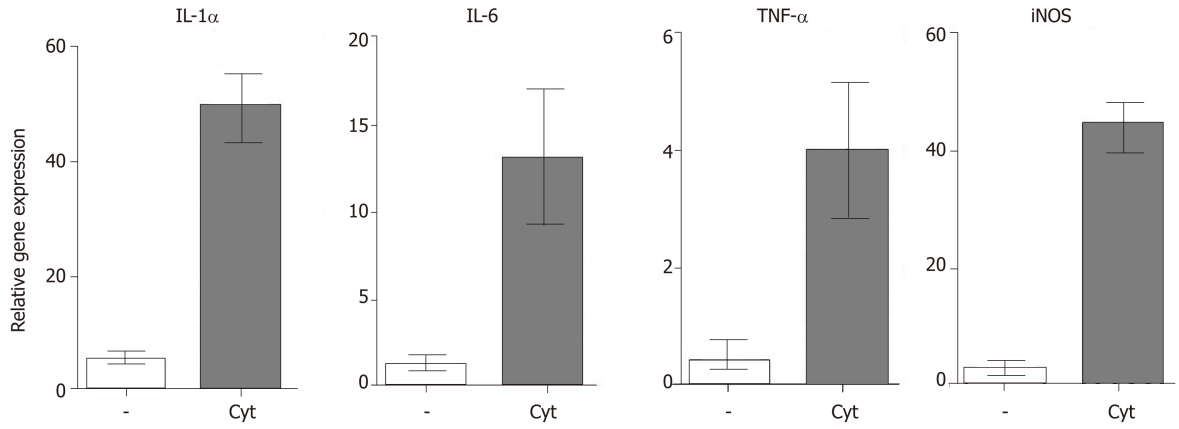

Figure 2 Expression of genes for immunoregulatory molecules and growth factors in the retina after intravitreal injection of pro-inflammatory cytokines. Mice were injected intravitreally with pro-inflammatory cytokines IL-lα, IFN-γ and TNF-α (10 ng of each, in a total volume of 4 μL), and the retinas from the control and treated eyes were harvested after 72 h. The expression of genes for IL-lα, IL-6, TNF-α and iNOS was determined in the retinas by real-time PCR.

suppression, one of the most important effects could be the spontaneous production of TGF-β by MSCs. TGF-β is a strong negative regulator of immune reactions, and it is the main cytokine determining the development of Tregs which can indirectly contribute to immunosuppression mediated by MSCs[60]. However, in the presence of pro-inflammatory cytokines, MSCs also produce IL-6, which is a pro-inflammatory cytokine and attenuates the development of Tregs. This plasticity of MSCs should be kept in the mind when MSCs are delivered into the inflamed site with the aim to attenuate the inflammation.

In addition to the production of immunoregulatory cytokines, MSCs are also potent producers of numerous growth and trophic factors, such as HGF, LIF, VEGF, IGF-I, nerve growth factor, brain-derived neurotrophic factor, CDTF, glial cell line-derived neurotrophic factor or platelet-derived growth factor[56,61,62]. After the application of MSCs into the eye, the factors produced by MSCs support the survival and viability of various types of retinal cells[2,3]. Ezquer *et al*[63] showed that intravitreal injection of MSCs in diabetic mice increased intraocular levels of nerve growth factor, basic fibroblast growth factor and glial cell line-derived neurotrophic factor and triggered an effective cytoprotective microenvironment. The factors produced by MSCs have a wider spectrum of trophic effects. For example, it has been shown that HGF supports the growth and differentiation of numerous cell types[64], LIF is a highly pleiotropic factor promoting cell differentiation and tissue growth[65], and IGF-I is also a pleiotropic factor with multiple effects on cell differentiation, survival and growth[66].

The angiogenic effects of VEGF are also well known[67]. These molecules and numerous other growth and trophic factors produced by MSCs can contribute to the regeneration of the diseased retina. However, some of these factors have both positive and negative impacts on the diseased retinal tissue. For example, VEGF supports tissue healing by stimulating the formation of blood vessels, but inadequate neovascularization can contribute to the development of some types of retinal degeneration[67]. Thus, local concentrations of particular factors decide their effects, and the stage and extent of the disease are very important for the formation of the cytokine microenvironment and for therapeutic possibilities.

CONCLUSION

With the persistent absence of treatment protocols for retinal degenerative diseases, stem cell-based therapy offers a promising approach. Among the various stem cell types, MSCs provide several advantages. They can be prepared in a sufficient number as autologous stem cells and administered repeatedly, and there is no danger of uncontrolled cell growth or a formation of teratomas. Numerous experimental therapeutic protocols and clinical studies using intravitreal administration of MSCs have demonstrated the safety of this therapy without any harmful side effects[68,69].

Retinal degenerative diseases are associated with local inflammation, cytokine disbalance and a loss of specialized retinal cells, and MSCs can display therapeutic effects for all of these dysfunctions by multiple mechanisms. They are the producers of numerous growth and trophic factors that support the survival and growth of the remaining retinal cells. They also possess potent immunosuppressive properties to

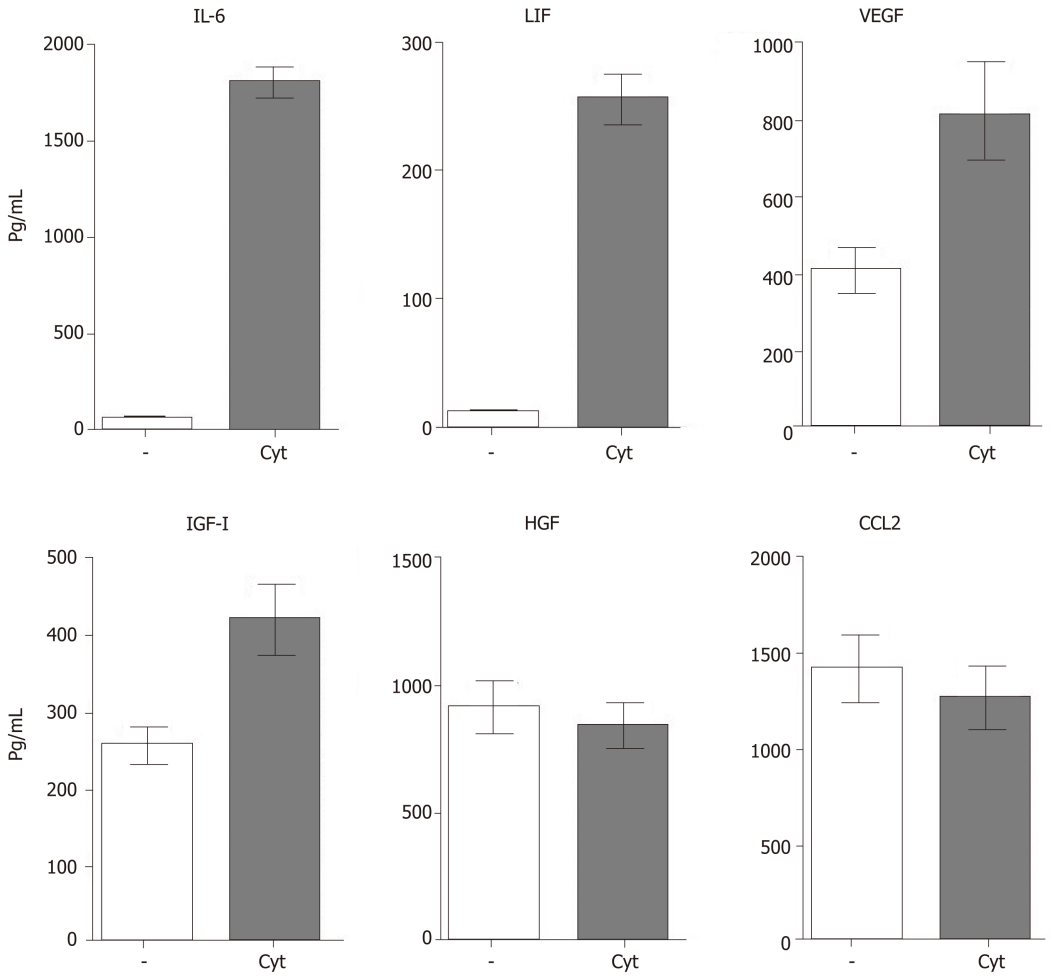

Figure 3 Spontaneous and cytokine induced production of immunoregulatory cytokines and growth factors by mouse mesenchymal stem cells. Mouse mesenchymal stem cells (8×10^4 cells in 1 mL of culture medium) were cultured for 48 h unstimulated (-) or in the presence of 10 ng/mL of IL-1β and TNF-α (Cyt.) The concentrations of IL-6, LIF, VEGF, IGF-I, HGF and CCL2 in supernatants were determined by ELISA. IL-6: Interleukin-6; LIF: Leukemia inhibitory factor; VEGF: Vascular endothelial growth factor; IGF-I: Insulin-like growth factor-I; HGF: hepatocyte growth factor.

attenuate inflammatory reactions and have the ability to differentiate into many other cell types. It has been shown that in the presence of retinal cells, supernatant from cultures of retinal cells or in the presence of retinal cell extracts MSCs differentiate into cells expressing genes and markers typical for retinal cells[18,20,70].

While there is abundant data from various experimental models that demonstrate the positive effects of intraocularly administered MSCs on retinal healing, the results still have to be taken with a precaution. In some studies, human MSCs were injected intravitreally in rodents and their biocompatibility and positive therapeutic effects were described[71-73]. However, a recent study by Lohan *et al*[74] demonstrated that human MSCs administered into immunocompetent rats do not have the same therapeutic effects as rat MSCs and that human MSCs do not suppress the proliferation of rat T lymphocytes. Moreover, human MSCs are not activated by rat pro-inflammatory cytokines[74]. These interspecies incompatibilities have to be taken into account when the results from preclinical animal studies utilizing human MSCs are considered in the context of translation to clinical trials.

Another issue that deserves attention is the secretory profile of MSCs after their delivery into the inflammatory environment of the diseased retina. Such a secretory profile could be quite distinct from that in MSCs during their cultivation *in vitro*. We have shown in a mouse model that highly purified MSCs spontaneously produce significant levels of TGF-β but do not produce IL-6. However, in the presence of pro-inflammatory cytokines, such as IL-1β, TNF-α or IFN-γ, MSCs simultaneously secrete a significant amount of IL-6[25]. IL-6 is a pro-inflammatory cytokine, which together with TGF-β determine the development of the highly pro-inflammatory Th17 cells. A crucial role in the shift between inhibitory Tregs and pro-inflammatory Th17 cells is played by the ratio in concentrations of TGF-β and IL-6[25,75]. This plasticity in the secretory potential of MSCs represents limitations when cultured MSCs producing

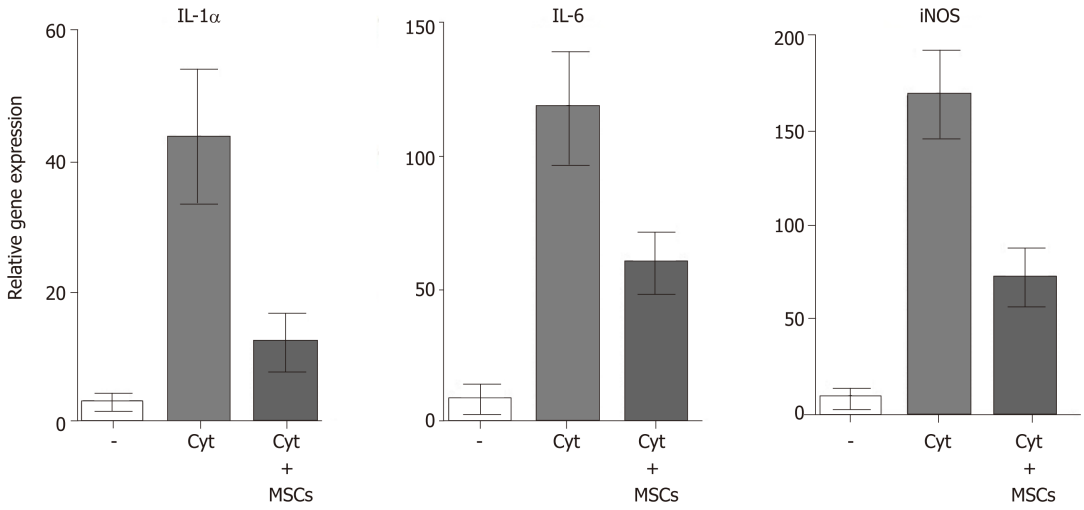

Figure 4 The expression of genes for pro-inflammatory molecules in stimulated retinal explants and suppression of the gene expression by MSCs. Retinal explants were cultured for 48 h untreated (-), stimulated with IL-Iα, IFN-γ and TNF-α (Cyt.) or stimulated with the cytokines in cultures containing MSCs (3×10^4 in 1 mL). The expression of genes for IL-Iα, IL-6 and iNOS in retinal explants was determined by real-time PCR. MSC: Mesenchymal stem cell; IL-6: Interleukin-6; IL-Iα: Interleukin-Iα; iNOS: Inducible nitrox oxide synthase; MSC: Mesenchymal stem cell.

TGF-β are transferred into the inflammatory environment of the diseased retina. To prevent activation of pro-inflammatory cells, MSCs could be co-administrated with antibody anti-IL-6 or anti-IL-6 receptor, as this has been tested in patients with kidney allografts[76,77]. It has been shown that blocking of the IL-6 pathway can attenuate inflammation, support the activation of Tregs and enhance allograft survival. It can be proposed that such interventions into cytokine pathways or the regulation of cytokine interactions can significantly improve the efficiency of stem cell-based therapy for retinal diseases.

While there is still a debate about the origin and characterization of MSCs[21] and many unknown interactions among cytokines and other growth and trophic factors remain to be recognized, stem cell-based therapy represents a great promise and hope for the patients with visual problems and for the treatment of so far incurable retinal degenerative diseases.

REFERENCES

1 **Zhao T**, Zhang ZN, Rong Z, Xu Y. Immunogenicity of induced pluripotent stem cells. *Nature* 2011; **474**: 212-215 [PMID: 21572395 DOI: 10.1038/nature10135]
2 **Arnhold S**, Absenger Y, Klein H, Addicks K, Schraermeyer U. Transplantation of bone marrow-derived mesenchymal stem cells rescue photoreceptor cells in the dystrophic retina of the rhodopsin knockout mouse. *Graefes Arch Clin Exp Ophthalmol* 2007; **245**: 414-422 [PMID: 16896916 DOI: 10.1007/s00417-006-0382-7]
3 **Inoue Y**, Iriyama A, Ueno S, Takahashi H, Kondo M, Tamaki Y, Araie M, Yanagi Y. Subretinal transplantation of bone marrow mesenchymal stem cells delays retinal degeneration in the RCS rat model of retinal degeneration. *Exp Eye Res* 2007; **85**: 234-241 [PMID: 17570362 DOI: 10.1016/j.exer.2007.04.007]
4 **Wang S**, Lu B, Girman S, Duan J, McFarland T, Zhang QS, Grompe M, Adamus G, Appukuttan B, Lund R. Non-invasive stem cell therapy in a rat model for retinal degeneration and vascular pathology. *PLoS One* 2010; **5**: e9200 [PMID: 20169166 DOI: 10.1371/journal.pone.0009200]
5 **Tasso R**, Ilengo C, Quarto R, Cancedda R, Caspi RR, Pennesi G. Mesenchymal stem cells induce functionally active T-regulatory lymphocytes in a paracrine fashion and ameliorate experimental autoimmune uveitis. *Invest Ophthalmol Vis Sci* 2012; **53**: 786-793 [PMID: 22232435 DOI: 10.1167/iovs.11-8211]
6 **Zhao PT**, Zhang LJ, Shao H, Bai LL, Yu B, Su C, Dong LJ, Liu X, Li XR, Zhang XM. Therapeutic effects of mesenchymal stem cells administered at later phase of recurrent experimental autoimmune uveitis. *Int J Ophthalmol* 2016; **9**: 1381-1389 [PMID: 27803852 DOI: 10.18240/ijo.2016.10.03]
7 **Lee MJ**, Ko AY, Ko JH, Lee HJ, Kim MK, Wee WR, Khwarg SI, Oh JY. Mesenchymal stem/stromal cells protect the ocular surface by suppressing inflammation in an experimental dry eye. *Mol Ther* 2015; **23**: 139-146 [PMID: 25152016 DOI: 10.1038/mt.2014.159]
8 **Holan V**, Trosan P, Cejka C, Javorkova E, Zajicova A, Hermankova B, Chudickova M, Cejkova J. A Comparative Study of the Therapeutic Potential of Mesenchymal Stem Cells and Limbal Epithelial Stem Cells for Ocular Surface Reconstruction. *Stem Cells Transl Med* 2015; **4**: 1052-1063 [PMID: 26185258 DOI: 10.5966/sctm.2015-0039]
9 **Mittal SK**, Omoto M, Amouzegar A, Sahu A, Rezazadeh A, Katikireddy KR, Shah DI, Sahu SK, Chauhan SK. Restoration of Corneal Transparency by Mesenchymal Stem Cells. *Stem Cell Reports* 2016; **7**: 583-

590 [PMID: 27693426 DOI: 10.1016/j.stemcr.2016.09.001]
10 **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA. Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. *Invest Ophthalmol Vis Sci* 2013; **54**: 7544-7556 [PMID: 24150755 DOI: 10.1167/iovs.13-13045]
11 **Tomita M**, Adachi Y, Yamada H, Takahashi K, Kiuchi K, Oyaizu H, Ikebukuro K, Kaneda H, Matsumura M, Ikehara S. Bone marrow-derived stem cells can differentiate into retinal cells in injured rat retina. *Stem Cells* 2002; **20**: 279-283 [PMID: 12110696 DOI: 10.1634/stemcells.20-4-279]
12 **Zwart I**, Hill AJ, Al-Allaf F, Shah M, Girdlestone J, Sanusi AB, Mehmet H, Navarrete R, Navarrete C, Jen LS. Umbilical cord blood mesenchymal stromal cells are neuroprotective and promote regeneration in a rat optic tract model. *Exp Neurol* 2009; **216**: 439-448 [PMID: 19320003 DOI: 10.1016/j.expneurol.2008.12.028]
13 **Friedenstein AJ**, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970; **3**: 393-403 [PMID: 5523063 DOI: 10.1111/j.1365-2184.1970.tb00347.x]
14 **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]
15 **Musina RA**, Bekchanova ES, Sukhikh GT. Comparison of mesenchymal stem cells obtained from different human tissues. *Bull Exp Biol Med* 2005; **139**: 504-509 [PMID: 16027890 DOI: 10.1007/s10517-005-0331-1]
16 **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]
17 **Kicic A**, Shen WY, Wilson AS, Constable IJ, Robertson T, Rakoczy PE. Differentiation of marrow stromal cells into photoreceptors in the rat eye. *J Neurosci* 2003; **23**: 7742-7749 [PMID: 12944502 DOI: 10.1523/JNEUROSCI.23-21-07742.2003]
18 **Huang C**, Zhang J, Ao M, Li Y, Zhang C, Xu Y, Li X, Wang W. Combination of retinal pigment epithelium cell-conditioned medium and photoreceptor outer segments stimulate mesenchymal stem cell differentiation toward a functional retinal pigment epithelium cell phenotype. *J Cell Biochem* 2012; **113**: 590-598 [PMID: 21948619 DOI: 10.1002/jcb.23383]
19 **Mathivanan I**, Trepp C, Brunold C, Baerlocher G, Enzmann V. Retinal differentiation of human bone marrow-derived stem cells by co-culture with retinal pigment epithelium. *in vitro Exp Cell Res* 2015; **333**: 11-20 [PMID: 25724900 DOI: 10.1016/j.yexcr.2015.02.001]
20 **Hermankova B**, Kossl J, Javorkova E, Bohacova P, Hajkova M, Zajicova A, Krulova M, Holan V. The Identification of Interferon-γ as a Key Supportive Factor for Retinal Differentiation of Murine Mesenchymal Stem Cells. *Stem Cells Dev* 2017; **26**: 1399-1408 [PMID: 28728472 DOI: 10.1089/scd.2017.0111]
21 **Sipp D**, Robey PG, Turner L. Clear up this stem-cell mess. *Nature* 2018; **561**: 455-457 [PMID: 30258150 DOI: 10.1038/d41586-018-06756-9]
22 **Le Blanc K**, Ringdén O. Immunomodulation by mesenchymal stem cells and clinical experience. *J Intern Med* 2007; **262**: 509-525 [PMID: 17949362 DOI: 10.1111/j.1365-2796.2007.01844.x]
23 **Abumaree M**, Al Jumah M, Pace RA, Kalionis B. Immunosuppressive properties of mesenchymal stem cells. *Stem Cell Rev Rep* 2012; **8**: 375-392 [PMID: 21892603 DOI: 10.1007/s12015-011-9312-0]
24 **Oh JY**, Kim MK, Shin MS, Wee WR, Lee JH. Cytokine secretion by human mesenchymal stem cells cocultured with damaged corneal epithelial cells. *Cytokine* 2009; **46**: 100-103 [PMID: 19223198 DOI: 10.1016/j.cyto.2008.12.011]
25 **Svobodova E**, Krulova M, Zajicova A, Pokorna K, Prochazkova J, Trosan P, Holan V. The role of mouse mesenchymal stem cells in differentiation of naive T-cells into anti-inflammatory regulatory T-cell or proinflammatory helper T-cell 17 population. *Stem Cells Dev* 2012; **21**: 901-910 [PMID: 21663543 DOI: 10.1089/scd.2011.0157]
26 **English K**, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett* 2007; **110**: 91-100 [PMID: 17507101 DOI: 10.1016/j.imlet.2007.04.001]
27 **Holan V**, Hermankova B, Bohacova P, Kossl J, Chudickova M, Hajkova M, Krulova M, Zajicova A, Javorkova E. Distinct Immunoregulatory Mechanisms in Mesenchymal Stem Cells: Role of the Cytokine Environment. *Stem Cell Rev Rep* 2016; **12**: 654-663 [PMID: 27665290 DOI: 10.1007/s12015-016-9688-y]
28 **Forrester JV**. Privilege revisited: an evaluation of the eye's defence mechanisms. *Eye (Lond)* 2009; **23**: 756-766 [PMID: 18989350 DOI: 10.1038/eye.2008.259]
29 **Chen KH**, Wu CC, Roy S, Lee SM, Liu JH. Increased interleukin-6 in aqueous humor of neovascular glaucoma. *Invest Ophthalmol Vis Sci* 1999; **40**: 2627-2632 [PMID: 10509659]
30 **Yuan L**, Neufeld AH. Tumor necrosis factor-alpha: a potentially neurodestructive cytokine produced by glia in the human glaucomatous optic nerve head. *Glia* 2000; **32**: 42-50 [PMID: 10975909 DOI: 10.1002/1098-1136(200010)32:1<42::AID-GLIA40>3.0.CO;2-3]
31 **Neufeld AH**, Kawai Si, Das S, Vora S, Gachie E, Connor JR, Manning PT. Loss of retinal ganglion cells following retinal ischemia: the role of inducible nitric oxide synthase. *Exp Eye Res* 2002; **75**: 521-528 [PMID: 12457864 DOI: 10.1006/exer.2002.2042]
32 **Chua J**, Vania M, Cheung CM, Ang M, Chee SP, Yang H, Li J, Wong TT. Expression profile of inflammatory cytokines in aqueous from glaucomatous eyes. *Mol Vis* 2012; **18**: 431-438 [PMID: 22355254]
33 **Nagineni CN**, Kommineni VK, William A, Hooks JJ, Detrick B. IL-11 expression in retinal and corneal cells is regulated by interferon-gamma. *Biochem Biophys Res Commun* 2010; **391**: 287-292 [PMID: 19913506 DOI: 10.1016/j.bbrc.2009.11.051]
34 **Sappington RM**, Chan M, Calkins DJ. Interleukin-6 protects retinal ganglion cells from pressure-induced death. *Invest Ophthalmol Vis Sci* 2006; **47**: 2932-2942 [PMID: 16799036 DOI: 10.1167/iovs.05-1407]
35 **Eastlake K**, Banerjee PJ, Angbohang A, Charteris DG, Khaw PT, Limb GA. Müller glia as an important source of cytokines and inflammatory factors present in the gliotic retina during proliferative vitreoretinopathy. *Glia* 2016; **64**: 495-506 [PMID: 26556395 DOI: 10.1002/glia.22942]
36 **Xi H**, Katschke KJ, Li Y, Truong T, Lee WP, Diehl L, Rangell L, Tao J, Arceo R, Eastham-Anderson J, Hackney JA, Iglesias A, Cote-Sierra J, Elstrott J, Weimer RM, van Lookeren Campagne M. IL-33 amplifies an innate immune response in the degenerating retina. *J Exp Med* 2016; **213**: 189-207 [PMID:

26755704 DOI: 10.1084/jem.20150894]
37 **Holtkamp GM**, Kijlstra A, Peek R, de Vos AF. Retinal pigment epithelium-immune system interactions: cytokine production and cytokine-induced changes. *Prog Retin Eye Res* 2001; **20**: 29-48 [PMID: 11070367 DOI: 10.1016/S1350-9462(00)00017-3]
38 **Madeira MH**, Boia R, Santos PF, Ambrósio AF, Santiago AR. Contribution of microglia-mediated neuroinflammation to retinal degenerative diseases. *Mediators Inflamm* 2015; **2015**: 673090 [PMID: 25873768 DOI: 10.1155/2015/673090]
39 **Kiang L**, Ross BX, Yao J, Shanmugam S, Andrews CA, Hansen S, Besirli CG, Zacks DN, Abcouwer SF. Vitreous Cytokine Expression and a Murine Model Suggest a Key Role of Microglia in the Inflammatory Response to Retinal Detachment. *Invest Ophthalmol Vis Sci* 2018; **59**: 3767-3778 [PMID: 30046818 DOI: 10.1167/iovs.18-24489]
40 **Yao K**, Qiu S, Wang YV, Park SJH, Mohns EJ, Mehta B, Liu X, Chang B, Zenisek D, Crair MC, Demb JB, Chen B. Restoration of vision after de novo genesis of rod photoreceptors in mammalian retinas. *Nature* 2018; **560**: 484-488 [PMID: 30111842 DOI: 10.1038/s41586-018-0425-3]
41 **Forrester JV**, Xu H. Good news-bad news: the Yin and Yang of immune privilege in the eye. *Front Immunol* 2012; **3**: 338 [PMID: 23230433 DOI: 10.3389/fimmu.2012.00338]
42 **Carwile ME**, Culbert RB, Sturdivant RL, Kraft TW. Rod outer segment maintenance is enhanced in the presence of bFGF, CNTF and GDNF. *Exp Eye Res* 1998; **66**: 791-805 [PMID: 9657912 DOI: 10.1006/exer.1998.0488]
43 **Simó R**, Carrasco E, García-Ramírez M, Hernández C. Angiogenic and antiangiogenic factors in proliferative diabetic retinopathy. *Curr Diabetes Rev* 2006; **2**: 71-98 [PMID: 18220619 DOI: 10.2174/157339906775473671]
44 **de Oliveira Dias JR**, Rodrigues EB, Maia M, Magalhães O Jr, Penha FM, Farah ME. Cytokines in neovascular age-related macular degeneration: fundamentals of targeted combination therapy. *Br J Ophthalmol* 2011; **95**: 1631-1637 [PMID: 21546514 DOI: 10.1136/bjo.2010.186361]
45 **Popescu M**, Bogdan C, Pintea A, Rugină D, Ionescu C. Antiangiogenic cytokines as potential new therapeutic targets for resveratrol in diabetic retinopathy. *Drug Des Devel Ther* 2018; **12**: 1985-1996 [PMID: 30013318 DOI: 10.2147/DDDT.S156941]
46 **Wang JC**, Cao S, Wang A, To E, Law G, Gao J, Zhang D, Cui JZ, Matsubara JA. CFH Y402H polymorphism is associated with elevated vitreal GM-CSF and choroidal macrophages in the postmortem human eye. *Mol Vis* 2015; **21**: 264-272 [PMID: 25814824]
47 **Ambati J**, Atkinson JP, Gelfand BD. Immunology of age-related macular degeneration. *Nat Rev Immunol* 2013; **13**: 438-451 [PMID: 23702979 DOI: 10.1038/nri3459]
48 **Mugisho OO**, Rupenthal ID, Squirrell DM, Bould SJ, Danesh-Meyer HV, Zhang J, Green CR, Acosta ML. Intravitreal pro-inflammatory cytokines in non-obese diabetic mice: Modelling signs of diabetic retinopathy. *PLoS One* 2018; **13**: e0202156 [PMID: 30133488 DOI: 10.1371/journal.pone.0202156]
49 **Kutty RK**, Samuel W, Boyce K, Cherukuri A, Duncan T, Jaworski C, Nagineni CN, Redmond TM. Proinflammatory cytokines decrease the expression of genes critical for RPE function. *Mol Vis* 2016; **22**: 1156-1168 [PMID: 27733811]
50 **Mahajan VB**, Vallone JG, Lin JH, Mullins RF, Ko AC, Folk JC, Stone EM. T-cell infiltration in autosomal dominant neovascular inflammatory vitreoretinopathy. *Mol Vis* 2010; **16**: 1034-1040 [PMID: 20596252]
51 **He C**, Lai P, Wang J, Zhou T, Huang Z, Zhou L, Liu X. TLR2/4 deficiency prevents oxygen-induced vascular degeneration and promotes revascularization by downregulating IL-17 in the retina. *Sci Rep* 2016; **6**: 27739 [PMID: 27297042 DOI: 10.1038/srep27739]
52 **Johnsen-Soriano S**, Sancho-Tello M, Arnal E, Navea A, Cervera E, Bosch-Morell F, Miranda M, Javier Romero F. IL-2 and IFN-gamma in the retina of diabetic rats. *Graefes Arch Clin Exp Ophthalmol* 2010; **248**: 985-990 [PMID: 20213480 DOI: 10.1007/s00417-009-1289-x]
53 **Geiger K**, Howes E, Gallina M, Huang XJ, Travis GH, Sarvetnick N. Transgenic mice expressing IFN-gamma in the retina develop inflammation of the eye and photoreceptor loss. *Invest Ophthalmol Vis Sci* 1994; **35**: 2667-2681 [PMID: 8188461]
54 **Ding SLS**, Kumar S, Mok PL. Cellular Reparative Mechanisms of Mesenchymal Stem Cells for Retinal Diseases. *Int J Mol Sci* 2017; 18 [PMID: 28788088 DOI: 10.3390/ijms18081406]
55 **Holan V**, Hermankova B, Kossl J. Perspectives of Stem Cell-Based Therapy for Age-Related Retinal Degenerative Diseases. *Cell Transplant* 2017; **26**: 1538-1541 [PMID: 29113466 DOI: 10.1177/0963689717721227]
56 **Park SS**, Moisseiev E, Bauer G, Anderson JD, Grant MB, Zam A, Zawadzki RJ, Werner JS, Nolta JA. Advances in bone marrow stem cell therapy for retinal dysfunction. *Prog Retin Eye Res* 2017; **56**: 148-165 [PMID: 27784628 DOI: 10.1016/j.preteyeres.2016.10.002]
57 **Oh JY**, Kim MK, Shin MS, Lee HJ, Ko JH, Wee WR, Lee JH. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. *Stem Cells* 2008; **26**: 1047-1055 [PMID: 18192235 DOI: 10.1634/stemcells.2007-0737]
58 **Mathew B**, Poston JN, Dreixler JC, Torres L, Lopez J, Zelkha R, Balyasnikova I, Lesniak MS, Roth S. Bone-marrow mesenchymal stem-cell administration significantly improves outcome after retinal ischemia in rats. *Graefes Arch Clin Exp Ophthalmol* 2017; **255**: 1581-1592 [PMID: 28523456 DOI: 10.1007/s00417-017-3690-1]
59 **Cejkova J**, Trosan P, Cejka C, Lencova A, Zajicova A, Javorkova E, Kubinova S, Sykova E, Holan V. Suppression of alkali-induced oxidative injury in the cornea by mesenchymal stem cells growing on nanofiber scaffolds and transferred onto the damaged corneal surface. *Exp Eye Res* 2013; **116**: 312-323 [PMID: 24145108 DOI: 10.1016/j.exer.2013.10.002]
60 **Ma OK**, Chan KH. Immunomodulation by mesenchymal stem cells: Interplay between mesenchymal stem cells and regulatory lymphocytes. *World J Stem Cells* 2016; **8**: 268-278 [PMID: 27679683 DOI: 10.4252/wjsc.v8.i9.268]
61 **Meirelles Lda S**, Fontes AM, Covas DT, Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev* 2009; **20**: 419-427 [PMID: 19926330 DOI: 10.1016/j.cytogfr.2009.10.002]
62 **Kolomeyer AM**, Zarbin MA. Trophic factors in the pathogenesis and therapy for retinal degenerative diseases. *Surv Ophthalmol* 2014; **59**: 134-165 [PMID: 24417953 DOI: 10.1016/j.survophthal.2013.09.004]
63 **Ezquer M**, Urzua CA, Montecino S, Leal K, Conget P, Ezquer F. Intravitreal administration of multipotent mesenchymal stromal cells triggers a cytoprotective microenvironment in the retina of diabetic mice. *Stem Cell Res Ther* 2016; **7**: 42 [PMID: 26983784 DOI: 10.1186/s13287-016-0299-y]

64 **Conway K**, Price P, Harding KG, Jiang WG. The molecular and clinical impact of hepatocyte growth factor, its receptor, activators, and inhibitors in wound healing. *Wound Repair Regen* 2006; **14**: 2-10 [PMID: 16476066 DOI: 10.1111/j.1524-475X.2005.00081.x]

65 **Nicola NA**, Babon JJ. Leukemia inhibitory factor (LIF). *Cytokine Growth Factor Rev* 2015; **26**: 533-544 [PMID: 26187859 DOI: 10.1016/j.cytogfr.2015.07.001]

66 **Mourkioti F**, Rosenthal N. IGF-1, inflammation and stem cells: interactions during muscle regeneration. *Trends Immunol* 2005; **26**: 535-542 [PMID: 16109502 DOI: 10.1016/j.it.2005.08.002]

67 **Ferrara N**, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003; **9**: 669-676 [PMID: 12778165 DOI: 10.1038/nm0603-669]

68 **Ng TK**, Fortino VR, Pelaez D, Cheung HS. Progress of mesenchymal stem cell therapy for neural and retinal diseases. *World J Stem Cells* 2014; **6**: 111-119 [PMID: 24772238 DOI: 10.4252/wjsc.v6.i2.111]

69 **Labrador-Velandia S**, Alonso-Alonso ML, Alvarez-Sanchez S, González-Zamora J, Carretero-Barrio I, Pastor JC, Fernandez-Bueno I, Srivastava GK. Mesenchymal stem cell therapy in retinal and optic nerve diseases: An update of clinical trials. *World J Stem Cells* 2016; **8**: 376-383 [PMID: 27928464 DOI: 10.4252/wjsc.v8.i11.376]

70 **Salehi H**, Amirpour N, Razavi S, Esfandiari E, Zavar R. Overview of retinal differentiation potential of mesenchymal stem cells: A promising approach for retinal cell therapy. *Ann Anat* 2017; **210**: 52-63 [PMID: 27986614 DOI: 10.1016/j.aanat.2016.11.010]

71 **Tzameret A**, Sher I, Belkin M, Treves AJ, Meir A, Nagler A, Levkovitch-Verbin H, Rotenstreich Y, Solomon AS. Epiretinal transplantation of human bone marrow mesenchymal stem cells rescues retinal and vision function in a rat model of retinal degeneration. *Stem Cell Res* 2015; **15**: 387-394 [PMID: 26322852 DOI: 10.1016/j.scr.2015.08.007]

72 **Ji S**, Lin S, Chen J, Huang X, Wei CC, Li Z, Tang S. Neuroprotection of Transplanting Human Umbilical Cord Mesenchymal Stem Cells in a Microbead Induced Ocular Hypertension Rat Model. *Curr Eye Res* 2018; **43**: 810-820 [PMID: 29505314 DOI: 10.1080/02713683.2018.1440604]

73 **Labrador Velandia S**, Di Lauro S, Alonso-Alonso ML, Tabera Bartolomé S, Srivastava GK, Pastor JC, Fernandez-Bueno I. Biocompatibility of intravitreal injection of human mesenchymal stem cells in immunocompetent rabbits. *Graefes Arch Clin Exp Ophthalmol* 2018; **256**: 125-134 [PMID: 29168045 DOI: 10.1007/s00417-017-3842-3]

74 **Lohan P**, Treacy O, Morcos M, Donohoe E, O'donoghue Y, Ryan AE, Elliman SJ, Ritter T, Griffin MD. Interspecies Incompatibilities Limit the Immunomodulatory Effect of Human Mesenchymal Stromal Cells in the Rat. *Stem Cells* 2018; **36**: 1210-1215 [PMID: 29726063 DOI: 10.1002/stem.2840]

75 **Zhou L**, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* 2008; **453**: 236-240 [PMID: 18368049 DOI: 10.1038/nature06878]

76 **Granofszky N**, Farkas AM, Muckenhuber M, Mahr B, Unger L, Maschke S, Pilat N, Holly R, Wiletel M, Regele H, Wekerle T. Anti-Interleukin-6 Promotes Allogeneic Bone Marrow Engraftment and Prolonged Graft Survival in an Irradiation-Free Murine Transplant Model. *Front Immunol* 2017; **8**: 821 [PMID: 28769930 DOI: 10.3389/fimmu.2017.00821]

77 **Jordan SC**, Choi J, Kim I, Wu G, Toyoda M, Shin B, Vo A. Interleukin-6, A Cytokine Critical to Mediation of Inflammation, Autoimmunity and Allograft Rejection: Therapeutic Implications of IL-6 Receptor Blockade. *Transplantation* 2017; **101**: 32-44 [PMID: 27547870 DOI: 10.1097/TP.0000000000001452]

World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2019 November 26; 11(11): 968-981

DOI: 10.4252/wjsc.v11.i11.968

ISSN 1948-0210 (online)

REVIEW

Developments in cell culture systems for human pluripotent stem cells

Weiwei Liu, Chunhao Deng, Carlos Godoy-Parejo, Yumeng Zhang, Guokai Chen

ORCID number: Weiwei Liu (0000-0001-6997-2412); Chunhao Deng (0000-0001-6551-9741); Carlos Godoy-Parejo (0000-0002-4324-3593); Yumeng Zhang (0000-0001-5220-0491); Guokai Chen (0000-0003-0921-8244).

Author contributions: Liu W, Deng C, Godoy-Parejo C, Zhang Y, and Chen G wrote the manuscript; Deng C drew the figure; Godoy-Parejo C compiled the tables.

Supported by University of Macau Multi-Year Research Grants, No. MYRG2015-00228-FHS and MYRG2018-00135-FHS; and Macao Science and Technology Development Fund, No. FDCT/131/2014/A3 and FDCT/056/2015/A2.

Conflict-of-interest statement: Dr. Chen has a patent 9644186 with royalties paid to Wisconsin Alumni Research Foundation. The other authors have no conflict of interest to declare.

Weiwei Liu, Chunhao Deng, Carlos Godoy-Parejo, Yumeng Zhang, Guokai Chen, Centre of Reproduction, Development and Aging, Faculty of Health Sciences, University of Macau, Macau 999078, China

Weiwei Liu, Chunhao Deng, Carlos Godoy-Parejo, Yumeng Zhang, Guokai Chen, Institute of Translational Medicine, Faculty of Health Sciences, University of Macau, Macau 999078, China

Weiwei Liu, Bioimaging and Stem Cell Core Facility, Faculty of Health Sciences, University of Macau, Macau 999078, China

Corresponding author: Guokai Chen, PhD, Associate Professor, Centre of Reproduction, Development and Aging, Faculty of Health Sciences, University of Macau, E12-4013, Taipa, Macau 999078, China. guokaichen@umac.mo
Telephone: +853-88224985

Abstract

Human pluripotent stem cells (hPSCs) are important resources for cell-based therapies and pharmaceutical applications. In order to realize the potential of hPSCs, it is critical to develop suitable technologies required for specific applications. Most hPSC technologies depend on cell culture, and are critically influenced by culture medium composition, extracellular matrices, handling methods, and culture platforms. This review summarizes the major technological advances in hPSC culture, and highlights the opportunities and challenges in future therapeutic applications.

Key words: Human pluripotent stem cells; Human embryonic stem cells; Cell culture; Culture medium; Stem cell niche; Signal transduction; Embryoid bodies

Core tip: This review summarizes recent developments in cell culture systems for human pluripotent stem cells, including signal transduction requirements at different pluripotency stages, advances in extracellular matrices and handling methods, establishment of chemically defined conditions, and various cell culture platforms for specific purposes.

Citation: Liu W, Deng C, Godoy-Parejo C, Zhang Y, Chen G. Developments in cell culture systems for human pluripotent stem cells. *World J Stem Cells* 2019; 11(11): 968-981

Manuscript source: Invited manuscript

Received: March 26, 2019
Peer-review started: March 28, 2019
First decision: April 16, 2019
Revised: August 21, 2019
Accepted: August 27, 2019
Article in press: August 27, 2019
Published online: November 26, 2019

P-Reviewer: Grawish ME, Tanabe S
S-Editor: Ma RY
L-Editor: Wang TQ
E-Editor: Wu YXJ

URL: https://www.wjgnet.com/1948-0210/full/v11/i11/968.htm
DOI: https://dx.doi.org/10.4252/wjsc.v11.i11.968

INTRODUCTION

Human pluripotent stem cells (hPSCs), including mainly human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), have the capacity to differentiate to all cell types in the human body[1-4]. Since they were first derived in 1998, hESCs have provided an unparalleled model to understand human embryogenesis, and has sparked a revolution in regenerative medicine[1,5]. Based on hESC culture conditions, hiPSCs were first derived in 2007, which again created an unprecedented opportunity to generate patient-specific hPSCs for disease modeling and therapeutic applications[3,4]. Because of their enormous potential, hPSCs have garnered vast interest in both basic research and clinical applications[6]. Unlike somatic cell types, pluripotent stem cells only transiently exist in the first few days of embryogenesis, and there is no natural environment that is capable of maintaining pluripotency *in vivo* for extended periods of time, so hPSCs in the lab are all artifacts of *in vitro* cell culture conditions[1]. It is commonly recognized that cell culture quality is a major limiting factor of hPSC applications. In the past 20 years, cell culture is a main research focus in the hPSC field, and many technological improvements have been made to realize the potential of hPSCs.

In order to maintain their pluripotency, hPSCs require proper combinations of extrinsic signal stimuli to establish a stem cell niche in cell culture systems[7]. Suitable methods and culture platforms are required to sustain cell survival and promote specific functions in various applications. hPSCs are traditionally cultured as a 2-dimensional (2D) monolayer on mouse embryonic fibroblast feeder cells (MEFs) in medium supplemented with either fetal bovine serum (FBS) or components extracted from serum[1,2]. This traditional culture is sufficient for hPSC maintenance and general characterization but cannot satisfy the needs of numerous potential applications, such as cell therapy and gene targeting. At the same time, new knowledge from basic research also leads to new questions and challenges for further technology development in cell culture[8-10]. This review will discuss five areas in hPSC culture development that includes: (1) Stage-specific signaling requirements; (2) Essential extracellular matrix; (3) Handling methods; (4) Defined culture composition; and (5) Culture platforms (Figure 1).

STAGE-SPECIFIC SIGNALING REQUIREMENTS FOR HPSC PLURIPOTENCY

After almost four decades of research, people have realized that mammalian PSCs could be maintained at distinctive developmental stages. hPSCs at each stage require a specific and different combination of growth factor stimulations. Three stages of pluripotency have been reported in hPSCs, including primed, naïve, and extended pluripotency. However, most hPSCs are derived and maintained as primed PSCs.

The pluripotency stages are defined according to the differentiation potential and developmental timing during mouse embryogenesis. In mouse embryogenesis, primed ESCs are derived from post-implantation epiblasts[11], and naïve ESCs come from the inner cell mass of preimplantation blastocysts[12,13], both of which show limited ability to contribute to the extraembryonic placental tissues *in vivo*. Recently, extended pluripotent stem cells (EPSCs) have been reported with extended ability to contribute to both extraembryonic and embryonic tissues[14,15]. Human and mouse PSCs share similar growth factor signal stimulations that are required to maintain PSCs at each specific stage. The hPSC pluripotency stages are determined according to signal requirements and corresponding gene expression.

Primed ESC stage - common hESC culture

Thomson *et al*[1] first established hESCs from the inner cell mass (ICM) of blastocysts in MEF feeder cell culture with FBS. In the following 20 years, researchers have endeavored to understand the essential signals from FBS and feeder cells that promote pluripotency (Table 1). Many cell culture systems have been established to maintain hESCs. FGF, TGF-β family, and insulin master the three essential signaling pathways for hPSC survival and pluripotency, and they have been combined together

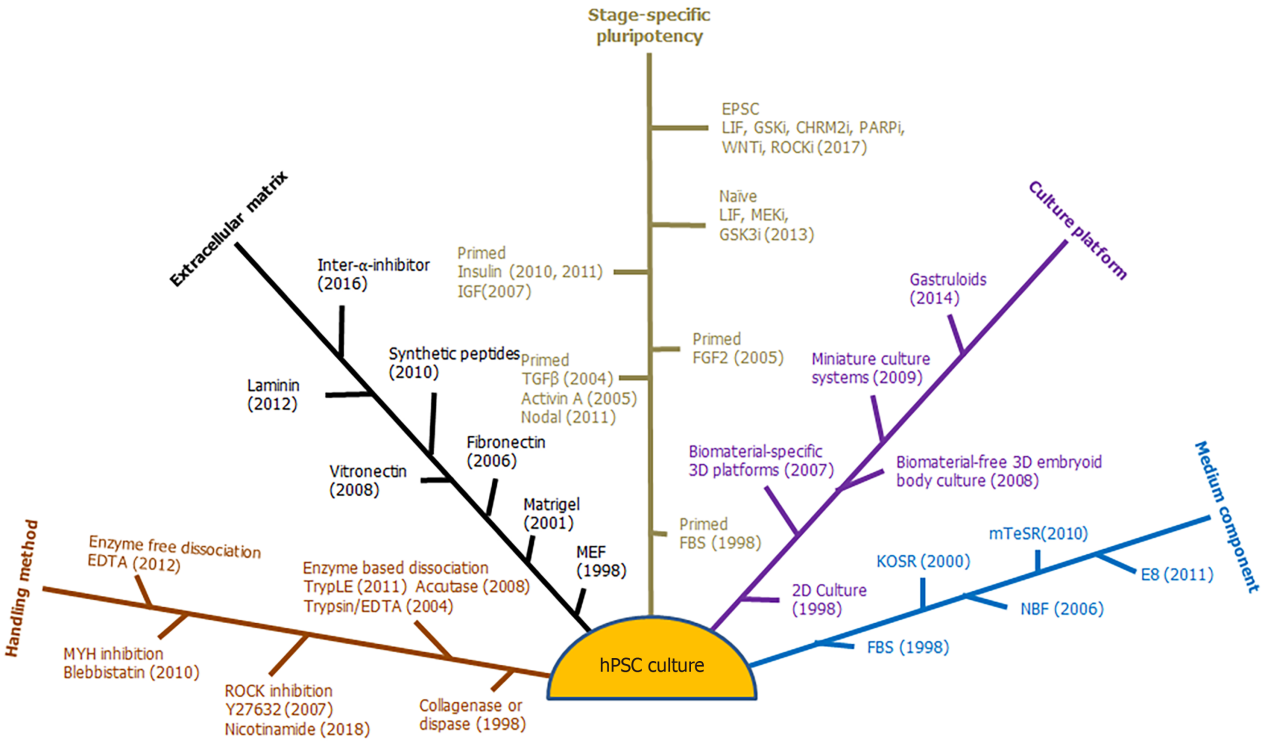

Figure 1 Five areas in human pluripotent stem cell culture development. hPSC: Human pluripotent stem cell.

as the most common extrinsic stimuli to derive and maintain hESCs and hiPSCs[16-18]. FGF family members, with FGF2 as the main effector, activate the ERK pathway to promote cell survival, proliferation, and pluripotency[19-21]. TGF-β family members, including TGF-β, Activin, and Nodal, induce SMAD2/3-dependent transcription to sustain pluripotency[16,17,22]. Insulin is a promiscuous factor present in most hESC media, and is required for cell survival, metabolism, and pluripotency[17,18]. Insulin can be replaced by insulin-like growth factor (IGF), which activates the IGF receptor pathway to support hESCs[23]. Based on the hESC culture conditions, Tesar *et al*[11] derived primed mouse ESCs (mESCs) from post-implantation epiblasts, which are also called mouse epiblast stem cells (mEpiSCs). These primed mESCs exhibit similar growth factor preference as hESCs. If without specification, hESC culture condition usually refers to conditions that can sustain primed hPSCs and primed mPSCs.

In addition to the growth factors ruled by the three mentioned pathways, other signaling pathways are also reported to support pluripotency. For example, FLT3, heregulin, heparin, heparan sulfate, S1P, and PDGF promote hESC pluripotency, and all are found to promote ERK pathway activation[24-29]. Beneficial effects are also observed with other factors such as pipecolic acid and GABA, but their molecular mechanisms remain unknown[30].

Besides the beneficial factors, some growth factor pathways need to be suppressed to maintain primed pluripotency in hESCs. Exogenous BMP signal from serum or serum products leads to the exit of self-renewal[31]. The inhibition of BMP pathway promotes hESC pluripotency even without addition of TGF-β in medium containing knockout serum replacement (KOSR)[20]. At the same time, WNT inhibition is also beneficial for cell pluripotency, while WNT activation leads to cell differentiation[32]. Multiple groups reported earlier that hESC pluripotency was promoted by WNT pathway activators, such as WNT3A, LiCl, and BIO[22,30,33], but those observations could be artifacts from differential culture background, coming from different medium composition. Presently, WNT activators are usually used to induce hPSC differentiation to mesoderm or neural crest lineages[34-36].

Based on the knowledge of hESC culture conditions, various primed stage hPSCs have been derived from different sources. In addition to the inner cell mass of embryos[1], hESCs are also derived from a single cell of an 8-cell blastomere without embryo destruction[37]. In recent years, patient-specific nuclear transfer-ESCs (hNT-ESCs) have been created through somatic cell nuclear transfer (SCNT) to caffeine-treated oocytes[38]. At the same time, hiPSCs are generated directly from somatic cells through somatic reprogramming by defined factors[3,4]. No matter the sources, the hPSCs derived under hESC conditions always reflect characteristics that resemble

primed mouse ESCs.

Naïve hPSC stage

Immediately after the derivation of hESCs, researchers noticed that hESCs required a different growth factor regulation from preimplantation blastocyst-derived mESCs[39]. Smith and colleagues show that mouse ESCs can be maintained by leukemia inhibitory factor (LIF) in media containing FBS, by LIF and BMP4 in serum-free media, or by the dual inhibition of MEK and GSK3 (2i) in serum-free cultures[40-42]. These cells are called naïve mESCs in contrast with primed mESCs, because the naïve mESCs represent an earlier developmental stage. The naïve mESC conditions cannot be used to maintain primed hPSCs and mESCs. Examples include BMP4, ERK inhibitor, and GSK3 inhibitor, all of which induce the differentiation of primed hPSCs.

Naïve mESCs contribute to blastocyst chimeras more effectively, demonstrating a unique epigenetic signature with genome-wide DNA hypomethylation, and with the silent-X chromosome reactivated in female cells[43]. Compared to primed mESCs, naïve mESCs exhibit more genomic consistency and differentiation potentials. Naïve hPSCs could presumably gain similar advantages, yet hPSC pluripotency could not be maintained by mouse naïve conditions. It is reported that key genes such as ESRRB, KLF2, and BMP4 are not expressed in human naïve epiblasts[44]. By overexpressing KLF4, naïve hPSCs can be maintained under mouse 2i conditions with an additional PKA inhibitor[45]. This suggests that additional signal modulation is necessary to maintain hPSCs at the naïve stage.

In the past 5 years, major efforts have been devoted to developing culture conditions to maintain naïve hESCs. Six combinations of extrinsic stimuli are reported to maintain naïve hPSC, which are summarized in Table 2[46-51]. Similar to mouse culture, all naïve conditions require MEK and GSK3 inhibitors (2i), and LIF is applied in five of the six media. All the conditions include additional supplements besides base medium, such as KOSR, B27, N2, and TeSR1 supplements[52]. Interestingly, four conditions also contain FGF2 that is not required in mESC culture. At the same time, various additional small chemicals are used to modulate pathways, such as LCK/SRC, Raf, FGFR, HDAC, and PKC. These additional factors help induce gene expression that are beneficial to naïve pluripotency. Currently, there remains no consensus concerning which pathways are essential for the maintenance of naïve hPSCs. It is conceivable that a more unified culture system will be developed in the near future.

It is important to point out that naïve pluripotency requires different maintenance signals, which sometimes have opposite effects on primed hPSCs. For example, the FGF and ERK pathway is inhibitory to naïve pluripotency, while it also promotes primed pluripotency. At the same time, BMP4 and WNT signals promote naïve hPSCs, but induce differentiation of primed hPSCs. Such phenomena demonstrate that hPSCs at each stage require distinctive signals for exiting pluripotency as well as for cell fate determination.

Extended pluripotency

Recently, two new mouse cell culture conditions have been reported to sustain ESCs with extended pluripotency. These cells can contribute to not only embryonic, but also extraembryonic lineages, giving them the name of extended pluripotent stem cells (EPSCs)[14,15]. Surprisingly, these two conditions only share GSK3 inhibition. Deng and colleagues showed that mouse EPSCs are maintained by the EPS-LCDM medium, which contains the combination of LIF, CHIR99021 (GSK inhibitor), dimethindene maleate (M2 muscarinic receptor inhibitor), and minocycline hydrochloride (PARP inhibitor)[15]. With the help of WNT inhibitor (IWR-endo-1) and ROCK inhibitor (Y27632), EPS-LCDM can be used to maintain human EPSCs. In contrast, Liu and colleagues reported a different formula to maintain EPSCs, which includes inhibitors of various pathways including MEK, GSK3, p38, JNK, SRC, and Tankyrase[14]. More work is necessary to determine whether the two EPSCs are at similar developmental stages, and what central regulation is shared by these two conditions.

Naïve hPSC and hEPSC cultures allow better cell survival after individualization than primed hPSCs, which is beneficial for applications such as gene targeting and expansion. They also provide alternative model systems to understand human embryogenesis, and more studies are needed to explore their potential. However, currently most studies and applications use primed hESC conditions, so the remainder of this review will focus on the technology development related to primed hPSCs.

CELL ADHESION FOR CELL SURVIVAL AND EXPANSION

Table 1 Primed human pluripotent stem cell culture conditions

	Thomson *et al*[1]	Xu *et al*[53]	Xu *et al*[20]	Vallier *et al*[16]	Li *et al*[26]	Ludwig *et al*[30,80]	Liu *et al*[52]	Lu *et al*[33]	Yao *et al*[117]	Wang *et al*[23]	Chen *et al*[17]	Frank *et al*[118]
FGF2	-	+	+	+	+	+	+	+	+	+	+	+
TGF-β family	-	-	-	Activin	-	TGF-β	-	TGF-β	-	Activin	TGF-β/Nodal	TGF-β/Activin
Serum	FBS	KOSR	KOSR	XF	-	-	-	-	-	-	-	-
Other factors	-	-	Noggin	Insulin	FLT3	Insulin	-	Insulin, BAFF, WNT3A	-	LR3-IGF1, HRG1β	Insulin	Dorsomorphin, IWP-2
Common culture supplements	-	-	NEAA, BME, L-glutamine	BSA, L-glutamine, transferrin, BME, F12, monothio-glycerol	BME, NEAA, L-glutamine	BSA, BME, transferrin, cholesterol, pipecolic acid, GABA	BME, N2, B27, L-glutamine	BME, transferrin, cholesterol, albumin, L-glutamine	BME, N2, B27, NEAA, BSA-Fraction-V, L-glutamine	BSA-Fraction-V, NEAA, Vc, trans-ferrin, BME, trace elements A, B, C	Vc, transferrin, selenium, NaHCO3	N2, B27, BSA, L-glutamine, NEAA, BME
Base medium	DMEM/F12	DMEM	DMEM/F12	IMDM	XVIVO-10	DMEM/F12	DMEM/F12	DMEM/F12	DMEM/F12	DMEM/F12	DMEM/F12	DMEM/F12
ECM	-	Matrigel	Matrigel	Matrigel	Matrigel	Matrigel	Fibronectin	Matrigel	Matrigel	Matrigel	Matrigel/ Vitronectin	Matrigel
Feeder	MEF	-	-	-	-	-	-	-	-	-	-	-
Markers	Surface markers[119]: SSEA3, SSEA4, TRA1-60, TRA1-81, CD24, CD57, CD90 Nuclear markers: OCT4, NANOG, SOX2											

Table 2 Naïve pluripotent stem cell and extended pluripotent stem cell culture conditions

	Gafni *et al*[48] Naive hESC	Chan *et al*[49] Naive hESC	Valamehr *et al*[50] Naive hESC	Ware *et al*[51] Naive hESC	Theunissen *et al*[120] Naive hESC	Guo *et al*[47] Naive hESC	Ying *et al*[42] Naive mESC	Yang *et al*[15] hEPSC	Yang *et al*[15] mEPSC	Yang *et al*[14] mEPSC
MEK inhibitor	PD0325901	PD0325901	PD0325901	PD0325901	PD0325901	PD0325901	PD0325901	-	-	PD0325901
GSK3 inhibitor	CHIR 99021	BIO	CHIR99021	CHIR99021	IM-12	CHIR99021	CHIR99021	CHIR99021	CHIR99021	CHIR99021
LIF	+	+	+	+	+	+	+	+	+	-
FGF2	+	+	+	+	-	-	-	-	-	-
TGFβ	+	+	-	Activin	Activin	-	-	-	-	-
ROCK Inhibitor	Y27632	Thiazovivin	Thiazovivin	Y27632	Y27632	Y27632	-	Y27632	-	-
Insulin	+	+	-	-	-	+	-	-	-	-
Other factors	JNKi; p38i; PKCi	Dorsomorphin	TGFi	TGFi; FGFRi; HDACi, STAT3i	B-RAFi; LCK/SRCi	PKCi	-	IWR1, dimethindene maleate, minocycline hydrochloride	Dimethindene maleate, minocycline hydrochloride	p38i, JNKi; SRCi; Tanki, WNTi
Base medium	KO DMEM	DMEM/F12	DMEM/F12	DMEM/F12	DMEM/F12/ Neurobasal	DMEM/F12/ Neurobasal	DMEM/F12	DMEM/F12/ Neurobasal	DMEM/F12/ Neurobasal	DMEM/F12
Medium Supplement	KOSR, NEAA, AlbuMAX I, L-glutamine, BME	TeSR1 components	KOSR, NEAA, BME, L-glutamine	GlutaMAX, KOSR, NEAA, BME, sodium pyruvate	B27/N2, L-glutamine, NEAA, BME, BSA	N2/B27, L-glutamine, BME, Vc, selenium, putrescine, proges-terone	N2/B27	N2/B27, GlutaMAX, BME, BSA, KOSR	N2/B27, GlutaMAX, BME, BSA, KOSR	BME, GPS, NEAA, KOSR
Feeder	MEF optional	MEF	-	-	MEF	MEF	-	MEF	MEF	MEF
Coating	Gelatin/vitronectin	Matrigel	Matrigel/vitronectin	Matrigel	-	-	Gelatin	-	-	-
Markers	Surface markers[121]: CD7, CD77, CD75, CD130 Nuclear markers[119]: OCT4, NANOG, SOX2, KLF4,5,17, TFCP2L1, DPPA3,5						Oct4, Nanog, Rex1	OCT4, KLF4 NANOG	Oct4, Nanog, Sox2, Sall4	Oct4, Nanog, Sox2, Klf4, Rex1, Esrrb

mESC: Mouse embryonic stem cell; hEPSC: Human extended pluripotent stem cell; mEPSC: Mouse extended pluripotent stem cell.

In order to maintain hPSC pluripotency, a stem cell niche requires not only growth factor signals but also cell adhesion. Without the support of exogenous adhesion proteins, hPSCs either die or differentiate[17,53]. In original hESC derivation, cell adhesion signals are provided by MEF feeder cells and their secreted extracellular matrix (ECM)[1]. Matrigel was later found sufficient to support hESC survival and self-renewal without feeder cells[53]. Matrigel is isolated from mouse Engelbreth-Holm-Swarm teratocarcinoma cells, so it is not ideal for hPSCs that have applications in clinical therapies[54,55]. The establishment of feeder-free culture permits the possibility to optimize ECM and medium composition in parallel, and greatly accelerates technology development.

Matrigel is a mixture of mainly laminin and collagen, which can activate integrin signaling. Various integrin-activating ECM proteins and their recombinant derivatives can support hPSCs, which include laminin, vitronectin, fibronectin, and inter-α-inhibitor[33,56-59]. Recombinant vitronectin and laminin domains can be produced in bacteria or in a cell free system, and they are becoming popular choices for cell culture that needs defined recombinant ECM components[17,56,59].

Besides cell-ECM interaction, cell-cell interaction mediated by E-cadherin is another key component of hPSC niche. E-cadherins facilitate hPSC expansion in the form of colonies[60]. Recombinant E-cadherin is produced as a fusion protein that contains N-terminal E-cadherin and C-terminal IgG-Fc domains. This protein sustains hPSC survival and self-renewal in the absence of integrin-stimulating ECM[61]. When E-Cadherin and laminin are combined to create an artificial matrix, the clonal expansion of hPSCs is significantly improved in comparison to single-component matrices[62]. These data suggest that integrin- and E-cadherin-mediated adhesions provide the principle ECM cues in hPSC niche.

Based on hPSC niche composition, specific peptides have been identified to sustain hPSC culture[10,63]. The most popular peptides are RGD domain-based peptides, which activate integrin pathways. These peptides are sufficient for hPSC survival and expansion, and the efficiency is similar to that of matrigel and vitronectin[63,64]. Such peptides can be chemically produced in large scale, and are very attractive materials for tissue engineering and technology development. The recombinant proteins and peptides can be conjugated to various matrices to construct a synthetic ECM environment. With improved hPSC handling methods, hPSCs are more tolerant to various materials, which greatly expand the choices of ECM materials for different culture platforms and applications.

HANDLING METHODS

Compared to naïve hPSCs and hEPSCs, primed hPSCs are more prone to cell death after dissociation. Most hPSC culture manipulations involve cell dissociation or individualization. Efforts have been focused on promoting cell survival in hPSC handling methods that are essential for various applications.

In regular maintenance, hPSCs proliferate quickly and are usually passaged every 4-7 d. Traditionally, hPSCs are manually split, or dissociated with collagenase or dispase, and cells are collected as clumps[1,2]. Collagenase and dispase cause minimal disruption of hPSC niches, and cells survive well as aggregates of uneven sizes. Unfortunately, such a dissociation method is not suitable for gene targeting or other experiments that require individualized cells. After hPSCs are individualized with trypsin/EDTA[65], most cells die within 24 h after passaging, and fewer than 1% of hPSCs can survive clonally without exogenous intervention. After individualization, the loss of cell adhesion activates the Rho-associated protein kinase (ROCK) /Actinomyosin axis that leads to increased actin-myosin contractility and cell death. Cell survival of individualized cells is significantly improved by the inhibition of ROCK, myosin heavy chain (MYH), and actin proteins[66,67]. Vitamin B3 is sometimes used in hPSC expansion, and it was recently found that nicotinamide promotes cell survival by inhibiting ROCK activity[68]. Interestingly, even though caspase cascades are activated by dissociation, caspase inhibitors could not rescue cell survival. Researchers typically use ROCK inhibitors and the MYH inhibitor blebbistatin to facilitate single cell clonal formation and expansion[66,67,69].

Dissociation reagents are also important for the cell survival after dissociation. TrypLE and accutase are recombinant proteases that have gentler effects on cells than traditional trypsin/EDTA, which greatly improves cell survival after dissociation, whether or not ROCK inhibitors are present[70,71]. hPSCs can also be dissociated as small aggregates with enzyme-free EDTA/PBS or citric acid solutions[72,73]. After attaching to ECM coated surfaces, these small aggregates quickly re-establish colonies and can achieve good survival ratios even without ROCK inhibitors. This enzyme-free

method does not digest the ECM, and does not need enzyme neutralization and removal. It provides some unique advantages in clonal hPSC expansion over enzyme-based dissociation methods[72].

Cryopreservation is the essential final stage of hPSC culture. It has long been problematic to efficiently revive hPSCs after cryopreservation. When hPSCs are harvested using dispase or collagenase, fewer than 5% of colonies could be recovered. In order to achieve good cryopreservation in these conditions, hPSCs need to be individualized, and later recovered in the presence of ROCK inhibitors or blebbistatin[74,75]. However, when enzyme-free PBS/EDTA is used to dissociate cells, hPSCs can be efficiently cryopreserved even without ROCK inhibitors[72].

With the emergence of new genome recombination technologies, increasingly hPSCs are being used in gene targeting that requires individualization and clonal expansion. For these purposes, TrypLE, accutase, and enzyme-free dissociation are usually used to harvest cells, and cells are often treated with ROCK inhibitor during electroporation and plating[76,77]. If antibiotics, such as puromycin and neomycin, are applied to select positive clones, ROCK inhibitor usually needs to be present to improve clonal hPSC expansion.

CULTURE CONSISTENCY, FROM SERUM TO CHEMICALLY DEFINED CONDITIONS

Even though growth factors and ECM are vital for hPSC maintenance, they are only a small part of cell culture components. Cell culture systems also include other equally important factors, such as water, nutrients, salts, vitamins, lipids, air, and temperature control[78]. Optimal culture composition is essential for cell survival, pluripotency, and therapeutic applications.

Most medium nutrients, salts, vitamins and water are provided through various basic media such as DMEM/F12. Additional medium components are usually supplied in the form of fetal bovine serum (FBS), KOSR, or defined mixture such as B27 supplement[52,79]. At the same time, MEF feeder cells are often used to provide unspecified beneficial factors[18]. FBS and KOSR contain undefined components of animal origins, and even B27 has bovine serum albumin (BSA) as a major component. These supplements pose major obstacles for hPSC applications. First, the exposure to animal cells and animal products leads to risk of immune rejection to hPSCs by contaminated animal components. This would make the hPSC product unsuitable for therapeutic applications. Second, secretion from feeder cells or serum components often lead to complications when trying to interpret a phenomenon or molecular mechanism. Third, the uncertainty of undefined composition also leads to complications in large-scale production which can cause a significant batch to batch variation. It is more desirable to have a robust culture system with defined composition while free of animal products.

Many defined cell culture systems have been developed to culture primed hPSCs. Individual labs and international collaboration have been involved in defining the components in cell culture media[16,23,26,33,80]. In 2010, the International Stem Cell Initiative systematically evaluated a few popular defined hPSC media, and mTeSR1 and STEMPRO demonstrated a more consistent ability to main hPSCs[18]. Besides FGF2, all these media contain BSA. Albumin is a principal serum protein, and makes up 3.5 to 5.0 g/dL in serum. Most cell culture contains albumin[81,82]. Even though BSA can be replaced by human serum albumin or recombinant albumin, the sheer amount of the albumin in medium significantly affects consistency which relies on the quality of albumin in each production batch. Chen *et al*[18] showed that the essential role of albumin is to block the toxicity of antioxidant 2-mercaptoethanol that is traditionally added into hPSC culture. When 2-mercaptoethanol is removed from medium, albumin is no longer required for hPSCs. Based on this finding, E8 medium was developed to sustain hPSC pluripotency with eight essential components[17]. E8 components include growth factors (insulin, FGF, and TGFβ), nutritional support (DMEM/F12 base medium), antioxidants (selenium, vitamin C, and transferrin) and pH modulator ($NaHCO_3$). The E8 medium formula has a significantly simplified composition, which is a good platform to understand hPSC physiology. When albumin is no longer necessary, E8-based culture system can facilitate hPSC production for therapeutic applications, and hPSCs can be cultured in E8 medium for >50 passages without any signs of karyotypic abnormalities while maintaining their pluripotency. hESCs and hiPSCs maintained in E8 medium have been efficiently induced into many somatic cell types and tissues under adherent and suspension culture conditions[83-86].

When researchers use albumin-free media, many cellular treatments and

manipulations need to be reevaluated. First, as a carrier protein, albumin is a strong adsorbent and blocker, so the concentrations of small chemicals used for cell manipulations need to be re-examined in albumin-free condition. Second, it is found that stem cells are more sensitive to suboptimal conditions or environmental changes in albumin-free medium, such as toxins, small chemical treatments, and medium acidosis[87].

Besides albumin, there are other factors that could also affect hPSC culture consistency. FGF2 is essential for pluripotency, but it is thermally unstable, which can result in precipitation and conformational changes of proteins, so high concentrations of FGF2 must be added in defined culture, and it needs to be replenished regularly. FGF2 can be stabilized by heparin, a specific point mutation, or slow release mechanism to help resolve this issue[28,88], and mutant forms of FGF2 that are stable against thermal denaturation have been established, such as K128N (Chen *et al*[89], 2016). It was also found that defined media produce different patterns of cellular metabolism compared to KOSR-containing media. Due to the lack of lipid components from KOSR, cells in E8 and TeSR demonstrate increased oxidative pentose phosphate pathway metabolism.

Although primed hPSCs can be maintained in E8 medium, there remains no similar albumin-free condition capable of naïve hPSC and EPSC maintenance. The media for the latter two hPSCs require albumin-containing supplements and additional small molecule modulators. This remains not only an interesting biological question but also a practical problem in the development of potential hPSC applications in the future.

CULTURE PLATFORMS FOR SPECIFIC PURPOSES

As discussed in previous sections, traditional hPSC culture systems are established on a 2D monolayer with suitable ECMs. However, conventional 2D monolayer culture does not accurately replicate the *in vivo* physiological environment, and often fails to meet the demands of research and therapeutic applications[90]. With the advances in culture medium, ECM, and handling methods, various culture platforms have been developed to utilize hPSCs beyond the usual 2D monolayer[91]. ROCK/MYH inhibitors promote cell survival and make hPSCs more tolerant to various treatments, which facilitates the fast development of hPSC culture platforms. We will briefly discuss biomaterial-free embryoid body culture as well as biomaterial-specific 2D and 3D platforms.

Biomaterial-free 3D embryoid body culture

When no ECM is supplemented to hPSCs, cells in suspension form embryoid bodies through E-cadherins. Many different methods have been developed to make embryoid bodies[92,93]. When hPSCs are harvested as individualized cells, ROCK inhibitor greatly promoted cell survival during the formation of embryoid bodies independent of the dissociation method.

The 3D suspension culture provides multiple advantages in large scale production, storage, and differentiation[94,95]. Clinical applications often require 10^7-10^{10} or more hPSCs. However, 2D culture cannot constantly produce uniform hPSCs in such large quantities. The embryoid bodies can be grown in stirred-suspension bioreactors, spinner flasks, or bag, greatly increasing cell culture capacity[96]. The suspension culture can now produce more than 10^{13} hPSCs[97]. Bioreactors provide a homogenous growth environment with real-time monitoring of oxygen level, medium acidosis, and metabolite concentrations[98]. The shear stress and slowed growth rate are common issues that need to be considered when cells are expanded.

The embryoid body structure mimics cell interaction in embryogenesis, and hPSCs can spontaneously differentiate to cell types of three germ layers in the absence of growth factors. Embryoid body can be used to evaluate pluripotency *in vitro*[99,100], and it has become an attractive alternative to a traditional teratoma assay[101]. The 3D culture can also be used for lineage-specific differentiation. In recent years, organoid is becoming a powerful model to understand embryogenesis and lineage-specific differentiation[102]. hPSC embryoid bodies can be adapted to organoid differentiation for specific cell types[103,104].

hPSCs in suspension display altered metabolic status and slower cell proliferation. Xu and colleagues utilized the altered hPSC physiology in suspension to develop the spheropreservation method[105]. In suspension, hPSC embryoid bodies can maintain cell viability and pluripotency at room temperature for several days. This allows the cells to be transported at room temperature without cryopreservation. It is a convenient way to transport cells without conventional methods that require either

dry ice for frozen cells or a 37 °C container for live cell culture vessels.

Biomaterial-specific 3D platforms

3D scaffolds are increasingly used for hPSC maintenance and differentiation with the assistance of natural and synthetic materials[106]. These materials are usually biocompatible and biodegradable, and provide various biological signals and mechanical strength for specific applications. Many natural materials, such as hyaluronic acid (HA) and alginate, are functional for hPSCs maintenance, but they are difficult to control due to undefined polymer size and the potential to influence cellular signal transduction[107]. Synthetic polymers such as polyethylene glycol (PEG) are readily polymerized, and can be functionalized with specific ligands. This allows fine-tuning of the stem cell niche to meet the requirements of various applications. PEG-based hydrogel has been used to study the effect of N-cadherin peptide on mESC growth and neural differentiation[108], and also shown to successfully support the development of neural tube structure from single mESCs[109]. Many ligands have been discussed in previous sections. With the proper choice of biomaterials, hPSCs can be maintained, differentiated, and cryopreserved efficiently in 3D platforms[110].

Miniature culture systems

Besides large-scale production for therapeutic applications, new platforms have been developed as miniature culture systems that can be used for basic research and drug screening.

Microfluidic systems feature automatic operation, precise control of treatment parameters, as well as integrated functional modules. It has been used to interrogate the effect of cell patterning, physical factors, chemical factors as well as cell-cell and cell-ECM interactions in hPSCs. Microfluidic chips were designed for analysis of hPSC response to treatments as single colonies[111,112]. Similar systems have been used to understand how growth factor signals could impact cell fate determination[113,114], comparable to organ-on-a-chip platforms for cancer and somatic cells. Microfluidic devices are powerful tools for research in cellular function, cell fate determination as well as disease modeling.

Recently, gastruloid culture platforms were developed for pluripotent stem cells as a multicellular *in vitro* model of the gastrulating embryo. Generation of geometrically confined stem cell colonies significantly improves the reproducibility and quantitative analysis of differentiation. 2D micropatterned hESC colonies are generated on a surface coated with patterned ECM proteins or ligands[115]. The pattern can be precisely controlled in size, shape, and ligand. This platform not only improves the reproducibility of differentiation, but also provides a platform for microscopic imaging and screening. Recently, gastruloid culture has been used to study how geometric constrains could affect cell fate determination with specific spatial distribution[116]. An important advantage of this system compared to the embryoid bodies platform is the better control over cell number, being important when reproducing early developmental stages influenced by cell number and patterning.

CONCLUSION

In the past 20 years, hPSC culture technologies have evolved extensively on all fronts. We can expect to see hPSCs at all three embryonic stages efficiently maintained very soon. However, development of clinical therapies or disease models needs more than just pluripotent stem cells. The next great challenge is to efficiently differentiate or generate specific cell types from hPSCs in cell culture, which will require more complex signal transduction and medium composition for differentiation initiation, cell fate specification, and maturation. In addition, the cell culture platform will be involved in how differentiated cells could be used in various applications, which will require the infusion of bioengineering technologies and efficient cell handling methods. The principles of hPSC culture technology could be applied to differentiation and further applications. More exciting cell culture advances will eventually help to realize the great potential of hPSCs just as people imagined when the cells were first derived in 1998.

ACKNOWLEDGEMENTS

We thank Dr. Garry Wong for his comments and suggestions.

REFERENCES

1 **Thomson JA**, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: 9804556]
2 **Reubinoff BE**, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* 2000; **18**: 399-404 [PMID: 10748519 DOI: 10.1038/74447]
3 **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]
4 **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]
5 **Castelvecchi D**. Billion-star map of Milky Way set to transform astronomy. *Nature* 2018; **557**: 18 [PMID: 29717241 DOI: 10.1038/d41586-018-04979-4]
6 **Yamanaka S**. Induced pluripotent stem cells: past, present, and future. *Cell Stem Cell* 2012; **10**: 678-684 [PMID: 22704507 DOI: 10.1016/j.stem.2012.05.005]
7 **Pera MF**, Tam PP. Extrinsic regulation of pluripotent stem cells. *Nature* 2010; **465**: 713-720 [PMID: 20535200 DOI: 10.1038/nature09228]
8 **Chen KG**, Mallon BS, McKay RD, Robey PG. Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics. *Cell Stem Cell* 2014; **14**: 13-26 [PMID: 24388173 DOI: 10.1016/j.stem.2013.12.005]
9 **Ying QL**, Smith A. The Art of Capturing Pluripotency: Creating the Right Culture. *Stem Cell Reports* 2017; **8**: 1457-1464 [PMID: 28591647 DOI: 10.1016/j.stemcr.2017.05.020]
10 **Desai N**, Rambhia P, Gishto A. Human embryonic stem cell cultivation: historical perspective and evolution of xeno-free culture systems. *Reprod Biol Endocrinol* 2015; **13**: 9 [PMID: 25890180 DOI: 10.1186/s12958-015-0005-4]
11 **Tesar PJ**, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL, McKay RD. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 2007; **448**: 196-199 [PMID: 17597760 DOI: 10.1038/nature05972]
12 **Evans MJ**, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; **292**: 154-156 [PMID: 7242681 DOI: 10.1038/292154a0]
13 **Martin GR**. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 1981; **78**: 7634-7638 [PMID: 6950406 DOI: 10.1073/pnas.78.12.7634]
14 **Yang J**, Ryan DJ, Wang W, Tsang JC, Lan G, Masaki H, Gao X, Antunes L, Yu Y, Zhu Z, Wang J, Kolodziejczyk AA, Campos LS, Wang C, Yang F, Zhong Z, Fu B, Eckersley-Maslin MA, Woods M, Tanaka Y, Chen X, Wilkinson AC, Bussell J, White J, Ramirez-Solis R, Reik W, Göttgens B, Teichmann SA, Tam PPL, Nakauchi H, Zou X, Lu L, Liu P. Establishment of mouse expanded potential stem cells. *Nature* 2017; **550**: 393-397 [PMID: 29019987 DOI: 10.1038/nature24052]
15 **Yang Y**, Liu B, Xu J, Wang J, Wu J, Shi C, Xu Y, Dong J, Wang C, Lai W, Zhu J, Xiong L, Zhu D, Li X, Yang W, Yamauchi T, Sugawara A, Li Z, Sun F, Li X, Li C, He A, Du Y, Wang T, Zhao C, Li H, Chi X, Zhang H, Liu Y, Li C, Duo S, Yin M, Shen H, Belmonte JCI, Deng H. Derivation of Pluripotent Stem Cells with In Vivo Embryonic and Extraembryonic Potency. *Cell* 2017; **169**: 243-257.e25 [PMID: 28388409 DOI: 10.1016/j.cell.2017.02.005]
16 **Vallier L**, Alexander M, Pedersen RA. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J Cell Sci* 2005; **118**: 4495-4509 [PMID: 16179608 DOI: 10.1242/jcs.02553]
17 **Chen G**, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, Wagner R, Lee GO, Antosiewicz-Bourget J, Teng JM, Thomson JA. Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* 2011; **8**: 424-429 [PMID: 21478862 DOI: 10.1038/nmeth.1593]
18 **International Stem Cell Initiative Consortium**. Akopian V, Andrews PW, Beil S, Benvenisty N, Brehm J, Christie M, Ford A, Fox V, Gokhale PJ, Healy L, Holm F, Hovatta O, Knowles BB, Ludwig TE, McKay RD, Miyazaki T, Nakatsuji N, Oh SK, Pera MF, Rossant J, Stacey GN, Suemori H. Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. *In Vitro Cell Dev Biol Anim* 2010; **46**: 247-258 [PMID: 20186512 DOI: 10.1007/s11626-010-9297-z]
19 **Lanner F**, Rossant J. The role of FGF/Erk signaling in pluripotent cells. *Development* 2010; **137**: 3351-3360 [PMID: 20876656 DOI: 10.1242/dev.050146]
20 **Xu RH**, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods* 2005; **2**: 185-190 [PMID: 15782187 DOI: 10.1038/nmeth744]
21 **Wang X**, Lin G, Martins-Taylor K, Zeng H, Xu RH. Inhibition of caspase-mediated anoikis is critical for basic fibroblast growth factor-sustained culture of human pluripotent stem cells. *J Biol Chem* 2009; **284**: 34054-34064 [PMID: 19828453 DOI: 10.1074/jbc.M109.052290]
22 **Sato N**, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* 2004; **10**: 55-63 [PMID: 14702635 DOI: 10.1038/nm979]
23 **Wang L**, Schulz TC, Sherrer ES, Dauphin DS, Shin S, Nelson AM, Ware CB, Zhan M, Song CZ, Chen X, Brimble SN, McLean A, Galeano MJ, Uhl EW, D'Amour KA, Chesnut JD, Rao MS, Blau CA, Robins AJ. Self-renewal of human embryonic stem cells requires insulin-like growth factor-1 receptor and ERBB2 receptor signaling. *Blood* 2007; **110**: 4111-4119 [PMID: 17761519 DOI: 10.1182/blood-2007-03-082586]
24 **Avery K**, Avery S, Shepherd J, Heath PR, Moore H. Sphingosine-1-phosphate mediates transcriptional regulation of key targets associated with survival, proliferation, and pluripotency in human embryonic stem cells. *Stem Cells Dev* 2008; **17**: 1195-1205 [PMID: 18393631 DOI: 10.1089/scd.2008.0063]
25 **Wong RC**, Pera MF, Pébay A. Maintenance of human embryonic stem cells by sphingosine-1-phosphate and platelet-derived growth factor. *Methods Mol Biol* 2012; **874**: 167-175 [PMID: 22528447 DOI: 10.1007/978-1-61779-800-9_13]
26 **Li Y**, Powell S, Brunette E, Lebkowski J, Mandalam R. Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. *Biotechnol Bioeng* 2005; **91**: 688-698

[PMID: 15971228 DOI: 10.1002/bit.20536]
27 **Levenstein ME**, Berggren WT, Lee JE, Conard KR, Llanas RA, Wagner RJ, Smith LM, Thomson JA. Secreted proteoglycans directly mediate human embryonic stem cell-basic fibroblast growth factor 2 interactions critical for proliferation. *Stem Cells* 2008; **26**: 3099-3107 [PMID: 18802039 DOI: 10.1634/stemcells.2007-1056]
28 **Chen G**, Gulbranson DR, Yu P, Hou Z, Thomson JA. Thermal stability of fibroblast growth factor protein is a determinant factor in regulating self-renewal, differentiation, and reprogramming in human pluripotent stem cells. *Stem Cells* 2012; **30**: 623-630 [PMID: 22213113 DOI: 10.1002/stem.1021]
29 **Pébay A**, Wong RC, Pitson SM, Wolvetang EJ, Peh GS, Filipczyk A, Koh KL, Tellis I, Nguyen LT, Pera MF. Essential roles of sphingosine-1-phosphate and platelet-derived growth factor in the maintenance of human embryonic stem cells. *Stem Cells* 2005; **23**: 1541-1548 [PMID: 16081668 DOI: 10.1634/stemcells.2004-0338]
30 **Ludwig TE**, Bergendahl V, Levenstein ME, Yu J, Probasco MD, Thomson JA. Feeder-independent culture of human embryonic stem cells. *Nat Methods* 2006; **3**: 637-646 [PMID: 16862139 DOI: 10.1038/nmeth902]
31 **Xu RH**, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, Thomson JA. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 2002; **20**: 1261-1264 [PMID: 12426580 DOI: 10.1038/nbt761]
32 **Davidson KC**, Adams AM, Goodson JM, McDonald CE, Potter JC, Berndt JD, Biechele TL, Taylor RJ, Moon RT. Wnt/β-catenin signaling promotes differentiation, not self-renewal, of human embryonic stem cells and is repressed by Oct4. *Proc Natl Acad Sci U S A* 2012; **109**: 4485-4490 [PMID: 22392999 DOI: 10.1073/pnas.1118777109]
33 **Lu J**, Hou R, Booth CJ, Yang SH, Snyder M. Defined culture conditions of human embryonic stem cells. *Proc Natl Acad Sci U S A* 2006; **103**: 5688-5693 [PMID: 16595624 DOI: 10.1073/pnas.0601383103]
34 **Davis LA**, Zur Nieden NI. Mesodermal fate decisions of a stem cell: the Wnt switch. *Cell Mol Life Sci* 2008; **65**: 2658-2674 [PMID: 18528633 DOI: 10.1007/s00018-008-8042-1]
35 **Elkouby YM**, Frank D. 2010 [PMID: 21452442 DOI: 10.4199/C00015ED1V01Y201007DEB004]
36 **Moldes M**, Zuo Y, Morrison RF, Silva D, Park BH, Liu J, Farmer SR. Peroxisome-proliferator-activated receptor gamma suppresses Wnt/beta-catenin signalling during adipogenesis. *Biochem J* 2003; **376**: 607-613 [PMID: 12954078 DOI: 10.1042/BJ20030426]
37 **Chung Y**, Klimanskaya I, Becker S, Li T, Maserati M, Lu SJ, Zdravkovic T, Ilic D, Genbacev O, Fisher S, Krtolica A, Lanza R. Human embryonic stem cell lines generated without embryo destruction. *Cell Stem Cell* 2008; **2**: 113-117 [PMID: 18371431 DOI: 10.1016/j.stem.2007.12.013]
38 **Tachibana M**, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H, Kang E, Fulati A, Lee HS, Sritanaudomchai H, Masterson K, Larson J, Eaton D, Sadler-Fredd K, Battaglia D, Lee D, Wu D, Jensen J, Patton P, Gokhale S, Stouffer RL, Wolf D, Mitalipov S. Human embryonic stem cells derived by somatic cell nuclear transfer. *Cell* 2013; **153**: 1228-1238 [PMID: 23683578 DOI: 10.1016/j.cell.2013.05.006]
39 **Smith AG**. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol* 2001; **17**: 435-462 [PMID: 11687496 DOI: 10.1146/annurev.cellbio.17.1.435]
40 **Smith AG**, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* 1988; **336**: 688-690 [PMID: 3143917 DOI: 10.1038/336688a0]
41 **Ying QL**, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 2003; **115**: 281-292 [PMID: 14636556 DOI: 10.1016/s0092-8674(03)00847-x]
42 **Ying QL**, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell self-renewal. *Nature* 2008; **453**: 519-523 [PMID: 18497825 DOI: 10.1038/nature06968]
43 **Martello G**, Smith A. The nature of embryonic stem cells. *Annu Rev Cell Dev Biol* 2014; **30**: 647-675 [PMID: 25288119 DOI: 10.1146/annurev-cellbio-100913-013116]
44 **Blakeley P**, Fogarty NM, del Valle I, Wamaitha SE, Hu TX, Elder K, Snell P, Christie L, Robson P, Niakan KK. Defining the three cell lineages of the human blastocyst by single-cell RNA-seq. *Development* 2015; **142**: 3151-3165 [PMID: 26293300 DOI: 10.1242/dev.123547]
45 **Hanna J**, Cheng AW, Saha K, Kim J, Lengner CJ, Soldner F, Cassady JP, Muffat J, Carey BW, Jaenisch R. Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proc Natl Acad Sci USA* 2010; **107**: 9222-9227 [PMID: 20442331 DOI: 10.1073/pnas.1004584107]
46 **Theunissen TW**, Friedli M, He Y, Planet E, O'Neil RC, Markoulaki S, Pontis J, Wang H, Iouranova A, Imbeault M, Duc J, Cohen MA, Wert KJ, Castanon R, Zhang Z, Huang Y, Nery JR, Drotar J, Lungjangwa T, Trono D, Ecker JR, Jaenisch R. Molecular Criteria for Defining the Naive Human Pluripotent State. *Cell Stem Cell* 2016; **19**: 502-515 [PMID: 27424783 DOI: 10.1016/j.stem.2016.06.011]
47 **Guo G**, von Meyenn F, Santos F, Chen Y, Reik W, Bertone P, Smith A, Nichols J. Naive Pluripotent Stem Cells Derived Directly from Isolated Cells of the Human Inner Cell Mass. *Stem Cell Reports* 2016; **6**: 437-446 [PMID: 26947977 DOI: 10.1016/j.stemcr.2016.02.005]
48 **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]
49 **Chan YS**, Göke J, Ng JH, Lu X, Gonzales KA, Tan CP, Tng WQ, Hong ZZ, Lim YS, Ng HH. Induction of a human pluripotent state with distinct regulatory circuitry that resembles preimplantation epiblast. *Cell Stem Cell* 2013; **13**: 663-675 [PMID: 24315441 DOI: 10.1016/j.stem.2013.11.015]
50 **Valamehr B**, Robinson M, Abujarour R, Rezner B, Vranceanu F, Le T, Medcalf A, Lee TT, Fitch M, Robbins D, Flynn P. Platform for induction and maintenance of transgene-free hiPSCs resembling ground state pluripotent stem cells. *Stem Cell Reports* 2014; **2**: 366-381 [PMID: 24672758 DOI: 10.1016/j.stemcr.2014.01.014]
51 **Ware CB**, Nelson AM, Mecham B, Hesson J, Zhou W, Jonlin EC, Jimenez-Caliani AJ, Deng X, Cavanaugh C, Cook S, Tesar PJ, Okada J, Margaretha L, Sperber H, Choi M, Blau CA, Treuting PM, Hawkins RD, Cirulli V, Ruohola-Baker H. Derivation of naive human embryonic stem cells. *Proc Natl Acad Sci U S A* 2014; **111**: 4484-4489 [PMID: 24623855 DOI: 10.1073/pnas.1319738111]
52 **Liu Y**, Song Z, Zhao Y, Qin H, Cai J, Zhang H, Yu T, Jiang S, Wang G, Ding M, Deng H. A novel chemical-defined medium with bFGF and N2B27 supplements supports undifferentiated growth in human

embryonic stem cells. *Biochem Biophys Res Commun* 2006; **346**: 131-139 [PMID: 16753134 DOI: 10.1016/j.bbrc.2006.05.086]
53 **Xu C**, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 2001; **19**: 971-974 [PMID: 11581665 DOI: 10.1038/nbt1001-971]
54 **Kleinman HK**, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR. Basement membrane complexes with biological activity. *Biochemistry* 1986; **25**: 312-318 [PMID: 2937447]
55 **Kleinman HK**. Preparation of basement membrane components from EHS tumors. *Curr Protoc Cell Biol* 2001; **Chapter** 10: Unit 10.2 [PMID: 18228296 DOI: 10.1002/0471143030.cb1002s00]
56 **Braam SR**, Zeinstra L, Litjens S, Ward-van Oostwaard D, van den Brink S, van Laake L, Lebrin F, Kats P, Hochstenbach R, Passier R, Sonnenberg A, Mummery CL. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. *Stem Cells* 2008; **26**: 2257-2265 [PMID: 18599809 DOI: 10.1634/stemcells.2008-0291]
57 **Miyazaki T**, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M, Hayashi M, Kumagai H, Nakatsuji N, Sekiguchi K, Kawase E. Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. *Nat Commun* 2012; **3**: 1236 [PMID: 23212365 DOI: 10.1038/ncomms2231]
58 **Pijuan-Galitó S**, Tamm C, Schuster J, Sobol M, Forsberg L, Merry CL, Annerén C. Human serum-derived protein removes the need for coating in defined human pluripotent stem cell culture. *Nat Commun* 2016; **7**: 12170 [PMID: 27405751 DOI: 10.1038/ncomms12170]
59 **Rodin S**, Domogatskaya A, Ström S, Hansson EM, Chien KR, Inzunza J, Hovatta O, Tryggvason K. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. *Nat Biotechnol* 2010; **28**: 611-615 [PMID: 20512123 DOI: 10.1038/nbt.1620]
60 **Li L**, Bennett SA, Wang L. Role of E-cadherin and other cell adhesion molecules in survival and differentiation of human pluripotent stem cells. *Cell Adh Migr* 2012; **6**: 59-70 [PMID: 22647941 DOI: 10.4161/cam.19583]
61 **Nagaoka M**, Si-Tayeb K, Akaike T, Duncan SA. Culture of human pluripotent stem cells using completely defined conditions on a recombinant E-cadherin substratum. *BMC Dev Biol* 2010; **10**: 60 [PMID: 20525219 DOI: 10.1186/1471-213X-10-60]
62 **Rodin S**, Antonsson L, Niaudet C, Simonson OE, Salmela E, Hansson EM, Domogatskaya A, Xiao Z, Damdimopoulou P, Sheikhi M, Inzunza J, Nilsson AS, Baker D, Kuiper R, Sun Y, Blennow E, Nordenskjöld M, Grinnemo KH, Kere J, Betsholtz C, Hovatta O, Tryggvason K. Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. *Nat Commun* 2014; **5**: 3195 [PMID: 24463987 DOI: 10.1038/ncomms4195]
63 **Melkoumian Z**, Weber JL, Weber DM, Fadeev AG, Zhou Y, Dolley-Sonneville P, Yang J, Qiu L, Priest CA, Shogbon C, Martin AW, Nelson J, West P, Beltzer JP, Pal S, Brandenberger R. Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. *Nat Biotechnol* 2010; **28**: 606-610 [PMID: 20512120 DOI: 10.1038/nbt.1629]
64 **Kolhar P**, Kotamraju VR, Hikita ST, Clegg DO, Ruoslahti E. Synthetic surfaces for human embryonic stem cell culture. *J Biotechnol* 2010; **146**: 143-146 [PMID: 20132848 DOI: 10.1016/j.jbiotec.2010.01.016]
65 **Cowan CA**, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, Wang S, Morton CC, McMahon AP, Powers D, Melton DA. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 2004; **350**: 1353-1356 [PMID: 14999088 DOI: 10.1056/NEJMsr040330]
66 **Watanabe K**, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K, Sasai Y. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol* 2007; **25**: 681-686 [PMID: 17529971 DOI: 10.1038/nbt1310]
67 **Chen G**, Hou Z, Gulbranson DR, Thomson JA. Actin-myosin contractility is responsible for the reduced viability of dissociated human embryonic stem cells. *Cell Stem Cell* 2010; **7**: 240-248 [PMID: 20682449 DOI: 10.1016/j.stem.2010.06.017]
68 **Meng Y**, Ren Z, Xu F, Zhou X, Song C, Wang VY, Liu W, Lu L, Thomson JA, Chen G. Nicotinamide Promotes Cell Survival and Differentiation as Kinase Inhibitor in Human Pluripotent Stem Cells. *Stem Cell Reports* 2018; **11**: 1347-1356 [PMID: 30503259 DOI: 10.1016/j.stemcr.2018.10.023]
69 **Walker A**, Su H, Conti MA, Harb N, Adelstein RS, Sato N. Non-muscle myosin II regulates survival threshold of pluripotent stem cells. *Nat Commun* 2010; **1**: 71 [PMID: 20842192 DOI: 10.1038/ncomms1074]
70 **T'joen V**, Declercq H, Cornelissen M. Expansion of human embryonic stem cells: a comparative study. *Cell Prolif* 2011; **44**: 462-476 [PMID: 21951289 DOI: 10.1111/j.1365-2184.2011.00773.x]
71 **Bajpai R**, Lesperance J, Kim M, Terskikh AV. Efficient propagation of single cells Accutase-dissociated human embryonic stem cells. *Mol Reprod Dev* 2008; **75**: 818-827 [PMID: 18157870 DOI: 10.1002/mrd.20809]
72 **Beers J**, Gulbranson DR, George N, Siniscalchi LI, Jones J, Thomson JA, Chen G. Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions. *Nat Protoc* 2012; **7**: 2029-2040 [PMID: 23099485 DOI: 10.1038/nprot.2012.130]
73 **Nie Y**, Walsh P, Clarke DL, Rowley JA, Fellner T. Scalable passaging of adherent human pluripotent stem cells. *PLoS One* 2014; **9**: e88012 [PMID: 24498239 DOI: 10.1371/journal.pone.0088012]
74 **Claassen DA**, Desler MM, Rizzino A. ROCK inhibition enhances the recovery and growth of cryopreserved human embryonic stem cells and human induced pluripotent stem cells. *Mol Reprod Dev* 2009; **76**: 722-732 [PMID: 19235204 DOI: 10.1002/mrd.21021]
75 **Martin-Ibañez R**, Unger C, Strömberg A, Baker D, Canals JM, Hovatta O. Novel cryopreservation method for dissociated human embryonic stem cells in the presence of a ROCK inhibitor. *Hum Reprod* 2008; **23**: 2744-2754 [PMID: 18716037 DOI: 10.1093/humrep/den316]
76 **Santos DP**, Kiskinis E, Eggan K, Merkle FT. Comprehensive Protocols for CRISPR/Cas9-based Gene Editing in Human Pluripotent Stem Cells. *Curr Protoc Stem Cell Biol* 2016; **38**: 5B.6.1-5B.6.60 [PMID: 27532820 DOI: 10.1002/cpsc.15]
77 **Zou J**, Maeder ML, Mali P, Pruett-Miller SM, Thibodeau-Beganny S, Chou BK, Chen G, Ye Z, Park IH, Daley GQ, Porteus MH, Joung JK, Cheng L. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell* 2009; **5**: 97-110 [PMID: 19540188 DOI: 10.1016/j.stem.2009.05.023]
78 **Yao T**, Asayama Y. Animal-cell culture media: History, characteristics, and current issues. *Reprod Med Biol* 2017; **16**: 99-117 [PMID: 29259457 DOI: 10.1002/rmb2.12024]
79 **Amit M**, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA.

Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* 2000; **227**: 271-278 [PMID: 11071754 DOI: 10.1006/dbio.2000.9912]

80 **Ludwig TE**, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, Frane JL, Crandall LJ, Daigh CA, Conard KR, Piekarczyk MS, Llanas RA, Thomson JA. Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* 2006; **24**: 185-187 [PMID: 16388305 DOI: 10.1038/nbt1177]

81 **Lee P**, Wu X. Review: modifications of human serum albumin and their binding effect. *Curr Pharm Des* 2015; **21**: 1862-1865 [PMID: 25732553 DOI: 10.2174/1381612821666150302115025]

82 **Merlot AM**, Kalinowski DS, Richardson DR. Unraveling the mysteries of serum albumin-more than just a serum protein. *Front Physiol* 2014; **5**: 299 [PMID: 25161624 DOI: 10.3389/fphys.2014.00299]

83 **Wang Y**, Chou BK, Dowey S, He C, Gerecht S, Cheng L. Scalable expansion of human induced pluripotent stem cells in the defined xeno-free E8 medium under adherent and suspension culture conditions. *Stem Cell Res* 2013; **11**: 1103-1116 [PMID: 23973800 DOI: 10.1016/j.scr.2013.07.011]

84 **Uenishi G**, Theisen D, Lee JH, Kumar A, Raymond M, Vodyanik M, Swanson S, Stewart R, Thomson J, Slukvin I. Tenascin C promotes hematoendothelial development and T lymphoid commitment from human pluripotent stem cells in chemically defined conditions. *Stem Cell Reports* 2014; **3**: 1073-1084 [PMID: 25448067 DOI: 10.1016/j.stemcr.2014.09.014]

85 **Burridge PW**, Holmström A, Wu JC. Chemically Defined Culture and Cardiomyocyte Differentiation of Human Pluripotent Stem Cells. *Curr Protoc Hum Genet* 2015; **87**: 21.3.1-21.315 [PMID: 26439715 DOI: 10.1002/0471142905.hg2103s87]

86 **Lindborg BA**, Brekke JH, Vegoe AL, Ulrich CB, Haider KT, Subramaniam S, Venhuizen SL, Eide CR, Orchard PJ, Chen W, Wang Q, Pelaez F, Scott CM, Kokkoli E, Keirstead SA, Dutton JR, Tolar J, O'Brien TD. Rapid Induction of Cerebral Organoids From Human Induced Pluripotent Stem Cells Using a Chemically Defined Hydrogel and Defined Cell Culture Medium. *Stem Cells Transl Med* 2016; **5**: 970-979 [PMID: 27177577 DOI: 10.5966/sctm.2015-0305]

87 **Liu W**, Ren Z, Lu K, Song C, Cheung ECW, Zhou Z, Chen G. The Suppression of Medium Acidosis Improves the Maintenance and Differentiation of Human Pluripotent Stem Cells at High Density in Defined Cell Culture Medium. *Int J Biol Sci* 2018; **14**: 485-496 [PMID: 29805300 DOI: 10.7150/ijbs.24681]

88 **Lotz S**, Goderie S, Tokas N, Hirsch SE, Ahmad F, Corneo B, Le S, Banerjee A, Kane RS, Stern JH, Temple S, Fasano CA. Sustained levels of FGF2 maintain undifferentiated stem cell cultures with biweekly feeding. *PLoS One* 2013; **8**: e56289 [PMID: 23437109 DOI: 10.1371/journal.pone.0056289]

89 **Zhang H**, Badur MG, Divakaruni AS, Parker SJ, Jäger C, Hiller K, Murphy AN, Metallo CM. Distinct Metabolic States Can Support Self-Renewal and Lipogenesis in Human Pluripotent Stem Cells under Different Culture Conditions. *Cell Rep* 2016; **16**: 1536-1547 [PMID: 27477285 DOI: 10.1016/j.celrep.2016.06.102]

90 **McKee C**, Chaudhry GR. Advances and challenges in stem cell culture. *Colloids Surf B Biointerfaces* 2017; **159**: 62-77 [PMID: 28780462 DOI: 10.1016/j.colsurfb.2017.07.051]

91 **Mirbagheri M**, Adibnia V, Hughes BR, Waldman SD, Banquy X, Hwang DK. Advanced cell culture platforms: a growing quest for emulating natural tissues. *Materials Horizons* 2019; **6**: 45-71 [DOI: 10.1039/c8mh00803e]

92 **Pettinato G**, Wen X, Zhang N. Engineering Strategies for the Formation of Embryoid Bodies from Human Pluripotent Stem Cells. *Stem Cells Dev* 2015; **24**: 1595-1609 [PMID: 25900308 DOI: 10.1089/scd.2014.0427]

93 **Lin Y**, Chen G. Embryoid body formation from human pluripotent stem cells in chemically defined E8 media, in StemBook. 2008: Cambridge (MA).

94 **Adil MM**, Schaffer DV. Expansion of human pluripotent stem cells. *Curr Opin Chem Eng* 2017; **15**: 24-35 [DOI: 10.1016/j.coche.2016.11.002]

95 **Langhans SA**. Three-Dimensional *in Vitro* Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol* 2018; **9**: 6 [PMID: 29410625 DOI: 10.3389/fphar.2018.00006]

96 **Kropp C**, Massai D, Zweigerdt R. Progress and challenges in large-scale expansion of human pluripotent stem cells. *Process Biochem* 2017; **59**: 244-254 [DOI: 10.1016/j.procbio.2016.09.032]

97 **Li X**, Ma R, Gu Q, Liang L, Wang L, Zhang Y, Wang X, Liu X, Li Z, Fang J, Wu J, Wang Y, Li W, Hu B, Wang L, Zhou Q, Hao J. A fully defined static suspension culture system for large-scale human embryonic stem cell production. *Cell Death Dis* 2018; **9**: 892 [PMID: 30166524 DOI: 10.1038/s41419-018-0863-8]

98 **Kropp C**, Kempf H, Halloin C, Robles-Diaz D, Franke A, Scheper T, Kinast K, Knorpp T, Joos TO, Haverich A, Martin U, Zweigerdt R, Olmer R. Impact of Feeding Strategies on the Scalable Expansion of Human Pluripotent Stem Cells in Single-Use Stirred Tank Bioreactors. *Stem Cells Transl Med* 2016; **5**: 1289-1301 [PMID: 27369897 DOI: 10.5966/sctm.2015-0253]

99 **Fergus J**, Quintanilla R, Lakshmipathy U. Characterizing Pluripotent Stem Cells Using the TaqMan® hPSC Scorecard(TM) Panel. *Methods Mol Biol* 2016; **1307**: 25-37 [PMID: 25138722 DOI: 10.1007/7651_2014_109]

100 **Müller FJ**, Brändl B, Loring JF. Assessment of human pluripotent stem cells with PluriTest 2012. [PMID: 23658970]

101 **Avior Y**, Biancotti JC, Benvenisty N. TeratoScore: Assessing the Differentiation Potential of Human Pluripotent Stem Cells by Quantitative Expression Analysis of Teratomas. *Stem Cell Reports* 2015; **4**: 967-974 [PMID: 26070610 DOI: 10.1016/j.stemcr.2015.05.006]

102 **Editorial**. Method of the Year 2017: Organoids. *Nature Methods* 2018; **15(1)**: p. 1-1 [DOI: 10.1038/nmeth.4575]

103 **Mazerik J.N**, Becker S, Sieving P.A. Sieving, 3-D retina organoids: Building platforms for therapies of the future. *Cell Medicine* 2018; **10** [DOI: 10.1177/2155179018773758]

104 **Li Y**, Muffat J, Omer A, Bosch I, Lancaster MA, Sur M, Gehrke L, Knoblich JA, Jaenisch R. Induction of Expansion and Folding in Human Cerebral Organoids. *Cell Stem Cell* 2017; **20**: 385-396.e3 [PMID: 28041895 DOI: 10.1016/j.stem.2016.11.017]

105 **Jiang B**, Yan L, Miao Z, Li E, Wong KH, Xu RH. Spheroidal formation preserves human stem cells for prolonged time under ambient conditions for facile storage and transportation. *Biomaterials* 2017; **133**: 275-286 [PMID: 28460350 DOI: 10.1016/j.biomaterials.2017.03.050]

106 **Kraehenbuehl TP**, Langer R, Ferreira LS. Three-dimensional biomaterials for the study of human pluripotent stem cells. *Nat Methods* 2011; **8**: 731-736 [PMID: 21878920 DOI: 10.1038/nmeth.1671]

107 **Gerecht S**, Burdick JA, Ferreira LS, Townsend SA, Langer R, Vunjak-Novakovic G. Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. *Proc Natl Acad*

Sci USA 2007; **104**: 11298-11303 [PMID: 17581871 DOI: 10.1073/pnas.0703723104]
108 **Lim HJ**, Mosley MC, Kurosu Y, Smith Callahan LA. Concentration dependent survival and neural differentiation of murine embryonic stem cells cultured on polyethylene glycol dimethacrylate hydrogels possessing a continuous concentration gradient of n-cadherin derived peptide His-Ala-Val-Asp-Lle. *Acta Biomater* 2017; **56**: 153-160 [PMID: 27915022 DOI: 10.1016/j.actbio.2016.11.063]
109 **Ishihara K**, Ranga A, Lutolf MP, Tanaka EM, Meinhardt A. Reconstitution of a Patterned Neural Tube from Single Mouse Embryonic Stem Cells. *Methods Mol Biol* 2017; **1597**: 43-55 [PMID: 28361309 DOI: 10.1007/978-1-4939-6949-4_4]
110 **Serra M**, Correia C, Malpique R, Brito C, Jensen J, Bjorquist P, Carrondo MJ, Alves PM. Microencapsulation technology: a powerful tool for integrating expansion and cryopreservation of human embryonic stem cells. *PLoS One* 2011; **6**: e23212 [PMID: 21850261 DOI: 10.1371/journal.pone.0023212]
111 **Kamei K**, Guo S, Yu ZT, Takahashi H, Gschweng E, Suh C, Wang X, Tang J, McLaughlin J, Witte ON, Lee KB, Tseng HR. An integrated microfluidic culture device for quantitative analysis of human embryonic stem cells. *Lab Chip* 2009; **9**: 555-563 [PMID: 19190791 DOI: 10.1039/b809105f]
112 **Villa-Diaz LG**, Torisawa YS, Uchida T, Ding J, Nogueira-de-Souza NC, O'Shea KS, Takayama S, Smith GD. Microfluidic culture of single human embryonic stem cell colonies. *Lab Chip* 2009; **9**: 1749-1755 [PMID: 19495459 DOI: 10.1039/b820380f]
113 **Sikorski DJ**, Caron NJ, VanInsberghe M, Zahn H, Eaves CJ, Piret JM, Hansen CL. Clonal analysis of individual human embryonic stem cell differentiation patterns in microfluidic cultures. *Biotechnol J* 2015; **10**: 1546-1554 [PMID: 26059045 DOI: 10.1002/biot.201500035]
114 **Zhang J**, Wei X, Zeng R, Xu F, Li X. Stem cell culture and differentiation in microfluidic devices toward organ-on-a-chip. *Future Sci OA* 2017; **3**: FSO187 [PMID: 28670476 DOI: 10.4155/fsoa-2016-0091]
115 **Simunovic M**, Brivanlou AH. Embryoids, organoids and gastruloids: new approaches to understanding embryogenesis. *Development* 2017; **144**: 976-985 [PMID: 28292844 DOI: 10.1242/dev.143529]
116 **Warmflash A**, Sorre B, Etoc F, Siggia ED, Brivanlou AH. A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. *Nat Methods* 2014; **11**: 847-854 [PMID: 24973948 DOI: 10.1038/nmeth.3016]
117 **Yao S**, Chen S, Clark J, Hao E, Beattie GM, Hayek A, Ding S. Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. *Proceedings of the National Academy of Sciences of the United States of America* 2006; **103**: 6907-6912 [PMID: 16632596 DOI: 10.1073/pnas.0602280103]
118 **Frank S**, Zhang M, Scholer HR, Greber B. Small molecule-assisted, line-independent maintenance of human pluripotent stem cells in defined conditions. *PloS One* 2012; **7**: e41958 [PMID: 22860038 DOI: 10.1371/journal.pone.0041958]
119 **Collier AJ**, Rugg-Gunn PJ. Identifying Human Naïve Pluripotent Stem Cells - Evaluating State-Specific Reporter Lines and Cell-Surface Markers. *Bioessays* 2018; **40**: e1700239 [PMID: 29574793 DOI: 10.1002/bies.201700239]
120 **Theunissen TW**, Powell BE, Wang H, Mitalipova M, Faddah DA, Reddy J, Fan ZP, Maetzel D, Ganz K, Shi L, Lungjangwa T, Imsoonthornruksa S, Stelzer Y, Rangarajan S, D'Alessio A, Zhang J, Gao Q, Dawlaty MM, Young RA, Gray NS, Jaenisch R. Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. *Cell stem cell* 2014; **15**: 524-526 [PMID: 28903030 DOI: 10.1016/j.stem.2014.09.003]
121 **Collier AJ**, Panula SP, Schell JP, Chovanec P, Plaza Reyes A, Petropoulos S, Corcoran AE, Walker R, Douagi I, Lanner F, Rugg-Gunn PJ. Comprehensive Cell Surface Protein Profiling Identifies Specific Markers of Human Naive and Primed Pluripotent States. *Cell Stem Cell* 2017; **20**: 874-890.e7 [PMID: 28343983 DOI: 10.1016/j.stem.2017.02.014]

World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2019 November 26; 11(11): 982-989

DOI: 10.4252/wjsc.v11.i11.982

ISSN 1948-0210 (online)

MINIREVIEWS

Monitoring maturation of neural stem cell grafts within a host microenvironment

Olga Kopach

ORCID number: Olga Kopach (0000-0002-3921-3674).

Author contributions: Kopach O analyzed the literature, designed the outline and wrote the paper, prepared the figure.

Conflict-of-interest statement: There is no conflict of interest to disclose.

Manuscript source: Invited manuscript

Received: March 23, 2019
Peer-review started: March 26, 2019
First decision: August 1, 2019
Revised: September 8, 2019
Accepted: October 1, 2019
Article in press: October 1, 2019
Published online: November 26, 2019

P-Reviewer: Atwood CS, Schmidt NO
S-Editor: Dou Y
L-Editor: A
E-Editor: Xing YX

Olga Kopach, Department of Clinical and Experimental Epilepsy, Queen Square Institute of Neurology, University College London, London WC1 N3BG, United Kingdom

Corresponding author: Olga Kopach, PhD, Department of Clinical and Experimental Epilepsy, Queen Square Institute of Neurology, University College London, Queen Square House, London WC1N3BG, United Kingdom. o.kopach@ucl.ac.uk
Telephone: +44-20-3448447

Abstract

Neural stem cells (NSC) act as a versatile tool for neuronal cell replacement strategies to treat neurodegenerative disorders in which functional neurorestorative mechanisms are limited. While the beneficial effects of such cell-based therapy have already been documented in terms of neurodegeneration of various origins, a neurophysiological basis for improvement in the recovery of neurological function is still not completely understood. This overview briefly describes the cumulative evidence from electrophysiological studies of NSC-derived neurons, aimed at establishing the maturation of differentiated neurons within a host microenvironment, and their integration into the host circuits, with a particular focus on the neurogenesis of NSC grafts within the post-ischemic milieu. Overwhelming evidence demonstrates that the host microenvironment largely regulates the lineage of NSC grafts. This regulatory role, as yet underestimated, raises possibilities for the favoured maturation of a subset of neural phenotypes in order to gain timely remodelling of the impaired brain tissue and amplify the therapeutic effects of NSC-based therapy for recovery of neurological function.

Key words: Neural stem cells; Embryonic progenitors; Neurogenesis; Maturation of neurophysiological properties; Integration into network; Neural stem cell therapy; Neurodegeneration; Ischemic injury

Core tip: Electrophysiology combined with post-hoc immunohistochemistry was utilized for monitoring the maturation of neural stem cell (NSC)-derived hippocampal neurons within a host tissue, aimed at establishing the neurogenesis of NSC grafts between physiological and post-ischemic endogenous milieus. Understanding the timing maturation of the neurophysiological properties of differentiated neurons within the microenvironment of a host brain tissue will provide an assessment of the effects of cell-based therapy with regard to neurodegenerative disorders of varied aetiology.

Citation: Kopach O. Monitoring maturation of neural stem cell grafts within a host microenvironment. *World J Stem Cells* 2019; 11(11): 982-989
URL: https://www.wjgnet.com/1948-0210/full/v11/i11/982.htm
DOI: https://dx.doi.org/10.4252/wjsc.v11.i11.982

INTRODUCTION

Stem cell therapy has emerged to become a universal "rescue" tool for a broad range of neurological disorders which are as yet incurable with canonical treatment approaches. Since the discovery of stem cells, this avenue of research has become mainstream, not least due to the enigmatic nature of stem cells, but mostly because of the immense therapeutic potential of stem cells, unveiled either by experimental studies or clinical trials. Undoubtedly, the prominent therapeutic effects produced by neural stem cell (NSC) use in different types of neuropathology give credit to NSC as a multipurpose tool for curing neurodegeneration in a variety of diseases of the central nervous system[1-4]. Among all the advanced features of this cell type, the multi-lineage potential of NSC is probably the most attractive in terms of a cell-based therapy for the treatment of neurodegenerative disorders which are accompanied by extensive neuronal cell death that require a replacement of the pool of non-recoverable cells. These include such disorders as stroke, epilepsy, different forms of dementia, including Alzheimer's and Parkinson's diseases, among others[5,6]. The high capacity of NSC - either of fetal or adult brain origin - to differentiate between the lineages of neuronal or glial cell types, provides the damaged brain with a newly developed pool of cells consisting of a mixture of the entirely different phenotypes: *e.g.*, cholinergic, serotonergic, GABAergic, other neuronal subtypes, mixed with oligodendrocytes, astrocytes, reparative microglial subtypes, *etc*. This innate peculiarity of NSC to differentiate into diverse phenotypes, confirmed by several lines of evidence as a result of monitoring cell grafts in the post-stroke brain[7-11], can underlie the versatile beneficial effects when employed as cell-based therapy[12]. Consequently, such a therapy could lead to the "self-repair" of the damaged tissue by amplifying the remodelling of the injured brain through the rebuilding of damaged neuronal ensembles, neurite remodelling and the rewiring of the whole circuitry using one therapeutic approach. Together, this offers a potential advantage of NSC use in terms of boosting neurorestorative effects and amplifying the recovery of neurological function. In addition, employing NSC as a therapeutic approach has proved to be safe, owing to the restricted proliferation of neural precursors – unlike stem cells - that implies a lower risk of malignant transformation that could subsequently develop in the brain[9,11,13]. The latter is an essential requirement that cell-based therapies should meet.

NEURAL STEM CELL FOR MULTI-LINEAGE DIFFERENTIATION WITHIN A HOST TISSUE

Among two stem cell therapy approaches applicable at present – the transplantation of already differentiated cell phenotypes from induced pluripotent stem cells (iPSC) and the engraftment of neural progenitors into the injured brain - the use of NSC has emerged as one that opens a door for "self-repair" of the damaged tissue. The rationale for this lies with the multi-lineage differentiation of NSC regulated by a host (endogenous) microenvironment. This implies that after engraftment into the injured tissue, NSC differentiation occurs in a way that is pertinent to impairments taking place within the damaged area. In the light of the microenvironment characteristics featuring the post-ischemic brain tissue, a high level of excitotoxicity which originates from overwhelming glutamate, necrotic, pro-apoptotic factors being released following massive ischemic cell death, is a critical determinant that dramatically lowers the cell viability of already differentiated iPSC-derived neurons after transplantation. Similarly, the overactivation of pro-inflammatory signalling pathways pertinent to the post-ischemic impairments would suppress the survival of vulnerable neurons transplanted into the post-stroke brain. In support of this, there has been a routine low survival rate of iPSC-derived neurons after transplantation into the post-ischemic brain - the process greatly exacerbated by the proximity of cell grafts to the stroke lesion[14-17]. Furthermore, the lowered viability of engineered iPSC-derived cell grafts following transplantation has been a general problem for gene

therapy applications in clinical trials for Alzheimer's and Parkinson's diseases[18-20]. Establishing how the transplanted neurons can be protected over time within a pathological milieu is a key prerequisite for achieving the optimal outcome of cell-based therapy. This is where the advantage of the high intrinsic plasticity of NSC over the differentiated neuronal phenotypes in terms of gaining cell viability while within a detrimental host microenvironment, has appeared.

Emerging data from functional studies of the NSC-derived neurons indicate that neuronal differentiation and maturation occur at a much faster rate within a host brain tissue than in *in vitro* cell cultures. For instance, the maturation of electrophysiological properties of the NSC-derived neurons in organotypic hippocampal tissue has been completed for up to 3 weeks after engraftment[21,22]. By contrast, the maturation of biophysical properties of stem cell-derived neurons in dissociated cell cultures normally requires months to achieve a similar result. Electrophysiological studies collectively suggest that it is often necessary to use enriched media (a "cocktail") composed of a mixture of transcriptional factors and master regulators to force the maturation of neurophysiological properties of iPSC-derived neurons[22,23]. In this context, the accelerated neuronal maturation of NSC grafts within an endogenous microenvironment is highly advantageous, since only mature neurons will contribute to neurological function and lead to tissue remodelling for functional recovery. Although the precise mechanisms that underlie the accelerated neuronal maturation of NSC within a host brain tissue remain largely unknown, the potential of NSC for achieving fast therapeutic outcomes argues the case for pursuing further research to explore this in detail.

Among the benefits of NSC use as a promising therapeutic approach, one may assume engaging other mechanism(s), which remain as yet enigmatic. It can include, in particular, triggering the pool of resident NSC to cause it to become activated. The resident NSC - the population of adult stem cells available across the mature brain at the subventricular and subgranular dentate gyrus zones of the hippocampus, cerebellum, forebrain, olfactory bulbs - revealed the innate therapeutic potential with regard to the regeneration of the impaired brain tissue[24-26]. Growing interest within this newly exploring research area piles up further arguments for the high intrinsic plasticity of NSC and the control of the NSC fate by a host endogenous environment.

MONITORING NEURONAL MATURATION - CHALLENGES AND IMPORTANCE

Despite the lengthy period of time since the therapeutic effects of stem cell applications in brain injuries were first documented, to date neurophysiological mechanisms mediating these effects are still beyond our comprehensive understanding. For decades, in innumerable attempts to assess how far competent NSC-derived cells become over time within the adult brain, most data across the field have generally illustrated many antigens/markers that differentiated cells can express. While a combination of immunocytochemical (histochemical) profiles firmly documents the cell lineage[10,27,28], along with an ample expression of various receptors, proteins, *etc*., across the pool of differentiated cells, it provides, however, no rigorous evidence for the functional properties of these cells. Cells displaying a clear immunoreactivity might yet possess neither functional receptors nor signalling pathways constituted to ensure appropriate neurophysiological activity. Given that the appropriate level of neurophysiological activity - of individual cells and integrated neuronal network activity - determines the function, the anticipated beneficial effects of cell-based therapy would ultimately rely on the timing maturation of the neurophysiological properties of differentiated neurons, followed by their functional integration into the host circuits. Eventually, this dictates the overall outcome of the therapy being applied.

Notwithstanding their importance, the functional studies investigating how far the stem cell-derived neurons are physiologically credible following neurogenesis, have been scarce. There have only been a few studies, with some exceptional examples as follows[7,11,22,29,30], which have performed meticulous investigations of the biophysical properties and the neurophysiological activity of stem cell-derived neurons. The depth to which the majority of works tested the maturation of stem cell-derived neurons consisted of basic patterns of firing and synaptic activity recorded from differentiated neurons, typically at the very late time-points after transplantation (a few months in the post-stroke brain). Certainly, major challenges lay in selecting the difficult electrophysiological technique. Among technical difficulties, the methodology of conventional whole-cell recordings carrying on *in vivo* demands the termination of an acute experiment; therefore, the assessment of neurological function

through behavioural testing commonly precedes studies at the neuronal level. Consequently, a huge leap exists for the time window between cell engraftment to when the neurophysiological properties of stem cell-differentiated neurons have been tested. Therefore, a number of important questions remain to be answered. First of all, the time window that stem cell-derived neurons require to set up their neurophysiological properties to match the level of functional activity displayed by endogenous neurons. Second, what is the time scale for differentiated neurons to become functionally integrated into the host circuits? Third, is there a difference in the timing of neuronal maturation between different milieus (*i.e.*, varied pathological microenvironments)? This knowledge is essential when it comes to making decisions with regard to a scheme for stem cell transplantation (timely initiation of the treatment) and the assessment of anticipated benefits, along with the potential risks associated with the therapy application, depending on the severity of the tissue damage[31].

Evidence-based advances of the *ex vivo* brain tissue preparations have attracted attention to this experimental approach as an alternative to *in vivo* studies. Brain slices fulfil expectations for functional studies at the sub-cellular, cellular and neuronal network levels due to the preserved tissue layer architecture consisting of innate cell assemblies. Over time, organotypic brain slices have been effectively used to discover important insights into the cellular and molecular mechanisms of neurodegeneration - first of all, because of feasibility for the long-term maintenance of viable tissue, with much fewer costs as compared to animal model use, and because varied combinations of advanced techniques and analytical tools become applicable to brain tissue at either immature or mature developmental stages[32-34]. One of the other problems in studies of neurodegenerative disorders is that the generated animal models do not replicate the neuropathological changes obtained from post-mortem studies of the brain neurodegeneration, for instance, in stroke (cerebral ischemia), Alzheimer's and Parkinson's diseases, other forms of dementia[35-38]. Whilst the use of animal models remains in a constant debate in terms of whether or not they are relevant to human neurodegenerative disorders associated with the clear clinicopathological profile of memory loss and cognitive decline (debatable in animal species), mechanistic studies require model systems for exploring the mechanisms of neurodegeneration and treatment strategies. In this context, once again organotypic brain slices perfectly fit these aims.

Taking all the above into account, monitoring the time-dependent maturation of NSC grafts within a host hippocampal tissue has recently been employed. Functional studies have been carried out in organotypic hippocampal slices, aimed at answering the questions as highlighted earlier. The experimental data from electrophysiological recordings, combined with electron microscopy and immunohistological approaches, have revealed that NSC-derived hippocampal neurons have matured electrophysiological properties, and have functionally integrated into the host circuits within 3 weeks of engraftment[21]. Moreover, the neurophysiological maturation of NSC-derived neurons achieved a similar level of activity as that exhibited by endogenous CA1 pyramidal neurons (varied electrophysiological parameters were quantitatively compared between the groups). Next, a morphological comparison has been performed with regard to the synapses which NSC-derived neurons constituted with endogenous cells. The visualised structures, either presynaptic terminals containing numerous vesicles or postsynaptic structures, revealed the typical morphology, confirmed by synaptic function (*i.e.*, recordings of the postsynaptic currents) detected as early as the first two weeks after engraftment[21]. Extrapolating from the experimental data from this and other studies[22], the maturation of neuronal excitability and synaptogenesis within a host tissue can be envisaged to last up to a few weeks - a time scale much faster than established in dissociated cell cultures across a vast literature (NSC-derived *vs* iPSC-derived neurons[23]). Consequently, the therapeutic outcome from NSC-based therapy could, therefore, be anticipated to emerge shortly after initiating the treatment - within only a few weeks. In the light of such a time range, accelerated NSC maturation can provide a mechanistic basis of the speedy therapeutic effects in a recovery of neurological function observed one week after stem cell transplantation into the stroke-damaged brain[11,39]. Collectively, the outlined *in vivo* and *in vitro* data suggest that NSC-based therapy is advantageous in promoting the remodeling of brain tissue to amplify a recovery of neurological function, given that no effective therapy currently exists.

GLIAL LINEAGE AT WORK

A substantial bias in the neurogenesis of NSC grafts to glial lineage has been found

while monitoring NSC neurogenesis within the ischemic-injured brain tissue[21]. In the post-ischemic environment (organotypic hippocampal slices subjected to ischemic conditions - oxygen-glucose deprivation[40]), NSC grafts have been largely differentiating into glia, with a prompt rise in NSC-derived oligodendrocytes, followed by astrocytes. Notably, NSC-derived oligodendrocytes have already been identified at week 1, and astrocytes - by two weeks. In the meantime, NSC-derived neurons matured in terms of their electrophysiological properties with a dramatically slower rate within the post-ischemic milieu than in a physiological environment[21]. Based on the experimental data from a direct comparison between electrophysiological parameters, the promoted glial lineage has been a hallmark of NSC neurogenesis within the post-ischemic tissue (approximately 70% of grafted NSC differentiated into glia), opposing the reduced neuronal lineage (a drop from approximately 70% to approximately 30% in the proportion of NSC-derived neurons; Figure 1). Similar effects with regard to both the differentiation and the maturation of fetal NSC grafts in the post-stroke brain were observed *in vivo*[7]. The rationale for such a strong influence of the post-ischemic environment on NSC neurogenesis rests in how far the post-ischemic milieu is overburdened with extracellular glutamate[41], potassium, mediators of inflammation[42], pro-apoptotic factors, enzymes, and other compounds[43,44] that produce long-lasting excitotoxic actions, resulting in delayed neuronal cell death[40,45].

Oligodendrocytes and astrocytes are thought to have diverse roles in brain physiology and neuropathology, and both can actively communicate with neurons and other cell types[46-49]. Therefore, the peculiarity of NSC neurogenesis within post-ischemic tissue may mirror the numerous roles that these glial cell types would play there. Promoted glial lineage implies the glia-mediated neuroprotective and neurotrophic supports of the oxygen-glucose-deprived endogenous neurons as the first steps of defence against the ischemic impairments. Owing to the neuroprotective role of oligodendrocytes, protecting, in particular, the survival of CA1 hippocampal neurons, the NSC-derived oligodendrocytes may constitute endogenously-driven neuroprotection by providing a metabolic supply (paracrine signaling action), for instance, *via* the production of lactate, oligodendrocyte-derived trophic factors, GDNF[46,47]. In addition to this mechanism, the revealed impact of oligodendrocytes on astroglial development[39,48] may explain that NSC-derived oligodendrocytes precede the derivation of astrocytic phenotype[21]. As the most abundant cell type in the mammalian brain[49], astrocytes are highly secretory cells, able to produce large amounts of proteins in order to provide trophic support. The astrocytic-mediated surveillance of neurotoxic inflammation[50], together with a high capability of taking up glutamate and potassium[51] are essential to lower excitotoxicity within the post-ischemic tissue. The stem cell-derived astrocytes have been shown to replicate the functional properties of astroglia, including the uptake of glutamate and promoting synaptogenesis[48,52]. All the aforementioned lines of evidence support the possibility that NSC-derived oligodendrocytes and astrocytes provide the post-ischemic tissue clearance off debris, lower down the high level of excitotoxicity, and eventually improve the survival of oxygen-glucose-deprived endogenous neurons in post-ischemic conditions. These together favor the maturation of NSC-derived neurons within the endogenous post-ischemic environment as the subsequent step of NSC-based therapy to advance the remodelling of the ischemic-injured tissue and to facilitate its functional recovery.

SUMMARY AND SOME REMARKS

The great ability of NSC grafts to differentiate into neurons, astrocytes or oligodendrocytes within damaged brain tissue marks these cells as a versatile tool for neural replacement strategies in neurodegenerative disorders of various origins. The potential of NSC-based therapy with regard to brain neurodegeneration treatment is, therefore, mediated by multiple mechanisms to effectively amplify the therapeutic outcome[31,53,54]. While ethics restrict the use of embryonic NSC, the reprogramming of somatic cells can offer an alternative source for generating the progeny-restricted neural progenitors applicable for cell-based therapies. Given that iPSC feature a patient-specific phenotype, this will ultimately meet any safety concerns effectively. The phenotypic specificity appears particularly useful in generating *in vitro* human models of neurological disorders linked to genetic mutations, and iPSC have become widely exploited in this avenue of research. The iPSC capability of recapitulating both genetic and phenotypic profiles over the developmental stages *in vitro* as in the adult human brain has enabled functional studies in human cells directly for exploring the pathogenesis of genetically-triggered neurodegenerative disorders. Many protocols

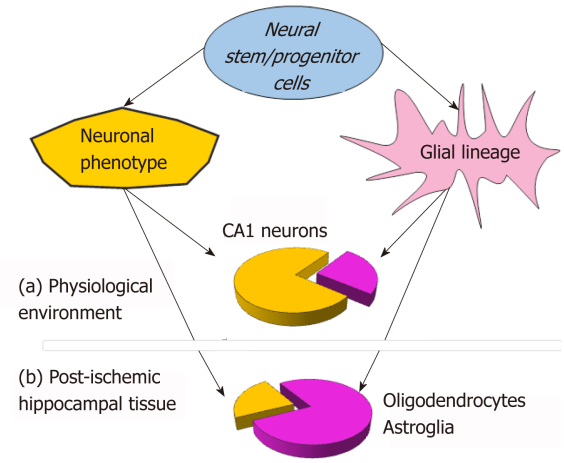

Figure 1 Schematic illustration for neurogenesis of neural stem cell (NSC) grafts within a host hippocampal tissue, showing the difference in relative proportions for neuronal (yellow) *vs* glial (purple) NSC lineage between physiological environment (a) and the post-ischemic milieu (tissue subjected to oxygen-glucose deprivation) (b) at an earlier time-window – first 2 weeks after engraftment into organotypic hippocampal tissue. NSC are fetal progenitors isolated from mouse hippocampus grafted into organotypic hippocampal slices for monitoring the time-dependent NSC neurogenesis within a host tissue.

for the manufacture of nerve cell phenotypes are being actively developed and made available, and the most recent advances in the technology of genome editing, including the CRISPR/Cas9-based correction of gene mutations, constantly refine stem cell clones to facilitate functional studies of brain neurodegeneration. This research direction has marked a new milestone in up-to-date strategies and therapeutic approaches tailored to amplify the remodeling of the injured brain tissue and boost the recovery of neurological function.

REFERENCES

1 **Goldman SA**. Stem and Progenitor Cell-Based Therapy of the Central Nervous System: Hopes, Hype, and Wishful Thinking. *Cell Stem Cell* 2016; **18**: 174-188 [PMID: 26849304 DOI: 10.1016/j.stem.2016.01.012]
2 **Lindvall O**, Kokaia Z. Stem cells for the treatment of neurological disorders. *Nature* 2006; **441**: 1094-1096 [PMID: 16810245 DOI: 10.1038/nature04960]
3 **Nedelec S**, Onteniente B, Peschanski M, Martinat C. Genetically-modified human pluripotent stem cells: new hopes for the understanding and the treatment of neurological diseases? *Curr Gene Ther* 2013; **13**: 111-119 [PMID: 23444870 DOI: 10.2174/1566523211313020005]
4 **Kokaia Z**, Llorente IL, Carmichael ST. Customized Brain Cells for Stroke Patients Using Pluripotent Stem Cells. *Stroke* 2018; **49**: 1091-1098 [PMID: 29669871 DOI: 10.1161/STROKEAHA.117.018291]
5 **Fricker M**, Tolkovsky AM, Borutaite V, Coleman M, Brown GC. Neuronal Cell Death. *Physiol Rev* 2018; **98**: 813-880 [PMID: 29488822 DOI: 10.1152/physrev.00011.2017]
6 **Kim JH**, Auerbach JM, Rodríguez-Gómez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sánchez-Pernaute R, Bankiewicz K, McKay R. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 2002; **418**: 50-56 [PMID: 12077607 DOI: 10.1038/nature00900]
7 **Bühnemann C**, Scholz A, Bernreuther C, Malik CY, Braun H, Schachner M, Reymann KG, Dihné M. Neuronal differentiation of transplanted embryonic stem cell-derived precursors in stroke lesions of adult rats. *Brain* 2006; **129**: 3238-3248 [PMID: 17018551 DOI: 10.1093/brain/awl261]
8 **Hayashi J**, Takagi Y, Fukuda H, Imazato T, Nishimura M, Fujimoto M, Takahashi J, Hashimoto N, Nozaki K. Primate embryonic stem cell-derived neuronal progenitors transplanted into ischemic brain. *J Cereb Blood Flow Metab* 2006; **26**: 906-914 [PMID: 16395293 DOI: 10.1038/sj.jcbfm.9600247]
9 **Jensen MB**, Yan H, Krishnaney-Davison R, Al Sawaf A, Zhang SC. Survival and differentiation of transplanted neural stem cells derived from human induced pluripotent stem cells in a rat stroke model. *J Stroke Cerebrovasc Dis* 2013; **22**: 304-308 [PMID: 22078778 DOI: 10.1016/j.jstrokecerebrovasdis.2011.09.008]
10 **Wei L**, Cui L, Snider BJ, Rivkin M, Yu SS, Lee CS, Adams LD, Gottlieb DI, Johnson EM, Yu SP, Choi DW. Transplantation of embryonic stem cells overexpressing Bcl-2 promotes functional recovery after transient cerebral ischemia. *Neurobiol Dis* 2005; **19**: 183-193 [PMID: 15837573 DOI: 10.1016/j.nbd.2004.12.016]
11 **Oki K**, Tatarishvili J, Wood J, Koch P, Wattananit S, Mine Y, Monni E, Tornero D, Ahlenius H, Ladewig J, Brüstle O, Lindvall O, Kokaia Z. Human-induced pluripotent stem cells form functional neurons and improve recovery after grafting in stroke-damaged brain. *Stem Cells* 2012; **30**: 1120-1133 [PMID: 22495829 DOI: 10.1002/stem.1104]
12 **Kopach O**, Pivneva T. Cell-based therapies for neural replacement strategies in stroke-related neurodegeneration: neurophysiological insights into stem progenitor cell neurogenesis within a host environment. *Neural Regen Res* 2018; **13**: 1350-1351 [PMID: 30106039 DOI: 10.4103/1673-5374.235224]

13 **Brederlau A**, Correia AS, Anisimov SV, Elmi M, Paul G, Roybon L, Morizane A, Bergquist F, Riebe I, Nannmark U, Carta M, Hanse E, Takahashi J, Sasai Y, Funa K, Brundin P, Eriksson PS, Li JY. Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation. *Stem Cells* 2006; **24**: 1433-1440 [PMID: 16556709 DOI: 10.1634/stemcells.2005-0393]

14 **Darsalia V**, Allison SJ, Cusulin C, Monni E, Kuzdas D, Kallur T, Lindvall O, Kokaia Z. Cell number and timing of transplantation determine survival of human neural stem cell grafts in stroke-damaged rat brain. *J Cereb Blood Flow Metab* 2011; **31**: 235-242 [PMID: 20531461 DOI: 10.1038/jcbfm.2010.81]

15 **Hicks AU**, Lappalainen RS, Narkilahti S, Suuronen R, Corbett D, Sivenius J, Hovatta O, Jolkkonen J. Transplantation of human embryonic stem cell-derived neural precursor cells and enriched environment after cortical stroke in rats: cell survival and functional recovery. *Eur J Neurosci* 2009; **29**: 562-574 [PMID: 19175403 DOI: 10.1111/j.1460-9568.2008.06599.x]

16 **Sakata H**, Niizuma K, Yoshioka H, Kim GS, Jung JE, Katsu M, Narasimhan P, Maier CM, Nishiyama Y, Chan PH. Minocycline-preconditioned neural stem cells enhance neuroprotection after ischemic stroke in rats. *J Neurosci* 2012; **32**: 3462-3473 [PMID: 22399769 DOI: 10.1523/JNEUROSCI.5686-11.2012]

17 **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 USA* 2004; **101**: 11839-11844 [PMID: 15280535 DOI: 10.1073/pnas.0404474101]

18 **Tuszynski MH**, Thal L, Pay M, Salmon DP, U HS, Bakay R, Patel P, Blesch A, Vahlsing HL, Ho G, Tong G, Potkin SG, Fallon J, Hansen L, Mufson EJ, Kordower JH, Gall C, Conner J. A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nat Med* 2005; **11**: 551-555 [PMID: 15852017 DOI: 10.1038/nm1239]

19 **Wahlberg LU**, Lind G, Almqvist PM, Kusk P, Tornøe J, Juliusson B, Söderman M, Selldén E, Seiger Å, Eriksdotter-Jönhagen M, Linderoth B. Targeted delivery of nerve growth factor via encapsulated cell biodelivery in Alzheimer disease: a technology platform for restorative neurosurgery. *J Neurosurg* 2012; **117**: 340-347 [PMID: 22655593 DOI: 10.3171/2012.2.JNS11714]

20 **Lindvall O**, Wahlberg LU. Encapsulated cell biodelivery of GDNF: a novel clinical strategy for neuroprotection and neuroregeneration in Parkinson's disease? *Exp Neurol* 2008; **209**: 82-88 [PMID: 17963752 DOI: 10.1016/j.expneurol.2007.08.019]

21 **Kopach O**, Rybachuk O, Krotov V, Kyryk V, Voitenko N, Pivneva T. Maturation of neural stem cells and integration into hippocampal circuits - a functional study in an *in situ* model of cerebral ischemia. *J Cell Sci* 2018; 131 [PMID: 29361548 DOI: 10.1242/jcs.210989]

22 **Morgan PJ**, Liedmann A, Hübner R, Hovakimyan M, Rolfs A, Frech MJ. Human neural progenitor cells show functional neuronal differentiation and regional preference after engraftment onto hippocampal slice cultures. *Stem Cells Dev* 2012; **21**: 1501-1512 [PMID: 21867424 DOI: 10.1089/scd.2011.0335]

23 **Telezhkin V**, Schnell C, Yarova P, Yung S, Cope E, Hughes A, Thompson BA, Sanders P, Geater C, Hancock JM, Joy S, Badder L, Connor-Robson N, Comella A, Straccia M, Bombau G, Brown JT, Canals JM, Randall AD, Allen ND, Kemp PJ. Forced cell cycle exit and modulation of GABAA, CREB, and GSK3β signaling promote functional maturation of induced pluripotent stem cell-derived neurons. *Am J Physiol Cell Physiol* 2016; **310**: C520-C541 [PMID: 26718628 DOI: 10.1152/ajpcell.00166.2015]

24 **Kempermann G**, Gage FH, Aigner L, Song H, Curtis MA, Thuret S, Kuhn HG, Jessberger S, Frankland PW, Cameron HA, Gould E, Hen R, Abrous DN, Toni N, Schinder AF, Zhao X, Lucassen PJ, Frisén J. Human Adult Neurogenesis: Evidence and Remaining Questions. *Cell Stem Cell* 2018; **23**: 25-30 [PMID: 29681514 DOI: 10.1016/j.stem.2018.04.004]

25 **Labusca L**, Mashayekhi K. Human adult pluripotency: Facts and questions. *World J Stem Cells* 2019; **11**: 1-12 [PMID: 30705711 DOI: 10.4252/wjsc.v11.i1.1]

26 **Roy NS**, Wang S, Jiang L, Kang J, Benraiss A, Harrison-Restelli C, Fraser RA, Couldwell WT, Kawaguchi A, Okano H, Nedergaard M, Goldman SA. In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus. *Nat Med* 2000; **6**: 271-277 [PMID: 10700228 DOI: 10.1038/73119]

27 **Rybachuk O**, Kopach O, Pivneva T, Kyryk V. Isolation of Neural Stem Cells from the Embryonic Mouse Hippocampus for in vitro Growth or Engraftment into a Host Tissue. *Bio-protocol* 2019; **9**: e3165 [DOI: 10.21769/BioProtoc.3165]

28 **Singh SP**, Tripathy NK, Nityanand S. Comparison of phenotypic markers and neural differentiation potential of multipotent adult progenitor cells and mesenchymal stem cells. *World J Stem Cells* 2013; **5**: 53-60 [PMID: 23671719 DOI: 10.4252/wjsc.v5.i2.53]

29 **Tornero D**, Tsupykov O, Granmo M, Rodriguez C, Grønning-Hansen M, Thelin J, Smozhanik E, Laterza C, Wattananit S, Ge R, Tatarishvili J, Grealish S, Brüstle O, Skibo G, Parmar M, Schouenborg J, Lindvall O, Kokaia Z. Synaptic inputs from stroke-injured brain to grafted human stem cell-derived neurons activated by sensory stimuli. *Brain* 2017; **140**: 692-706 [PMID: 28115364 DOI: 10.1093/brain/aww347]

30 **Shi Y**, Kirwan P, Smith J, Robinson HP, Livesey FJ. Human cerebral cortex development from pluripotent stem cells to functional excitatory synapses. *Nat Neurosci* 2012; **15**: 477-486, S1 [PMID: 22306606 DOI: 10.1038/nn.3041]

31 **Bacigaluppi M**, Pluchino S, Peruzzotti-Jametti L, Kilic E, Kilic U, Salani G, Brambilla E, West MJ, Comi G, Martino G, Hermann DM. Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain* 2009; **132**: 2239-2251 [PMID: 19617198 DOI: 10.1093/brain/awp174]

32 **Mewes A**, Franke H, Singer D. Organotypic brain slice cultures of adult transgenic P301S mice--a model for tauopathy studies. *PLoS One* 2012; **7**: e45017 [PMID: 22984603 DOI: 10.1371/journal.pone.0045017]

33 **Noraberg J**, Poulsen FR, Blaabjerg M, Kristensen BW, Bonde C, Montero M, Meyer M, Gramsbergen JB, Zimmer J. Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. *Curr Drug Targets CNS Neurol Disord* 2005; **4**: 435-452 [PMID: 16101559 DOI: 10.2174/1568007054546108]

34 **Holloway PM**, Gavins FN. Modeling Ischemic Stroke In Vitro: Status Quo and Future Perspectives. *Stroke* 2016; **47**: 561-569 [PMID: 26742797 DOI: 10.1161/STROKEAHA.115.011932]

35 **Götz J**, Bodea LG, Goedert M. Rodent models for Alzheimer disease. *Nat Rev Neurosci* 2018; **19**: 583-598 [PMID: 30194347 DOI: 10.1038/s41583-018-0054-8]

36 **Hainsworth AH**, Allan SM, Boltze J, Cunningham C, Farris C, Head E, Ihara M, Isaacs JD, Kalaria RN, Lesnik Oberstein SA, Moss MB, Nitzsche B, Rosenberg GA, Rutten JW, Salkovic-Petrisic M, Troen AM. Translational models for vascular cognitive impairment: a review including larger species. *BMC Med* 2017; **15**: 16 [PMID: 28118831 DOI: 10.1186/s12916-017-0793-9]

37 **McCabe C**, Arroja MM, Reid E, Macrae IM. Animal models of ischaemic stroke and characterisation of the ischaemic penumbra. *Neuropharmacology* 2018; **134**: 169-177 [PMID: 28923277 DOI: 10.1016/j.neuropharm.2017.09.022]

38 **Shih AY**, Hyacinth HI, Hartmann DA, van Veluw SJ. Rodent Models of Cerebral Microinfarct and Microhemorrhage. *Stroke* 2018; **49**: 803-810 [PMID: 29459393 DOI: 10.1161/STROKEAHA.117.016995]

39 **Jiang P**, Chen C, Wang R, Chechneva OV, Chung SH, Rao MS, Pleasure DE, Liu Y, Zhang Q, Deng W. hESC-derived Olig2+ progenitors generate a subtype of astroglia with protective effects against ischaemic brain injury. *Nat Commun* 2013; **4**: 2196 [PMID: 23880652 DOI: 10.1038/ncomms3196]

40 **Rybachuk O**, Kopach O, Krotov V, Voitenko N, Pivneva T. Optimized Model of Cerebral Ischemia *In situ* for the Long-Lasting Assessment of Hippocampal Cell Death. *Front Neurosci* 2017; **11**: 388 [PMID: 28729821 DOI: 10.3389/fnins.2017.00388]

41 **Rossi DJ**, Oshima T, Attwell D. Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* 2000; **403**: 316-321 [PMID: 10659851 DOI: 10.1038/35002090]

42 **Nathan C**, Ding A. Nonresolving inflammation. *Cell* 2010; **140**: 871-882 [PMID: 20303877 DOI: 10.1016/j.cell.2010.02.029]

43 **Kroemer G**, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev* 2007; **87**: 99-163 [PMID: 17237344 DOI: 10.1152/physrev.00013.2006]

44 **Puig B**, Brenna S, Magnus T. Molecular Communication of a Dying Neuron in Stroke. *Int J Mol Sci* 2018; 19 [PMID: 30235837 DOI: 10.3390/ijms19092834]

45 **Lipton P**. Ischemic cell death in brain neurons. *Physiol Rev* 1999; **79**: 1431-1568 [PMID: 10508238 DOI: 10.1152/physrev.1999.79.4.1431]

46 **Lee Y**, Morrison BM, Li Y, Lengacher S, Farah MH, Hoffman PN, Liu Y, Tsingalia A, Jin L, Zhang PW, Pellerin L, Magistretti PJ, Rothstein JD. Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* 2012; **487**: 443-448 [PMID: 22801498 DOI: 10.1038/nature11314]

47 **Wilkins A**, Majed H, Layfield R, Compston A, Chandran S. Oligodendrocytes promote neuronal survival and axonal length by distinct intracellular mechanisms: a novel role for oligodendrocyte-derived glial cell line-derived neurotrophic factor. *J Neurosci* 2003; **23**: 4967-4974 [PMID: 12832519 DOI: 10.1523/JNEUROSCI.23-12-04967.2003]

48 **Tsai HH**, Li H, Fuentealba LC, Molofsky AV, Taveira-Marques R, Zhuang H, Tenney A, Murnen AT, Fancy SP, Merkle F, Kessaris N, Alvarez-Buylla A, Richardson WD, Rowitch DH. Regional astrocyte allocation regulates CNS synaptogenesis and repair. *Science* 2012; **337**: 358-362 [PMID: 22745251 DOI: 10.1126/science.1222381]

49 **Oberheim NA**, Takano T, Han X, He W, Lin JH, Wang F, Xu Q, Wyatt JD, Pilcher W, Ojemann JG, Ransom BR, Goldman SA, Nedergaard M. Uniquely hominid features of adult human astrocytes. *J Neurosci* 2009; **29**: 3276-3287 [PMID: 19279265 DOI: 10.1523/JNEUROSCI.4707-08.2009]

50 **Sofroniew MV**. Astrocyte barriers to neurotoxic inflammation. *Nat Rev Neurosci* 2015; **16**: 249-263 [PMID: 25891508 DOI: 10.1038/nrn3898]

51 **Rothstein JD**, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 1996; **16**: 675-686 [PMID: 8785064 DOI: 10.1016/S0896-6273(00)80086-0]

52 **Krencik R**, Weick JP, Liu Y, Zhang ZJ, Zhang SC. Specification of transplantable astroglial subtypes from human pluripotent stem cells. *Nat Biotechnol* 2011; **29**: 528-534 [PMID: 21602806 DOI: 10.1038/nbt.1877]

53 **Andres RH**, Horie N, Slikker W, Keren-Gill H, Zhan K, Sun G, Manley NC, Pereira MP, Sheikh LA, McMillan EL, Schaar BT, Svendsen CN, Bliss TM, Steinberg GK. Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. *Brain* 2011; **134**: 1777-1789 [PMID: 21616972 DOI: 10.1093/brain/awr094]

54 **Zhang ZG**, Buller B, Chopp M. Exosomes - beyond stem cells for restorative therapy in stroke and neurological injury. *Nat Rev Neurol* 2019; **15**: 193-203 [PMID: 30700824 DOI: 10.1038/s41582-018-0126-4]

World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2019 November 26; 11(11): 990-1004

DOI: 10.4252/wjsc.v11.i11.990

ISSN 1948-0210 (online)

ORIGINAL ARTICLE

Basic Study

Ameliorating liver fibrosis in an animal model using the secretome released from miR-122-transfected adipose-derived stem cells

Kee-Hwan Kim, Jae Im Lee, Ok-Hee Kim, Ha-Eun Hong, Bong Jun Kwak, Ho Joong Choi, Joseph Ahn, Tae Yun Lee, Sang Chul Lee, Say-June Kim

ORCID number: Kee-Hwan Kim (0000-0001-6219-6027); Jae Im Lee (0000-0002-4074-3904); Ok-Hee Kim (0000-0002-9204-2587); Ha-Eun Hong (0000-0002-4361-4809); Bong Jun Kwak (0000-0003-2409-9386); Ho Joong Choi (0000-0002-0862-098X); Joseph Ahn (0000-0001-8528-4919); Tae Yun Lee (0000-0002-4414-7958); Sang Chul Lee (0000-0002-8681-7059); Say-June Kim (0000-0001-5171-4837).

Author contributions: Kim SJ was responsible for planning the study, data interpretation, and manuscript preparation; Kim KH and Kim SJ wrote paper, complemented *in vivo* experiments, and data interpretation; Kim OK and Hong HE performed *in vitro* experiments; Lee JI, Kwak BJ, Choi HJ, Ahn J, Lee TR and Lee SC were involved in various *in vivo* experiments and data interpretation. All authors read and approved the manuscript.

Supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT), No. NRF-2015R1D1A1A01060721.

Institutional review board statement: This research was approved by Institutional Review Board, IRB No. 700069-201407-BR-002-01, of Hurim BioCell Co. Ltd. (Seoul, Korea).

Institutional animal care and use committee statement: Animal studies were carried out in compliance with the guidelines of the Institute for Laboratory Animal

Kee-Hwan Kim, Jae Im Lee, Department of Surgery, Uijeongbu St. Mary's Hospital, College of Medicine, the Catholic University of Korea, Seoul 11765, South Korea

Kee-Hwan Kim, Ok-Hee Kim, Ha-Eun Hong, Say-June Kim, Catholic Central Laboratory of Surgery, Institute of Biomedical Industry, College of Medicine, the Catholic University of Korea, Seoul 06591, South Korea

Ok-Hee Kim, Ha-Eun Hong, Bong Jun Kwak, Ho Joong Choi, Joseph Ahn, Tae Yun Lee, Say-June Kim, Department of Surgery, Seoul St. Mary's Hospital, College of Medicine, the Catholic University of Korea, Seoul 06591, South Korea

Sang Chul Lee, Department of Surgery, Daejeon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul 34943, South Korea

Corresponding author: Say-June Kim, MD, PhD, Professor, Department of Surgery, Seoul St. Mary's Hospital, College of Medicine, the Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul 06591, South Korea. sayjunekim@gmail.com
Telephone: +82-2-22587636
Fax: +82-2-5350070

Abstract

BACKGROUND

Recently, the exclusive use of mesenchymal stem cell (MSC)-secreted molecules, called secretome, rather than cells, has been evaluated for overcoming the limitations of cell-based therapy, while maintaining its advantages. However, the use of naïve secretome may not fully satisfy the specificity of each disease. Therefore, it appears to be more advantageous to use the functionally reinforced secretome through a series of processes involving physico-chemical adjustments or genetic manipulation rather than to the use naïve secretome.

AIM

To determine the therapeutic potential of the secretome released from miR-122-transfected adipose-derived stromal cells (ASCs).

METHODS

We collected secretory materials released from ASCs that had been transfected with antifibrotic miR-122 (MCM) and compared their antifibrotic effects with those of the naïve secretome (CM). MCM and CM were intravenously administered to the mouse model of thioacetamide-induced liver fibrosis, and their therapeutic potentials were compared.

Research, Korea, IRB No: CUMC-2017-0317-04.

Conflict-of-interest statement: The authors have declared no potential conflicts of interest.

Data sharing statement: Requests for access to data should be addressed to the corresponding author.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Manuscript source: Unsolicited manuscript

Received: March 12, 2019
Peer-review started: March 15, 2019
First decision: July 31, 2019
Revised: September 2, 2019
Accepted: September 13, 2019
Article in press: September 13, 2019
Published online: November 26, 2019

P-Reviewer: Labusca L
S-Editor: Dou Y
L-Editor: A
E-Editor: Wu YXJ

RESULTS

MCM infusion provided higher therapeutic potential in terms of: (A) Reducing collagen content in the liver; (B) Inhibiting proinflammatory cytokines; and (C) Reducing abnormally elevated liver enzymes than the infusion of the naïve secretome. The proteomic analysis of MCM also indicated that the contents of antifibrotic proteins were significantly elevated compared to those in the naïve secretome.

CONCLUSION

We could, thus, conclude that the secretome released from miR-122-transfected ASCs has higher antifibrotic and anti-inflammatory properties than the naïve secretome. Because miR-122 transfection into ASCs provides a specific way of potentiating the antifibrotic properties of ASC secretome, it could be considered as an enhanced method for reinforcing secretome effectiveness.

Key words: Adipose-derived stem cells; Liver fibrosis; MicroRNAs; miR-122; Mesenchymal stem cells; Secretome

Core tip: We herein intended to determine the antifibrotic effects of the secretome released from miR-122-transfected adipose-derived stromal cells (miR-122-secretome). miR122-secrectome and naïve secretome were intravenously administered to the mice with liver fibrosis, respectively. miR122-secrectome infusion provided higher therapeutic potential in terms of reducing collagen content in the liver, inhibiting proinflammatory cytokines, and reducing abnormally elevated liver enzymes than the infusion of the naïve secretome. Proteomic analysis of the miR122-secrectome indicated that the contents of antifibrotic proteins were significantly elevated compared to those in the naïve secretome. Our results demonstrate that miR122-secrectome has higher antifibrotic and anti-inflammatory properties than the naïve secretome.

Citation: Kim KH, Lee JI, Kim OH, Hong HE, Kwak BJ, Choi HJ, Ahn J, Lee TY, Lee SC, Kim SJ. Ameliorating liver fibrosis in an animal model using the secretome released from miR-122-transfected adipose-derived stem cells. *World J Stem Cells* 2019; 11(11): 990-1004
URL: https://www.wjgnet.com/1948-0210/full/v11/i11/990.htm
DOI: https://dx.doi.org/10.4252/wjsc.v11.i11.990

INTRODUCTION

Stem cell research is one of the promising areas of biomedical research. However, notwithstanding remarkable achievements in the field of mesenchymal stem cells (MSCs), their clinical applications are still challenging, especially due to safety concerns. To date, increasing evidence has been accumulating in support of the notion that the principal action mechanism of MSCs is secretome-mediated[1-5]. Thus, to overcome the limitations of cell-based therapy, numerous researchers have focused on the exclusive use of MSC-secreted molecules rather than the cells *per se*. The total set of molecules secreted or surface-shed by cells is generally referred to as secretome. The secretome includes bioactive peptides, such as cytokines, chemokines, and growth factors[1,4]. These soluble factors are released from MSCs either alone or in the form of extracellular vesicles.

The therapeutic potential of secretome can be potentiated by adjusting the conditions under which MSCs are incubated. Among these conditions, the genetic modification of MSCs can offer enormous and persistent reinforcements of the MSC secretome. Literature supports that microRNAs (miRNAs) play a substantial role in the process of liver fibrosis[6-8]. MicroRNAs are small non-coding RNA molecules (containing about 22 nucleotides) that alter gene expression at the posttranscriptional level, resulting in altered protein synthesis[9]. Hence, miRNAs can exquisitely adjust the expression of numerous genes particularly responsible for fundamental cellular processes, such as proliferation, development, and differentiation[10]. The miRNAs responsible for liver fibrosis can largely be divided into fibrotic and antifibrotic miRNAs. Of these, miR-122 is one of the representative antifibrotic miRNAs that

negatively regulates collagen production in hepatic stellate cells (HSCs)[11,12]. Thus, harnessing MSCs to confer miR-122 to HSCs would be a potential novel therapeutic approach for reinforcing the antifibrotic effects of MSCs. In this study, we aimed to the determine the antifibrotic effects of the secretome released from miR-122-transfected ASCs in both *in vitro* and *in vivo* models of liver fibrosis.

MATERIALS AND METHODS

Isolation of ASCs

Human adipose-derived stromal cells (ASCs) were obtained from lipoaspirated fat with inform consent of the volunteers. This research was approved by Institutional Review Board (IRB number 700069-201407-BR-002-01) of Hurim BioCell Co. Ltd. (Seoul, South Korea). ASCs were isolated and cultured according to previous reports[13]. Lipoaspirated fat was digested by 0.1% collagenase (Sigma-Aldrich, St. Louis, MO, United States) in saline and collected after centrifugation. Cells were plated into culture flask in low-glucose Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Hemel Hempstead, United Kingdom) supplemented with 10% FBS (Thermo Fisher Scientific), 100 U/mL of penicillin (Thermo Fisher Scientific), and 0.1 mg/mL of streptomycin (Thermo Fisher Scientific). ASCs were incubated at 37 °C in humidified chamber containing 5% carbon dioxide and medium was changed every 3 d.

Transfection and attainment of secretome

ASCs were transfected with miR-122 (Exiqon, Germatown, MD) per well mixed with the Lipofectamine RNAiMAX Reagent (Thermo). After 72hr of transfection, the cells were morphologically observed by the inverted microscope. The cell numbers of the experimental groups were counted automatic cell counter (Countess®, Invitrogen, San Diego, CA, United States) using trypan blue solution. Transfected cells were processed for cell phenotyping or differentiated into three-lineage induction.

ASCs with or without miR-122 transfection were grown in a 100 mm cell dishes (Corning Glass Works, Corning, NY, United States). After reaching 70%-80% confluence, 1.0×10^6 ASCs were cultured in 5 mL serum-free low-glucose DMEM for 48 h. Therefore, to obtain 0.2 mL amount of secretome from 1.0×10^6 ASCs, the conditioned media were concentrated 25-fold using ultra filtration units with a 3-kDa molecular weight cutoff (Amicon Ultra-PL 3; Millipore, Bedford, MA, United States). We then injected 0.1 mL amount of secretome per mouse. This means that one mouse is injected with the secretome obtained from 5×10^5 ASCs. In this study, NCM refers to the secretome shed from ASCs after 48 h of incubation, and MCM refers to the secretome shed from miR-122-transfected ASCs after 48 h of incubation.

Cell phenotyping by FACS analysis

The immunophnotypes of the experimental groups were determined by flow cytometry analysis (Cytomics FC500 flow cytometer, Beckman Coulter, Fullerton, CA, United States) using FITC-conjugated CD31, CD45, and CD73 antibodies and PE-conjugated CD90 and CD105 antibodies (BD Pharmingen, San Jose, CA, United States). Isotype controls were performed with antibodies against IgG for samples.

Differentiation into adipocytes, osteocytes, and chondrocytes

Transfected cells were induced toward the three lineages for 21 d. The adipogenic, osteogenic and chondrogenic differentiation ability of MSCs was determined as previously described[14,15]. Briefly, the cells were plated at a density of 1×10^4 or 5×10^3 cells/cm^2 in growth medium for 3 d, and then cultured in adipocyte and osteocyte differentiation medium (StemPro™, Gibco) for 3 wk. For chondrogenic induction, expansion medium containing 8×10^4 cells was cultured for 2 h. Then, chondrogenesis differentiation medium (StemPro™, Gibco) was added and cultured for 3 wk. After differentiation, Lipid vesicles and calcium deposition were observed by oil Red O and Alizarin Red staining. For chondrogenic induction, micromass cultures were plated by seeding 5 μL droplets of 8×10^4 cells into the center of 48-well plate. After incubating micromass cultures for 2 h at 37 °C, chondrogenic medium (StemPro, GIBCO) was added to 400 μL per culture wells and cultured for 3 wk. Chondrocyte induction was determined by immunohistochemical staining for collagen type I and II and proteoglycan[16]. Primary antibodies were purchased from Millipore (Millipore, CA, United States) and reacted with sections. After incubation with primary antibodies, sections were incubated with PE-conjugated goat anti-rabbit immunoglobulin G (Abcam, Cambridge, MA, United Kingdom) and rabbit anti-mouse immunoglobulin G (Abcam). Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, Invitrogen).

Human HSC culture

The LX-2 human HSCs were obtained from were kindly donated by Dr. Won-il Jeong in KAIST Biomedical research of Korea. LX-2 cells were maintained in DMEM (Thermo, Carlsbad, CA, United States). The medium was supplemented with 10% FBS (GibcoBRL, Calsbad, CA, United States), 1% antibiotics (Thermo), at 37 °C.

Western blot analysis

LX-2 cells and liver specimens obtained from mice were lysed using the EzRIPA Lysis kit (ATTO Corporation; Tokyo, Japan), and quantified by Bradford reagent (Bio-RadHercules, CA, United States). Proteins were visualized by western analysis using the following primary antibodies (1:1000 dilution) at 4 °C overnight and then with HRP-conjugated secondary antibodies (1:2000 dilution) for 1 h at 25°C. From Cell Signaling Technology (Beverly, MA, United States), we obtained primary antibodies against Proliferating cell nuclear antigen (PCNA), transforming growth factor-β (TGF-β1), alpha-smooth muscle actin (α-SMA), metallopeptidase inhibitor 1 (TIMP-1), matrix metallopeptidase 2 (MMP2), collagen type- 1 alpha-1 (COL1A1), β-actin, and horseradish peroxidase (HRP)-conjugated secondary antibody. Specific immune complexes were detected using the Western Blotting Plus Chemiluminescence Reagent (Millipore, Bedford, MA, United States).

Animals and study design

Five-week male BALB/c mice (Orient Bio, Seongnam, Korea) were used in this study. Animal studies were carried out in compliance with the guidelines of the Institute for Laboratory Animal Research, Korea (IRB No: CUMC-2017-0317-04). We then compared the effects of the MCM in an *in vivo* model of Thioacetamide (TAA)-induced hepatic fibrosis model. The *in vivo* model was generated by subcutaneous injection of TAA (200 mg/kg, three times a week for 8 wk) into experimental mice. Each group included 10 mice, and these were further divided into two subgroups: those for Control mice (n = 30), and those for TAA-treated mice (n = 30). Subsequently, control mice and TAA-treated mice were intravenously (using tail vein) infused with normal saline, CM, and MCM, respectively.

Serology test and ELISA

Blood samples were collected from each mouse, centrifuged for 10 min at 9500 *g*, and serum was collected. We measured the concentrations of markers for liver injury and kidney injury, such as aspartate transaminase (AST), alanine transaminase (ALT), and creatine, using an IDEXX VetTest Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, ME, United States). The concentrations of mouse interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured by sandwich enzyme-linked immunosorbent assay (ELISA kits, Biolegend, San Diego, CA, United States) according to the manufacturer's instructions.

Immunohistochemistry, Sirius red staining and masson's trichrome staining

For immunohistochemical analysis, formalin-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated in an ethanol series and subjected to epitope retrieval using standard procedures. Antibodies against of PCNA, TIMP-1, Albumin, α-SMA, TGF-β1, MMP-2, SOD, Catalase and GPx (all from Cell Signaling Technology, MA, United States) were used for immunochemical staining. The samples were then examined under a laser-scanning microscope (Eclipse TE300; Nikon, Tokyo, Japan) to analyze the expression of PCNA, TIMP-1, Albumin, a-SMA, TGF-β1, MMP-2, SOD, Catalase and GPx. Sirius red staining and Trichrome staining were performed using the Sirius red staining kit and Masson's trichrome staining kit according to the manufacturer's protocol (Polysciences, Warrington, PA, United Kingdom).

Statistical analysis

All data were analyzed with SPSS 11.0 software (SPSS Inc., Chicago, IL, United States) and SigmaPlot® ver. 12.0 (Systat Software Inc., Chicago, IL, United States). The data are presented as mean ± standard deviation (SD). Statistical comparison among groups was determined using Kruskal–Wallis test followed by Dunnett's test as the post hoc analysis. Probability values of $P < 0.05$ were regarded as statistically significant.

RESULTS

Determination of differentiation potential of miR-122-transfected ASCs

We first determined whether miR-122 transfection impairs ASC functionality, especially their multilineage differentiation potential. Flow cytometric analysis

showed that miR-122 transfection did not alter the expression of surface markers of ASCs (Figure 1A). Gross cell morphology was also identical regardless of miR-122 transfection (Figure 1B). In addition, transfecting miRNA did not affect multilineage differentiation potential of ASCs, including the potentials of differentiating adipocytic (Figure 1B) or osteogenic (Figure 1C) lineages, and the expression of collagens (type I and type II) and proteoglycan (Figure 1D).

In vitro experiments validating the effects of miR-122transfection into ASCs

We investigated the expression of fibrosis-related markers in miR-122-transfected ASCs. miR-122-transfected ASCs showed a decreased expression of fibrosis-related proteins (TGF β1, MMP2, α-SMA, and TIMP) compared to control ASCs or ASCs transfected with miR-122 (Figure 2A). We obtained human HSCs (LX2 cells) and treated them with a varying concentration of TAA for determining *in vitro* model of liver fibrosis. TAA elicited a concentration-dependent increase of fibrosis markers to a certain extent, and we determined that 5.0 mmol TAA is appropriate for inducing fibrosis in LX2 cells (Figure 2B and C).

Next, we obtained the secretome from the CM of ASCs as described in the method. In this study, NCM refers to the secretome shed from ASCs after 48 h of incubation, and MCM refers to the secretome shed from miR-122-transfected ASCs after 48 h of incubation. The *in vitro* model of liver fibrosis was generated by treating human HSCs cells (LX2 cells) with a hepatotoxin (TAA). We then treated the TAA-treated LX2 cells with NCM or MCM, and investigated the expression of fibrosis-related markers using western blot analysis (Figure 2D). Overall, the addition of each secretome (NCM or MCM) to TAA-treated LX2 cells significantly decreased the expression of fibrotic markers (MMP2, TGF-β1, and α-SMA) ($P < 0.05$). When comparing the two kinds of secretome, MCM induced the more significant reduction of fibrotic markers than did NCM ($P < 0.05$).

Determination of antifibrotic effects of the secretome released from miR-122-transfected ASCs in the in vivo model of liver fibrosis

We generated an *in vivo* model of liver fibrosis in mouse by subcutaneous injection of TAA (200 mg/kg) three times a week for 5 wk and validated the effects of MCM in this model. The mice were divided into two groups: control ($n = 30$) and TAA-treated mice ($n = 30$), and the latter were intravenously infused normal saline ($n = 10$), NCM ($n = 10$), or MCM ($n = 10$) twice (200 mg/kg, three times a week for 8 wk). On the 7th d after infusion, the mice were euthanized and specimens were obtained for study. Sirius red and Masson trichrome stains were used for the estimation of fibrosis. These stains showed that, although both treatments (NCM and MCM) decreased the content of collagen, MCM significantly had the greatest effect (Figure 3A and B). In the western blot analysis of the liver specimens, MCM infusion significantly increased the expression of PCNA (a proliferation marker), and significantly decreased the expression of α-SMA, TGF-β1, and MMP1 (fibrotic markers) and increased an antifibrotic marker (TIMP-1) in the TAA-treated mice (Figure 3C).

Comparison of immunohistochemical stains

We compared the histological changes of the livers obtained from each mouse group. PCNA was used as the marker for hepatocyte proliferation; α-SMA, TGF-β1, and MMP1 for liver fibrosis; albumin for hepatic synthetic function; TIMP-1 for liver antifibrosis; and SOD, catalase, and GPx for liver antioxidant activity. Through immunohistochemical staining, the MCM group showed the highest expression of PCNA, albumin, and TIMP-1, and the lowest expression of α-SMA, TGF-β1, and MMP1 (Figure 4A and B). The MCM group also showed the highest expression of SOD, catalase, and GPx (Figure 5).

Comparison of systemic markers and liver enzymes after each treatment

We compared the expression of systemic inflammatory markers, such as IL-6 and TNF-α, in the serum of each mouse group. Secretome infusions (NCM and MCM) significantly decreased the expression of these markers, and MCM decreased their expression in a higher degree than NCM ($P < 0.05$) (Figure 6A). Finally, we compared the serum levels of liver enzymes (AST and ALT) in each mouse group. Secretome infusions significantly decreased the elevated levels of liver enzymes, and MCM had a higher effect than NCM ($P < 0.05$) (Figure 6B).

Using liquid chromatography–mass spectrometry (LC/MS), we analyzed and compared the protein contents of NCM and MCM (Figure 6C). The protein constituents and concentrations of various important proteins varied widely between NCM and MCM, validating the effects of miR-125 transfection. Specifically, MCM exhibited a significantly decreased concentration of essential intermediates of the TGF-β/Smad signaling, such as transgelin, PIN1, and profilin-1, compared to NCM.

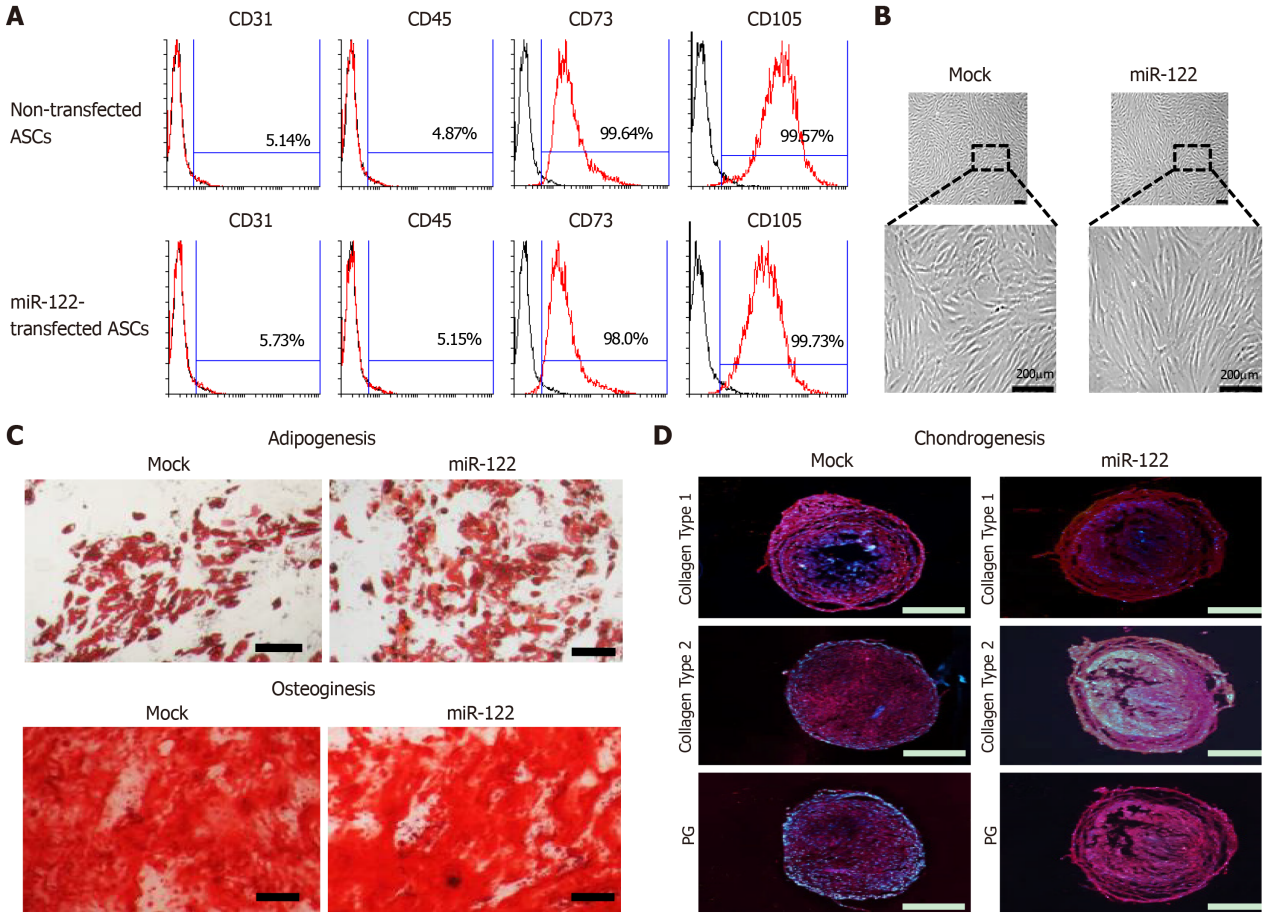

Figure 1 Assessment of multilineage differentiation potential of miR-122-transfected adipose-derived stem cells. A: Flow cytometric analysis showing that miR-122 transfection did not alter the expression of surface markers of adipose-derived stem cells (ASCs). ASCs were negative for CD31 and CD45 (hematopoietic stem cell-associated markers) expression and positive for CD73 and CD105 (mesenchymal stem cell-associated markers) expression regardless of miR-122 transfection; B: Comparison of gross cell morphology between ASCs with/without miR-122 transfection. Cells appear to be identical regardless of miR-122 transfection; C, D: Photomicrographs showing successful differentiation of ASCs into adipocytes, osteocytes, and chondrocytes regardless of miR-122 transfection. The differentiated cells were identified using four distinct staining methods (Oil Red O, Alizarin Red, collagen type 1, and proteoglycan). Scale bars = 100 μm. Values are presented as mean ± standard deviation of three independent experiments. $^{a}P < 0.05$. ASC: Adipose-derived stem cell; HSC: Hepatic stellate cell; PG: Proteoglycan.

DISCUSSION

In this study, we have shown that the secretome released from miR-122 transfected ASCs was superior to the naïve secretome in improving liver fibrosis while minimizing inflammatory processes in mice with TAA-induced liver fibrosis. Specifically, infusion of the secretome from miR-122-transfected ASCs provided higher therapeutic potential in terms of: (A) Reducing collagen content in the liver; (B) Inhibiting proinflammatory cytokines; and (C) Reducing abnormally elevated liver enzymes than infusion of the naïve secretome. Thus, it can be postulated that miR-122 transfection into ASCs reconditions them to have higher antifibrotic properties and to release a secretome with higher antifibrotic components. In reality, our proteomic analysis of the secretome released from miR-122-transfected ASCs indicated that it had significantly lesser contents of essential intermediates of liver fibrosis compared to the naïve secretome. We could, thus, conclude that the secretome released from miR-122-transfected ASCs has higher antifibrotic and anti-inflammatory properties than the naïve secretome.

Accumulating evidence indicates that various miRNAs are essentially involved in the process of fibrosis, particularly related with the action of HSCs[17]. Fibrogenic injury of the liver prompts HSCs to undergo proliferation, migrate to injured sites, and transform into myofibroblast-like cells which apparently lose their lipid droplets[18-20]. Subsequently, the activated HSCs, named fibroblast-like cells, produce large amounts of extracellular matrix proteins, such as collagen I and II, finally leading to liver fibrosis[19,21-24]. Of various cytokines, TGF-β plays essential roles in the process of liver fibrosis[25-28].

A number of miRNAs are involved in the processes of liver fibrosis, by either

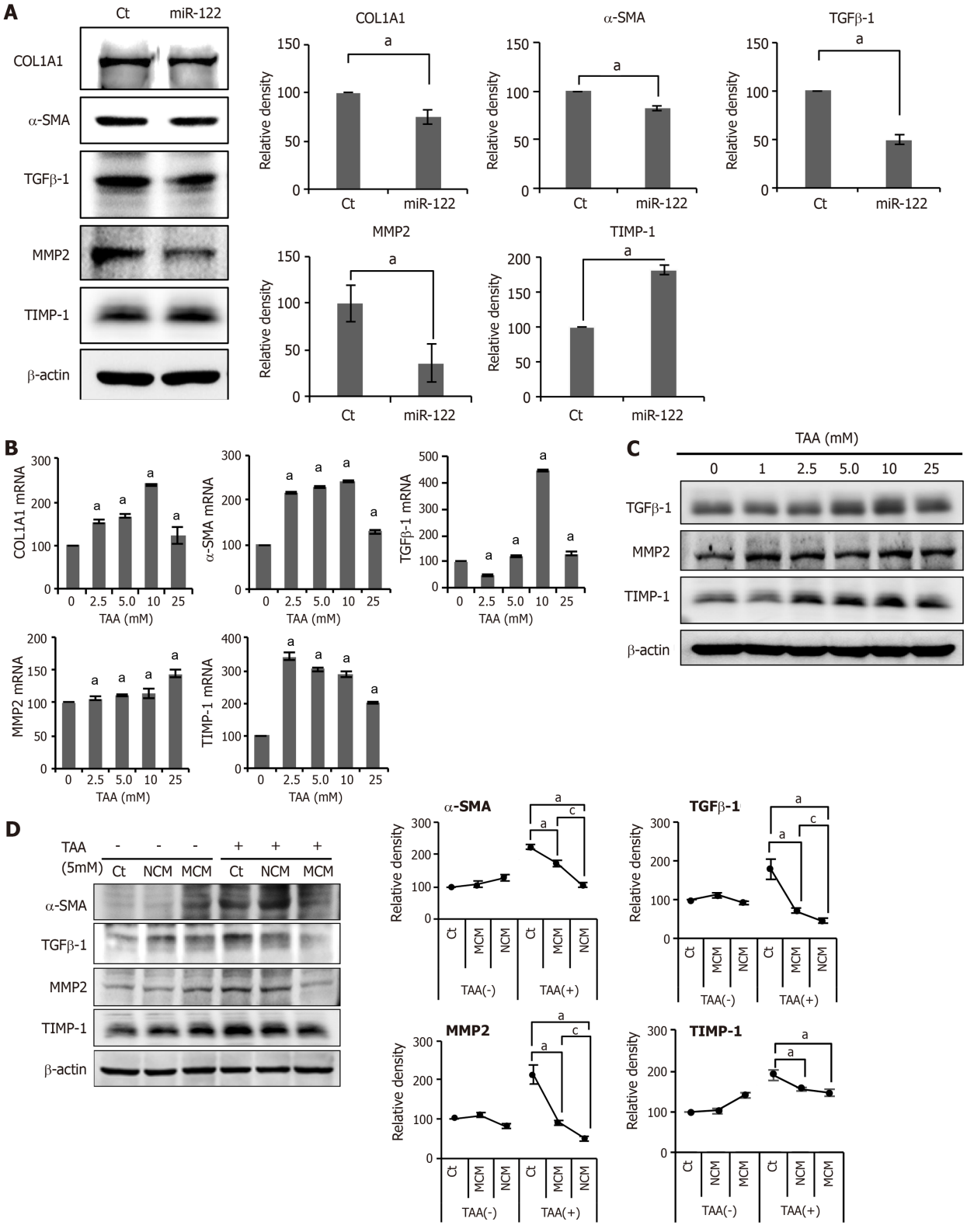

Figure 2 ***In vitro*** **experiments validating the effects of miR-122 transfection into Adipose-derived stem cells.** A: Western blot analysis showing the expression of fibrotic and antifibrotic markers in miR-122-transfected adipose-derived stem cells (ASCs). miR-122-transfected ASCs showed decreased expression of fibrotic proteins (TGF β1, MMP2, and α-SMA) and increased expression of an antifibrotic protein (TIMP-1) than control ASCs. The graphs below microscopic figures show the relative densities of these markers; B, C: RT-PCR (left) and western blot analysis (right) of LX2 cells for the determination of the thioacetamide (TAA) concentration used for generating *in vitro* model of liver fibrosis. A TAA concentration of 2.5 mM was used for inducting LX2 cells into fibrosis; D: Effects of MCM in the *in vitro* model of liver fibrosis. The *in vitro* model of liver fibrosis was generated by treating human HSCs cells (LX2 cells) with a hepatotoxin (TAA). In western blot analysis (Left), MCM induced the lowest expression of fibrotic markers (MMP2, TGF-β1, and α-SMA) in the TAA-treated LX2 cells. Relative densities of fibrosis-related markers in each group (Right). Values are presented as mean ± standard deviation of three independent experiments. $^{a}P < 0.05$ *vs* Ct. $^{c}P < 0.05$ between NCM and MCM. α-SMA: Alpha-smooth muscle actin; COL1A1: Collagen type-1 alpha-1; Ct: Control; CM: The secretome obtained from ASCs after 48-h-incubation; MCM: The secretome released from miR-122-transfected ASCs; MMP-1: Metalloproteinases-1; TAA: Thioacetamide; TGF-β: Transforming growth factor-β; TIMP-1: Tissue inhibitor of metalloproteinases-1.

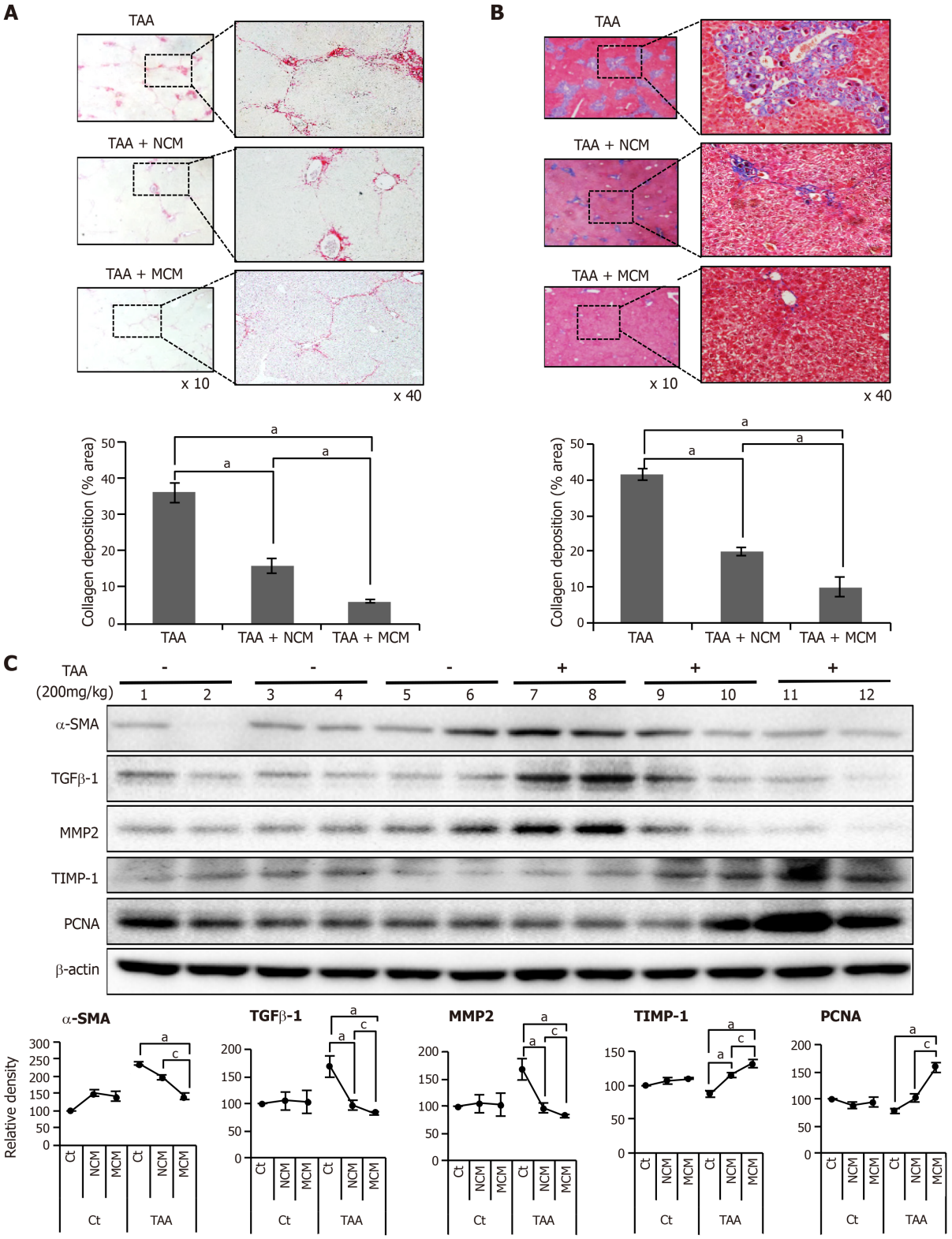

Figure 3 Determination of antifibrotic effects of MCM in the *in vivo* model of liver fibrosis. Control mice and thioacetamide (TAA)-treated mice (mouse model of liver fibrosis) were intravenously (using tail vein) infused with normal saline, CM, and MCM. A, B: Sirius red A and Masson's trichrome B stains showing that MCM infusion significantly decreased the collagen content of the liver in the mouse model of liver fibrosis. Magnification × 400. Percentages of fibrotic areas were measured using NIH image J and expressed as relative values to those in normal livers; C: Western blot analysis of liver specimens. MCM infusion significantly increased the expression of PCNA (a proliferation marker), and significantly decreased the expression of α-SMA, TGF-β1, and MMP1 (fibrotic markers) and increased an antifibrotic marker (TIMP-1) in the mouse model of liver fibrosis. The relative densities of individual markers had been quantified using Image Lab 3.0 (Bio-Rad) software and then were normalized to that of β-actin in each group. Values are presented as mean ± standard deviation of three independent experiments. $^{a}P < 0.05$ *vs* Ct (TAA). $^{c}P < 0.05$ between TAA + NCM and TAA + MCM. α-SMA: Alpha-smooth muscle actin; Ct: Control; CM: The secretome obtained from ASCs after 48-h-incubation; MCM: The secretome released from miR-122-transfected ASCs; MMP-1: Metalloproteinases-1; PCNA: Proliferating cell nuclear antigen; TAA: Thioacetamide; TGF-β: Transforming growth factor-β; TIMP-1: Tissue inhibitor of metalloproteinases-1.

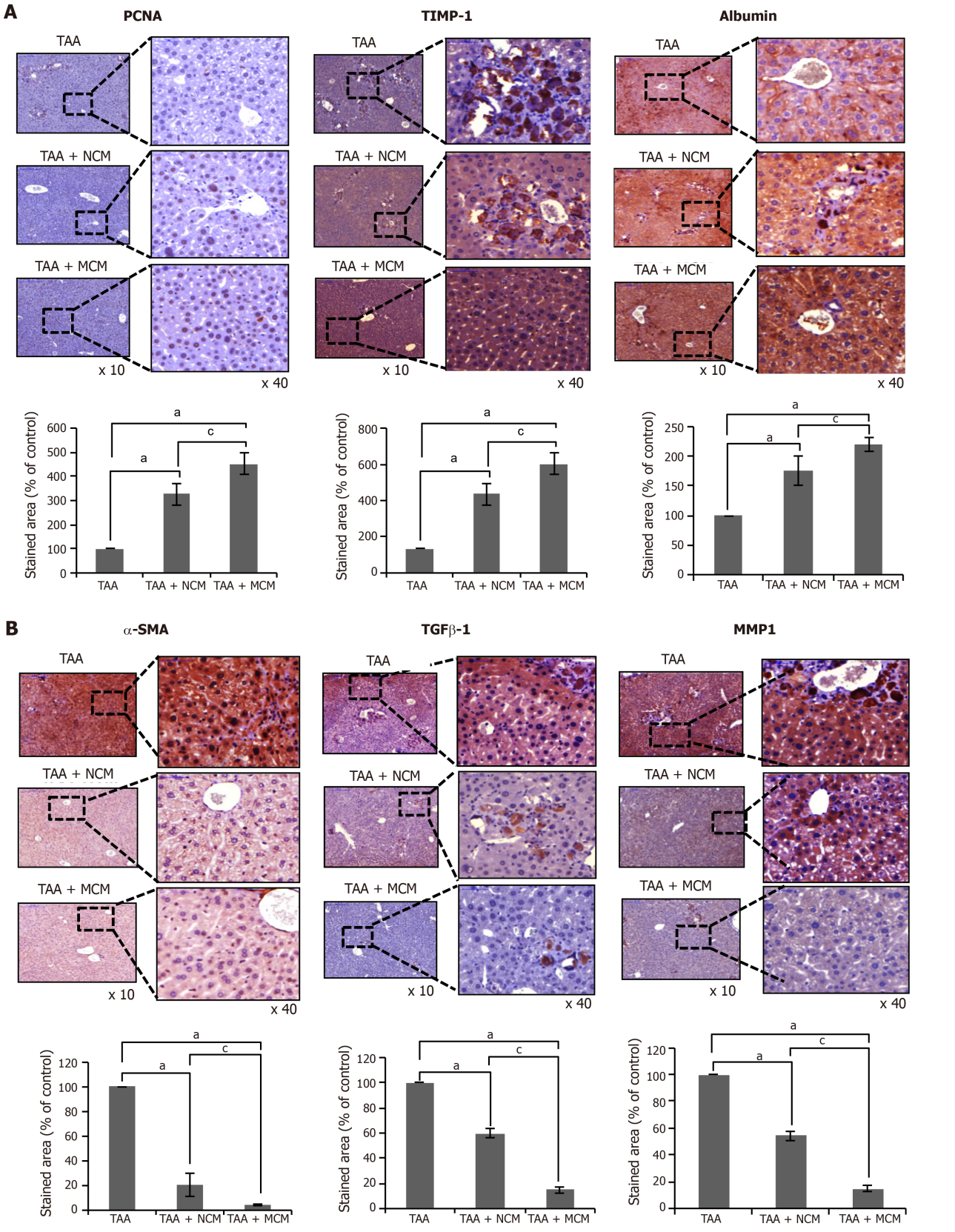

Figure 4 Immunohistochemical staining showing the effects of MCM on the expression of inflammatory and fibrotic markers in the livers. A, B: Upon comparing immunohistochemical staining patterns, MCM infusion led to higher expression of PCNA (an inflammatory marker), albumin, and TIMP-1 (an antifibrotic marker) A, and lower expression of α-SMA, TGF-β1, and MMP1 (fibrotic markers) B in the livers of TAA-treated mice. Percentages of immunoreactive areas were measured using NIH image J and expressed as relative values to those in normal livers. Magnification × 400. Values are presented as mean ± standard deviation of three independent experiments. $^{a}P < 0.05$ *vs* Ct (TAA). $^{c}P < 0.05$ between TAA + NCM and TAA + MCM. α-SMA: Alpha-smooth muscle actin; Ct: Control; CM: The secretome obtained from ASCs after 48-h-incubation; MCM: The secretome released from miR-122-transfected ASCs; MMP-1: Metalloproteinases-1; PCNA: Proliferating cell nuclear antigen; TAA: Thioacetamide; TGF-β: Transforming growth factor-β; TIMP-1: Tissue inhibitor of metalloproteinases-1.

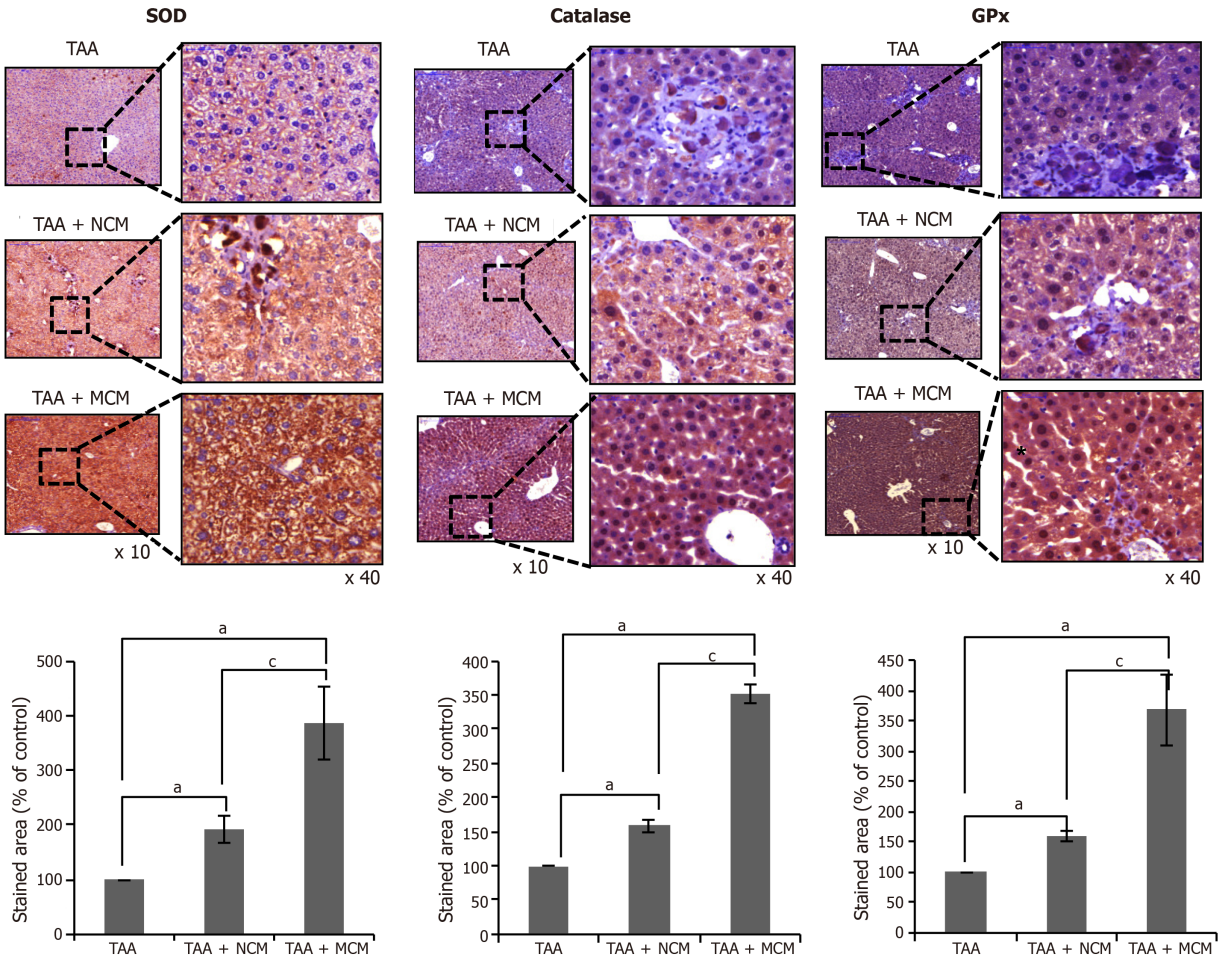

Figure 5 Effects of MCM on the expression of antioxidant enzymes in the liver. Upon comparing immunohistochemical staining patterns, MCM infusion was observed to lead to a higher expression of SOD, catalase, and GPx in the livers of thioacetamide (TAA)-treated mice. The graphs below microscopic figures show the relative densities of these markers. Percentages of immunoreactive areas were measured using NIH image J and expressed as relative values to those in normal livers. Magnification × 400. Values are presented as mean ± standard deviation of three independent experiments. [a]*P* < 0.05 *vs* Ct (TAA). [c]*P* < 0.05 between TAA + NCM and TAA + MCM. Ct: Control; CM: The secretome obtained from ASCs after 48-h-incubation; GPx: Slutathione peroxidase; MCM: The secretome released from miR-122-transfected ASCs; SOD: Superoxide dismutase; TAA: Thioacetamide.

promoting or preventing it. For instance, profibrotic miRNAs include miR-29b, miR-571, miR-199a, miR-200a, and miR-200b, and antifibrotic miRNAs include miR-122, miR-199, miR-200, miR-542, miR-652, and imR-181b[29-32]. Specifically, miR-29b exerts its antifibrotic properties by inhibiting activation of HSCs[31]. Increased serum level of miR-571 has been proposed as a potential biomarker of liver fibrosis, and serum levels of miR-542, miR-652, and imR-181b are decreased in cirrhosis. In addition, serum levels of miR-199a, miR-200a, and miR-200b were highly associated with progression of liver fibrosis in patients with chronic HCV infection[29].

miR-122 is highly expressed in liver, accounting for about 70% and 52% of total miRNAs in liver of adult mouse and human, respectively[33-35]. miR-122 is essentially involved in liver development, differentiation, homeostasis, and functions. Initially, investigators revealed the crucial role of miR-122 in the regulation of cholesterol and fatty acid metabolism in the adult liver[36-38]. Thereafter, anti-inflammatory and anti-fibrotic properties of miR-122 have been revealed by the generation of both germline knock-out (KO) mice and liver-specific KO[39-41]. Specifically, genetic deletion of miR-122 led to liver microsteatosis and inflammation, ultimately resulting in steatohepatitis and fibrosis[38,39]. Additionally, miR-122 expression was reduced in a carbon tetrachloride-induced liver fibrosis mouse model[11]. Interestingly, the restoration of miR-122 levels in miR-122 KO mice reversed the process of liver inflammation, by repressing two miR-122 targets, the chemokine Ccl2[39] and the pro-fibrogenic Krüppel-like factor 6 (KLF6)[40], demonstrating potential utility of miR-122 in therapeutics. We, thus, selected the delivery of miR-122 into ASCs as a mean of reinforcing the antifibrotic properties of ASCs in this study.

We have also shown that the expression of antioxidant enzymes in the liver specimens was significantly increased in the mice infused with the secretome released

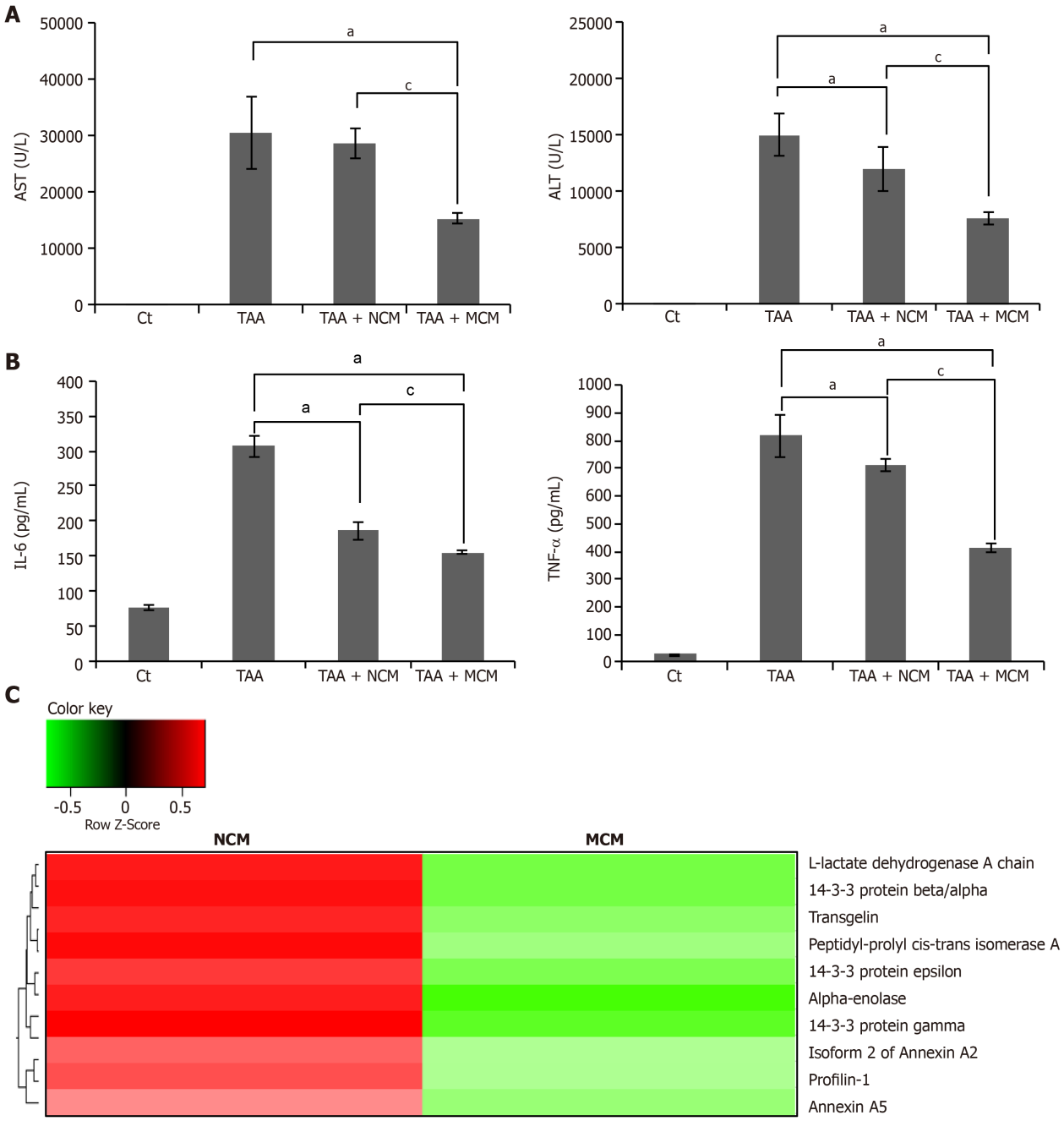

Figure 6 Determination of systemic effects of MCM and analysis of secretome components. A: Results of ELISA showing serum levels of inflammatory markers (IL-6 and TNF-α) in each group. MCM administration had the greatest effect on lowering the serum levels of IL-6 and TNF-α in thioacetamide (TAA)-treated mice; B: Serology tests of AST and ALT in the mouse model of liver fibrosis. MCM infusion had the greatest effect on decreasing the serum levels of AST and ALT; C: Heat map generated from label-free LC-MS for quantitative proteomics reflecting protein expression values of NCM and MCM. Samples are arranged in columns, proteins in rows. Red shading indicates increased expression in samples compared to control; green shading indicates reduced expression; and black shading indicates median expression. Each sample for LC-MS was pooled from three samples of the secretome. The components and concentrations of various essential proteins varied widely between NCM and MCM, validating the effects of miR-125 transfection. Specifically, MCM exhibited a significantly lower concentration of essential intermediates of TGF-β/Smad signaling, such as transgelin, PIN1, and Profilin-1, than NCM. Values are presented as mean ± standard deviation of three independent experiments. $^{a}P < 0.05$ *vs* Ct (TAA). $^{c}P < 0.05$ between TAA + NCM and TAA + MCM. ALT: Alanine transaminase; AST: Aspartate transaminase; TAA: Thioacetamide; TNF- α: Tumor necrosis factor-α.

from miR-122 transfected ASCs compared with the mice infused with the naïve secretome. Although a variety of functional capacities of MSCs or their secretome have been reported, the protective effects against oxidative stress have rarely been reported. Kim *et al*[42] reported that incubation with secretomes derived from ASCs aided human dental fibroblast cells to resist free radicals, and increased antioxidant enzymes, such as SOD and glutathione peroxidase. Recently, Arslan *et al*[43] showed that MSC-derived exosome treatment decreased oxidative stress in the mouse model of ischemia/reperfusion.

It has been demonstrated that oxidative stress involves in both onset and progression of fibrosis arising from a variety origin, such as alcohol, viruses, iron or copper overload, or cholestasis[44]. Both expression and synthesis of this inflammatory and profibrogenic cytokines are mainly modulated through redox-sensitive reactions[45,46]. Further, redox-sensitive reactions also involve in other essential processes of liver fibrosis, such as activation of HSCs and expression of metalloproteinases and of their specific inhibitors[47-49]. We thus think that reduction of oxidative stress could be another way of antifibrotic mechanisms exerted by the secretome released from miR-122 transfected ASCs.

Here, we have focused on the effects of secretome and not those of the stem cells, on liver fibrosis. The term secretome was first mentioned by Black *et al*[50] to refer to all the factors secreted by a cell, along with the secretory pathway constituents. The main constituents of a secretome include secretory proteins and extracellular vesicles. The secreted proteins in humans account for 13%-20% of the entire proteome and include growth factors, cytokines, chemokines, adhesion molecules, proteases, and shed receptors[51]. Extracellular vesicles are typically 30-2000 nm in diameter and can be subdivided into exosomes, microvesicles, and apoptotic bodies, according to their size. Extracellular vesicles usually contain and, thus, carry non-protein components, such as lipids, DNAs, micro-RNAs, and mRNAs. In this study, we focused on the effects of the whole secretome, not its individual constituents, such as exosomes. Exosomes, for example, can be obtained by protracted, complex, and expansive processes[52]. We expect that our results will help eliminate the laborious and expensive process of obtaining exosomes.

The concept of using miRNAs for enhancing the therapeutic potential of the secretome released from stem cells is quite different from how they have been used before. Previously used methods for potentiating secretome, which include physical and chemical stimulation methods, such as hypoxic preconditioning[53,54] or the use of lipopolysaccharides[55], can be categorized as nonspecific stimulation. By contrast, the concept of using miRNAs can be categorized as liver-specific stimulation. In the future, the clinical application of secretome is expected to be tailored according to the needs of patients, combining nonspecific and specific stimulations.

In conclusion, we have shown that the secretome released from miR-122-transfected ASCs was superior to the naïve secretome in improving liver fibrosis, while minimizing inflammatory processes, in mice with TAA-induced liver fibrosis. Hence, it can be postulated that miR-122 transfection into ASCs reconditioned them to have higher antifibrotic properties and to release a secretome with higher antifibrotic components. We could, thus, conclude that the secretome released from miR-122 transfected ASCs has higher antifibrotic and anti-inflammatory properties than the naïve secretome. Because miR-122 transfection into ASCs provides a specific way of potentiating the antifibrotic properties of the ASC secretome, it could be considered as an enhanced method of reinforcing secretome effectiveness.

ARTICLE HIGHLIGHTS

Research background

The therapeutic potential of mesenchymal stem cells (MSCs) is known to be mediated mainly by the secretome that refers to the total collection of secretory materials from MSCs. Basically, naïve secretome has anti-inflammatory, immunomodulatory, and tissue reparative properties. To increase the amount or to reinforce the potential of naïve secretome, researchers have attempted to adjust physico-chemical environment of MSCs or genetically manipulate MSCs. The former has the advantage of being simple but lacking persistence, while the latter has a strong persistence but has the disadvantage of a safety concern in the clinical application.

Research motivation

We have been considering genetic modification as a way of persistently potentiating the therapeutic potential of naïve secretome. In addition, contrasted by the use of genetically modified MSCs, we thought that the use of the secretome could significantly lower the safety concern. We also noted miRNAs as the materials to be used for genetic manipulation, because miRNA is critically involved in the process of liver fibrosis.

Research objectives

Our aim was to determine the antifibrotic potential of the secretome released from miR-122-transfected adipose-derived stromal cells (ASCs) in the model of liver fibrosis.

Research methods

Secretory materials released from ASCs that had been transfected with antifibrotic miR-122 were collected and termed as miR122-secretome. The *in vitro* model of liver fibrosis was generated by treating human hepatic stellate cells (LX2 cells) with a hepatotoxin (thioacetamide; TAA), and the *in vivo* model of liver fibrosis was generated by subcutaneous injection of TAA (200 mg/kg,

three times a week for 8 wk) into five-week male BALB/c mice. For determining *in vivo* effects of miR122-secretome, each secretome (miR122-secrectome and naïve secretome) was intravenously administered to the mice with liver fibrosis, respectively. The degree of liver fibrosis and other alternations in cells or tissues were determined using by molecular and histological investigations, including cell viability assay, western blotting, immunohistochemistry, serology tests, and sandwich enzyme-linked immunosorbent assays.

Research results

The addition of miR-122-secretome to fibrosis-induced LX2 cells significantly decreased the expression of fibrotic markers (MMP2, TGF-β1, TIMP-1, and α-SMA) and increased the expression of an antifibrotic marker (TIMP-1). The western blot analysis showed that miR122-secretome infusion significantly increased the expression of PCNA (a proliferation marker), significantly decreased the expression of α-SMA, TGF-β1, and MMP1 (fibrotic markers), and increased an antifibrotic marker (TIMP-1) in the livers of TAA-treated mice. In addition, miR122-secretome infusion significantly reduced collagen content in the livers, inhibited serum levels of proinflammatory cytokines, such as IL-6 and TNF-α, as well as serum levels of liver enzymes than infusion of the naïve secretome. Finally, our analysis of the components of miR-122-secretome showed that miR-122-secretome exhibited a significantly decreased concentration of essential intermediates of the TGF-β/Smad signaling, such as transgelin, PIN1, and profilin-1, compared to NCM.

Research conclusions

miR-122-secretome was found to be superior to the naïve secretome in improving liver fibrosis while minimizing inflammatory processes in mice with TAA-induced liver fibrosis. Our proteomic analysis of the miR-122-secretome also validated that miR-122-secretome had significantly lesser contents of essential intermediates of liver fibrosis. Therefore, transfecting miR-122 into ASCs is worth considering as a way of reinforcing antifibrotic properties of the secretome from ASCs.

ACKNOWLEDGEMENTS

We thank Byung-Rok Do and Ji-Hyang Kim in Hurim BioCell Company for providing human ASCs and technical assistance. We would like to thank the Francis Sahngun Nahm (a professional statistician) for his devoted assistance of statistical analysis. We would like to thank Hye-Jung Kim for photoshop works that improved the figure quality. We also would like to thank Ji-Hye Park for data processing and statistical analysis. We would like to thank Drug and Disease Target Team (Ochang), Korea Basic Science Institute.

REFERENCES

1 **Baglio SR**, Pegtel DM, Baldini N. Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy. *Front Physiol* 2012; **3**: 359 [PMID: 22973239 DOI: 10.3389/fphys.2012.00359]
2 **Gnecchi M**, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* 2008; **103**: 1204-1219 [PMID: 19028920 DOI: 10.1161/CIRCRESAHA.108.176826]
3 **Hoch AI**, Binder BY, Genetos DC, Leach JK. Differentiation-dependent secretion of proangiogenic factors by mesenchymal stem cells. *PLoS One* 2012; **7**: e35579 [PMID: 22536411 DOI: 10.1371/journal.pone.0035579]
4 **Ranganath SH**, Levy O, Inamdar MS, Karp JM. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell Stem Cell* 2012; **10**: 244-258 [PMID: 22385653 DOI: 10.1016/j.stem.2012.02.005]
5 **Yang Z**, Di Santo S, Kalka C. Current developments in the use of stem cell for therapeutic neovascularisation: is the future therapy "cell-free"? *Swiss Med Wkly* 2010; **140**: w13130 [PMID: 21170763 DOI: 10.4414/smw.2010.13130]
6 **Jiang XP**, Ai WB, Wan LY, Zhang YQ, Wu JF. The roles of microRNA families in hepatic fibrosis. *Cell Biosci* 2017; **7**: 34 [PMID: 28680559 DOI: 10.1186/s13578-017-0161-7]
7 **Kitano M**, Bloomston PM. Hepatic Stellate Cells and microRNAs in Pathogenesis of Liver Fibrosis. *J Clin Med* 2016; 5 [PMID: 26999230 DOI: 10.3390/jcm5030038]
8 **O'Reilly S**. MicroRNAs in fibrosis: opportunities and challenges. *Arthritis Res Ther* 2016; **18**: 11 [PMID: 26762516 DOI: 10.1186/s13075-016-0929-x]
9 **Chen SL**, Zheng MH, Shi KQ, Yang T, Chen YP. A new strategy for treatment of liver fibrosis: letting MicroRNAs do the job. *BioDrugs* 2013; **27**: 25-34 [PMID: 23329398 DOI: 10.1007/s40259-012-0005-2]
10 **Hu J**, Xu Y, Hao J, Wang S, Li C, Meng S. MiR-122 in hepatic function and liver diseases. *Protein Cell* 2012; **3**: 364-371 [PMID: 22610888 DOI: 10.1007/s13238-012-2036-3]
11 **Li J**, Ghazwani M, Zhang Y, Lu J, Li J, Fan J, Gandhi CR, Li S. miR-122 regulates collagen production via targeting hepatic stellate cells and suppressing P4HA1 expression. *J Hepatol* 2013; **58**: 522-528 [PMID: 23178710 DOI: 10.1016/j.jhep.2012.11.011]
12 **Zeng C**, Wang YL, Xie C, Sang Y, Li TJ, Zhang M, Wang R, Zhang Q, Zheng L, Zhuang SM. Identification of a novel TGF-β-miR-122-fibronectin 1/serum response factor signaling cascade and its implication in hepatic fibrogenesis. *Oncotarget* 2015; **6**: 12224-12233 [PMID: 25909171 DOI: 10.18632/oncotarget.3652]
13 **Zuk PA**, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; **13**: 4279-

4295 [PMID: 12475952 DOI: 10.1091/mbc.e02-02-0105]
14 **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]
15 **Swioklo S**, Constantinescu A, Connon CJ. Alginate-Encapsulation for the Improved Hypothermic Preservation of Human Adipose-Derived Stem Cells. *Stem Cells Transl Med* 2016; **5**: 339-349 [PMID: 26826163 DOI: 10.5966/sctm.2015-0131]
16 **Voga M**, Drnovsek N, Novak S, Majdic G. Silk fibroin induces chondrogenic differentiation of canine adipose-derived multipotent mesenchymal stromal cells/mesenchymal stem cells. *J Tissue Eng* 2019; **10**: 2041731419835056 [PMID: 30899447 DOI: 10.1177/2041731419835056]
17 **Szabo G**, Bala S. MicroRNAs in liver disease. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 542-552 [PMID: 23689081 DOI: 10.1038/nrgastro.2013.87]
18 **Elsharkawy AM**, Oakley F, Mann DA. The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis. *Apoptosis* 2005; **10**: 927-939 [PMID: 16151628 DOI: 10.1007/s10495-005-1055-4]
19 **Moreira RK**. Hepatic stellate cells and liver fibrosis. *Arch Pathol Lab Med* 2007; **131**: 1728-1734 [PMID: 17979495]
20 **Mormone E**, George J, Nieto N. Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. *Chem Biol Interact* 2011; **193**: 225-231 [PMID: 21803030 DOI: 10.1016/j.cbi.2011.07.001]
21 **Henderson NC**, Iredale JP. Liver fibrosis: cellular mechanisms of progression and resolution. *Clin Sci (Lond)* 2007; **112**: 265-280 [PMID: 17261089 DOI: 10.1042/CS20060242]
22 **Friedman SL**. Stellate cell activation in alcoholic fibrosis--an overview. *Alcohol Clin Exp Res* 1999; **23**: 904-910 [PMID: 10371412 DOI: 10.1111/j.1530-0277.1999.tb04201.x]
23 **Friedman SL**. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; **134**: 1655-1669 [PMID: 18471545 DOI: 10.1053/j.gastro.2008.03.003]
24 **Hautekeete ML**, Geerts A. The hepatic stellate (Ito) cell: its role in human liver disease. *Virchows Arch* 1997; **430**: 195-207 [PMID: 9099976 DOI: 10.1007/BF01324802]
25 **Czochra P**, Klopcic B, Meyer E, Herkel J, Garcia-Lazaro JF, Thieringer F, Schirmacher P, Biesterfeld S, Galle PR, Lohse AW, Kanzler S. Liver fibrosis induced by hepatic overexpression of PDGF-B in transgenic mice. *J Hepatol* 2006; **45**: 419-428 [PMID: 16842882 DOI: 10.1016/j.jhep.2006.04.010]
26 **Gressner AM**, Weiskirchen R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. *J Cell Mol Med* 2006; **10**: 76-99 [PMID: 16563223 DOI: 10.1111/j.1582-4934.2006.tb00292.x]
27 **Liu Y**, Wang Z, Kwong SQ, Lui ELH, Friedman SL, Li FR, Lam RWC, Zhang GC, Zhang H, Ye T. Inhibition of PDGF, TGF-β, and Abl signaling and reduction of liver fibrosis by the small molecule Bcr-Abl tyrosine kinase antagonist Nilotinib. *J Hepatol* 2011; **55**: 612-625 [PMID: 21251937 DOI: 10.1016/j.jhep.2010.11.035]
28 **Ogawa S**, Ochi T, Shimada H, Inagaki K, Fujita I, Nii A, Moffat MA, Katragadda M, Violand BN, Arch RH, Masferrer JL. Anti-PDGF-B monoclonal antibody reduces liver fibrosis development. *Hepatol Res* 2010; **40**: 1128-1141 [PMID: 20880061 DOI: 10.1111/j.1872-034X.2010.00718.x]
29 **Murakami Y**, Toyoda H, Tanaka M, Kuroda M, Harada Y, Matsuda F, Tajima A, Kosaka N, Ochiya T, Shimotohno K. The progression of liver fibrosis is related with overexpression of the miR-199 and 200 families. *PLoS One* 2011; **6**: e16081 [PMID: 21283674 DOI: 10.1371/journal.pone.0016081]
30 **Roderburg C**, Mollnow T, Bongaerts B, Elfimova N, Vargas Cardenas D, Berger K, Zimmermann H, Koch A, Vucur M, Luedde M, Hellerbrand C, Odenthal M, Trautwein C, Tacke F, Luedde T. Micro-RNA profiling in human serum reveals compartment-specific roles of miR-571 and miR-652 in liver cirrhosis. *PLoS One* 2012; **7**: e32999 [PMID: 22412969 DOI: 10.1371/journal.pone.0032999]
31 **Sekiya Y**, Ogawa T, Yoshizato K, Ikeda K, Kawada N. Suppression of hepatic stellate cell activation by microRNA-29b. *Biochem Biophys Res Commun* 2011; **412**: 74-79 [PMID: 21798245 DOI: 10.1016/j.bbrc.2011.07.041]
32 **Wang B**, Li W, Guo K, Xiao Y, Wang Y, Fan J. miR-181b promotes hepatic stellate cells proliferation by targeting p27 and is elevated in the serum of cirrhosis patients. *Biochem Biophys Res Commun* 2012; **421**: 4-8 [PMID: 22446332 DOI: 10.1016/j.bbrc.2012.03.025]
33 **Girard M**, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A. miR-122, a paradigm for the role of microRNAs in the liver. *J Hepatol* 2008; **48**: 648-656 [PMID: 18291553 DOI: 10.1016/j.jhep.2008.01.019]
34 **Hou W**, Tian Q, Zheng J, Bonkovsky HL. MicroRNA-196 represses Bach1 protein and hepatitis C virus gene expression in human hepatoma cells expressing hepatitis C viral proteins. *Hepatology* 2010; **51**: 1494-1504 [PMID: 20127796 DOI: 10.1002/hep.23401]
35 **Lagos-Quintana M**, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol* 2002; **12**: 735-739 [PMID: 12007417 DOI: 10.1016/S0960-9822(02)00809-6]
36 **Elmén J**, Lindow M, Schütz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjärn M, Hansen HF, Berger U, Gullans S, Kearney P, Sarnow P, Straarup EM, Kauppinen S. LNA-mediated microRNA silencing in non-human primates. *Nature* 2008; **452**: 896-899 [PMID: 18368051 DOI: 10.1038/nature06783]
37 **Esau C**, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S, Monia BP. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* 2006; **3**: 87-98 [PMID: 16459310 DOI: 10.1016/j.cmet.2006.01.005]
38 **Krützfeldt J**, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. Silencing of microRNAs in vivo with 'antagomirs'. *Nature* 2005; **438**: 685-689 [PMID: 16258535 DOI: 10.1038/nature04303]
39 **Hsu SH**, Wang B, Kota J, Yu J, Costinean S, Kutay H, Yu L, Bai S, La Perle K, Chivukula RR, Mao H, Wei M, Clark KR, Mendell JR, Caligiuri MA, Jacob ST, Mendell JT, Ghoshal K. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. *J Clin Invest* 2012; **122**: 2871-2883 [PMID: 22820288 DOI: 10.1172/JCI63539]
40 **Tsai WC**, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R, Huang Y, Chen HC, Lee CH, Tsai TF, Hsu MT, Wu JC, Huang HD, Shiao MS, Hsiao M, Tsou AP. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. *J Clin Invest* 2012; **122**: 2884-2897 [PMID: 22820290 DOI: 10.1172/JCI63455]

41 **Zeisel MB**, Pfeffer S, Baumert TF. miR-122 acts as a tumor suppressor in hepatocarcinogenesis in vivo. *J Hepatol* 2013; **58**: 821-823 [PMID: 23085250 DOI: 10.1016/j.jhep.2012.10.010]

42 **Kim WS**, Park BS, Kim HK, Park JS, Kim KJ, Choi JS, Chung SJ, Kim DD, Sung JH. Evidence supporting antioxidant action of adipose-derived stem cells: protection of human dermal fibroblasts from oxidative stress. *J Dermatol Sci* 2008; **49**: 133-142 [PMID: 17870415 DOI: 10.1016/j.jdermsci.2007.08.004]

43 **Arslan F**, Lai RC, Smeets MB, Akeroyd L, Choo A, Aguor EN, Timmers L, van Rijen HV, Doevendans PA, Pasterkamp G, Lim SK, de Kleijn DP. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res* 2013; **10**: 301-312 [PMID: 23399448 DOI: 10.1016/j.scr.2013.01.002]

44 **Poli G**. Pathogenesis of liver fibrosis: role of oxidative stress. *Mol Aspects Med* 2000; **21**: 49-98 [PMID: 10978499 DOI: 10.1016/S0098-2997(00)00004-2]

45 **Ahamed J**, Laurence J. Role of Platelet-Derived Transforming Growth Factor-β1 and Reactive Oxygen Species in Radiation-Induced Organ Fibrosis. *Antioxid Redox Signal* 2017; **27**: 977-988 [PMID: 28562065 DOI: 10.1089/ars.2017.7064]

46 **Gonzalez-Gonzalez FJ**, Chandel NS, Jain M, Budinger GRS. Reactive oxygen species as signaling molecules in the development of lung fibrosis. *Transl Res* 2017; **190**: 61-68 [PMID: 29080401 DOI: 10.1016/j.trsl.2017.09.005]

47 **Lee AY**, Jang Y, Hong SH, Chang SH, Park S, Kim S, Kang KS, Kim JE, Cho MH. Ephedrine-induced mitophagy via oxidative stress in human hepatic stellate cells. *J Toxicol Sci* 2017; **42**: 461-473 [PMID: 28717105 DOI: 10.2131/jts.42.461]

48 **Tsuchida T**, Friedman SL. Mechanisms of hepatic stellate cell activation. *Nat Rev Gastroenterol Hepatol* 2017; **14**: 397-411 [PMID: 28487545 DOI: 10.1038/nrgastro.2017.38]

49 **Zhang F**, Jin H, Wu L, Shao J, Zhu X, Chen A, Zheng S. Diallyl Trisulfide Suppresses Oxidative Stress-Induced Activation of Hepatic Stellate Cells through Production of Hydrogen Sulfide. *Oxid Med Cell Longev* 2017; **2017**: 1406726 [PMID: 28303169 DOI: 10.1155/2017/1406726]

50 **Black MW**, Boothroyd JC. Lytic cycle of Toxoplasma gondii. *Microbiol Mol Biol Rev* 2000; **64**: 607-623 [PMID: 10974128 DOI: 10.1128/MMBR.64.3.607-623.2000]

51 **Agrawal GK**, Jwa NS, Lebrun MH, Job D, Rakwal R. Plant secretome: unlocking secrets of the secreted proteins. *Proteomics* 2010; **10**: 799-827 [PMID: 19953550 DOI: 10.1002/pmic.200900514]

52 **Helwa I**, Cai J, Drewry MD, Zimmerman A, Dinkins MB, Khaled ML, Seremwe M, Dismuke WM, Bieberich E, Stamer WD, Hamrick MW, Liu Y. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLoS One* 2017; **12**: e0170628 [PMID: 28114422 DOI: 10.1371/journal.pone.0170628]

53 **Lee SC**, Jeong HJ, Lee SK, Kim SJ. Hypoxic Conditioned Medium From Human Adipose-Derived Stem Cells Promotes Mouse Liver Regeneration Through JAK/STAT3 Signaling. *Stem Cells Transl Med* 2016; **5**: 816-825 [PMID: 27102647 DOI: 10.5966/sctm.2015-0191]

54 **Lee SC**, Kim KH, Kim OH, Lee SK, Hong HE, Won SS, Jeon SJ, Choi BJ, Jeong W, Kim SJ. Determination of optimized oxygen partial pressure to maximize the liver regenerative potential of the secretome obtained from adipose-derived stem cells. *Stem Cell Res Ther* 2017; **8**: 181 [PMID: 28774345 DOI: 10.1186/s13287-017-0635-x]

55 **Lee SC**, Jeong HJ, Lee SK, Kim SJ. Lipopolysaccharide preconditioning of adipose-derived stem cells improves liver-regenerating activity of the secretome. *Stem Cell Res Ther* 2015; **6**: 75 [PMID: 25890074 DOI: 10.1186/s13287-015-0072-7]

World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2019 November 26; 11(11): 1005-1019

DOI: 10.4252/wjsc.v11.i11.1005

ISSN 1948-0210 (online)

SYSTEMATIC REVIEW

In vitro differentiation capacity of human breastmilk stem cells: A systematic review

Camila Maria Ribeiro Pacheco, Priscila Elias Ferreira, Claudia Sayuri Saçaki, Luana Alves Tannous, Idiberto José Zotarelli-Filho, Luiz Cesar Guarita-Souza, Katherine Athayde Teixeira de Carvalho

ORCID number: Camila Maria Ribeiro Pacheco (0000-0002-6698-6074); Priscila Elias Ferreira (0000-0002-2582-137X); Claudia Sayuri Saçaki (0000-0003-0312-2663); Luana Alves Tannous (0000-0003-3559-1712); Idiberto José Zotarelli-Filho (0000-0003-4328-1829); Luiz Cesar Guarita-Souza (0000-0003-2781-9705); Katherine Athayde Teixeira de Carvalho (0000-0003-4860-2855).

Author contributions: Pacheco CMR collected the data and drafted this manuscript; Ferreira PE collected the data and revised references; Saçaki CS collected the data; Tannous LA revised the draft; Zotarelli-Filho IJ revised the Prisma 2009; Guarita-Souza LC proposed some discussions in this work; de Carvalho KAT designed the systematic review and performed the data analysis; All authors read and approved the final manuscript.

Supported by Araucária Foundation (Paraná State-Brazil); and the Coordination for the Improvement of Higher Education Personnel-Brazil (Capes), Financial code 001

Conflict-of-interest statement: The authors have no potential conflicts of interests to declare.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

Camila Maria Ribeiro Pacheco, Priscila Elias Ferreira, Claudia Sayuri Saçaki, Katherine Athayde Teixeira de Carvalho, Cell Therapy and Biotechnology in Regenerative Medicine Department, Pelé Pequeno Príncipe Institute, Child and Adolescent Health Research and Pequeno Príncipe Faculty, Curitiba 80.240-020, Paraná, Brazil

Luana Alves Tannous, Luiz Cesar Guarita-Souza, PUCPR-Institute of Biological and Health Sciences, CCBS, Curitiba 80.215-901, Paraná, Brazil

Idiberto José Zotarelli-Filho, Post Graduate and Continuing Education (Unipos), Department of Scientific Production, São José do Rio Preto 15.020-040, São Paulo, Brazil

Corresponding author: Katherine Athayde Teixeira de Carvalho, MD, MsC, PhD, Professor, Cell Therapy and Biotechnology in Regenerative Medicine Department, Pelé Pequeno Príncipe Institute, Child and Adolescent Health Research and Pequeno Príncipe Faculty, Ave. Silva Jardim, n°1632, Curitiba 80.240-020, Paraná, Brazil. katherinecarv@gmail.com

Abstract

BACKGROUND

Mesenchymal stem cells are pluripotent cells that have the ability to generate cells from a cell line or in other cell types from different tissues but from the same origin. Although those cells have more limited differentiation capacity than embryonic stem cells, they are easily obtained from somatic tissue and can be grown in large quantities. This characteristic of undifferentiated stem cells differentiating into different cell lines arouses strategies in regenerative medicine for the treatment of different diseases such as neurodegenerative diseases.

AIM

To evaluate the cell differentiation capacity of human breastmilk stem cells for the three germ layers by a systematic review.

METHODS

The searched databases were PubMed, EMBASE, OVID, and COCHRANE LIBRARY, published between 2007 and 2018 in the English language. All were *in vitro* studies for analysis of the "cell differentiation potential" in the literature using the keywords "human breastmilk," "stem cells," and keywords combined with the Boolean operator "NOT" were used to exclude those articles that had the word "CANCER" and their respective synonyms, which were previously consulted according to medical subject heading terms. PRISMA 2009 guidelines were followed in this study.

Manuscript source: Invited manuscript

Received: April 3, 2019
Peer-review started: April 4, 2019
First decision: June 6, 2019
Revised: July 17, 2019
Accepted: September 4, 2019
Article in press: September 4, 2019
Published online: November 26, 2019

P-Reviewer: Bragança J, Grawish ME, Scarfì S
S-Editor: Ma YJ
L-Editor: Filipodia
E-Editor: Wu YXJ

RESULTS

A total of 315 titles and abstracts of articles were examined. From these, 21 were in common with more than one database, leaving 294 articles for analysis. Of that total, five publications met the inclusion criteria. When analyzing the publications, it was demonstrated that human breastmilk stem cells have a high cellular plasticity, exhibiting the ability to generate cells of all three germ layers, endoderm, mesoderm, and ectoderm, demonstrating their stemness. Those cells expressed the genes, TRA-1-60/81, octamer-binding transcription factor 4, and NANOG, of which NANOG, a critical regulator for self-renewal and maintenance, was the most highly expressed. Those cells have the ability to differentiate *in vitro* into adipocytes, chondrocytes, osteocytes, oligodendrocytes, astrocytes, and neurons as well hepatocytes, β-pancreatic cells, and cardiomyocytes.

CONCLUSION

Although the literature has been scarce, the pluripotentiality of these cells represents great potential for tissue engineering and cellular therapy. Further studies for safe clinical translation are needed.

Key words: Human breastmilk; Stem cells; Cell differentiation; Pluripotency; Stemness

Core tip: Human breastmilk stem cells present interesting features that make them an alternative source of stem cells, mainly because they do not require any invasive procedure to be obtained. The objective was to investigate the literature data on their ability to differentiate into other cell lines. It was possible to verify that these cells have a high capacity of differentiation, as they are able to generate cells of the endoderm, mesoderm, and ectoderm lineages. However, the number of publications on the subject is still scarce, demonstrating that this source needs more studies and has the potential to be explored in regenerative medicine.

Citation: Pacheco CMR, Ferreira PE, Saçaki CS, Tannous LA, Zotarelli-Filho IJ, Guarita-Souza LC, de Carvalho KAT. *In vitro* differentiation capacity of human breastmilk stem cells: A systematic review. *World J Stem Cells* 2019; 11(11): 1005-1019
URL: https://www.wjgnet.com/1948-0210/full/v11/i11/1005.htm
DOI: https://dx.doi.org/10.4252/wjsc.v11.i11.1005

INTRODUCTION

Adult stem cells (ASCs) are present in small quantities in several mature tissues where they are quiescent, that is, not in the process of cell division. These cells are activated during the process of cellular replacement of tissues, being responsible for maintaining the biological homeostasis of the organism, thereby preserving the integrity of the tissues in which they are found. Similar to all other stem cells (SCs), ASCs have the property of self-renewal in that they make identical copies of themselves for long periods. Also, they may give rise to mature cell types with characteristic morphology and specialized functions[1].

Typically, SCs generate an intermediate cell type, called progenitor or precursor cells, before they become fully differentiated. These intermediate-stage cells are partially differentiated in fetal or adult tissues and give rise to different lineages after cell division. ASCs generally reach differentiation through a specific cellular development pathway; however, research has shown that their characteristics are not as definitive as previously thought[2,3].

A well-known type of ASC is mesenchymal stem cells (MSCs), which are present in several adult tissues. Among the most studied are the bone marrow (BM), adipose tissue, dental pulp, and vessel wall of the umbilical cord. MSCs are considered pluripotent cells by many researchers, because they are able to differentiate into osteoblasts, chondrocytes, adipocytes, as well as neural precursors, neurons, and glial cells. This cell population can be easily isolated and expanded *in vitro* because its potential of differentiation reflects the stimulatory and inhibitory factors to which

they are subjected. Moreover, they are able to differentiate into specialized cells, with distinct phenotypes from their precursor, through inhibition and/or activation of certain molecular pathways[4].

Recently, the mammary epithelium has been the focus of a large number of studies due to its remarkable population of SCs as human breastmilk stem cells (hBSCs). It is believed that the existence of these cells in this organ is related to the ability of the mammary gland to expand significantly and regress over the adult life (Figure 1)[5]. The hBSCs are found in the mammary gland in a state of latency and in low numbers; however, during gestation and lactation, they are activated and then transform the ductal structure into a secretory organ. Afterwards, when weaning occurs, milk production decays, inducing apoptosis of the mammary parenchyma cells. As a result, the ductal structure returns until a next pregnancy stimulates modifications of the tissue architecture once again[6,7].

Breastmilk has several cellular components together with hBSCs. In 2007, this population of stem cells in breastmilk was identified for the first time and when analyzed, showed Nestin (marker of neural stem cells) and different cytokeratins (CKs) such as CK5, CK14, CK18, and CK19 on their surfaces[8]. CKs are intermediate filaments expressed in the mammary epithelium depending on the differentiation that the cell undergoes. For example, CK5 expression is indicative of hBSCs of mammary origin. Cells in culture were found to be positive only for Nestin or double positive for CK5/Nestin, but no co-expression of CK14, CK18, or CK19 and Nestin was observed. It is also noteworthy that the analysis of fresh milk by real-time polymerase chain reaction (RT-PCR) only detected Nestin and CK18, indicating that breastmilk contains SCs and differentiated cells[9].

Research has shown that hBSCs have the ability to generate the following three lineages: (1) Alveolar lobe, also named myoepithelial cells, a structure of the adult gland, which constitutes the basal layer of the ducts and alveoli; (2) Ductal epithelial cells, which coat the lumen of the ducts; and (3) Alveolar epithelial cells, which are responsible for protein synthesis in breastmilk[10]. This evident ability of differentiation into cell types different from their tissue of origin raises the question of the real potential of differentiation of these cells. In this context, the purpose of this systematic review was to address the potential of differentiation of hBSCs into the three germ layers.

MATERIALS AND METHODS

Data sources and search strategy

In order to select papers that addressed the topic "potential of cell differentiation of hBSCs" in the literature, PubMed, EMBASE, OVID, AND COCHRANE LIBRARY databases were searched for analysis of the "potential of cell differentiation of hBSCs." The keywords used were "HUMAN BREAST MILK" and "STEM CELLS." Also, a combination of the keywords with AND, and the Boolean operator "NOT" were used to exclude those papers that had the word "CANCER" and its respective synonyms, which were previously consulted in medical subject heading (MeSH) terms. The title and abstracts were examined in all conditions. Papers on cell differentiation were selected manually from this broader primary search by adding the term "cell differentiation" and synonyms, and a very small number of papers were generated. The research structure used in the databases is represented in Table 1.

All duplicate papers were excluded, and the remaining ones were sorted t by title, abstract, and full text. Furthermore, papers in which the abstract was not available were analyzed in their entirety.

Study eligibility criteria

The inclusion criteria were all papers that: (1) Addressed the plasticity of stem cells derived from human breastmilk, that is, the ability of the cell to differentiate into more than one cell line; (2) Were published between 2007 and 2018; (3) Were written in the English language; and (4) Were *in vitro* studies. Three authors independently assessed all papers, and the reviewers reached consensus on the eligibility of the studies after discussion. The exclusion criteria were as follows: (1) Studies in which the origin of the stem cells obtained were from other animal species; (2) Studies that used stem cells from other body tissues; (3) *In vivo* studies; and (4) Written in languages other than English. Subsequently, using the same criteria, the abstracts were selected. Lastly, the complete texts of the papers considered relevant in the previous steps were evaluated according to the following exclusion criteria: (1) Unpublished literature; (2) Only summary of the congress available; (3) Full text not available; and (4) Does not contemplate the process of cell differentiation.

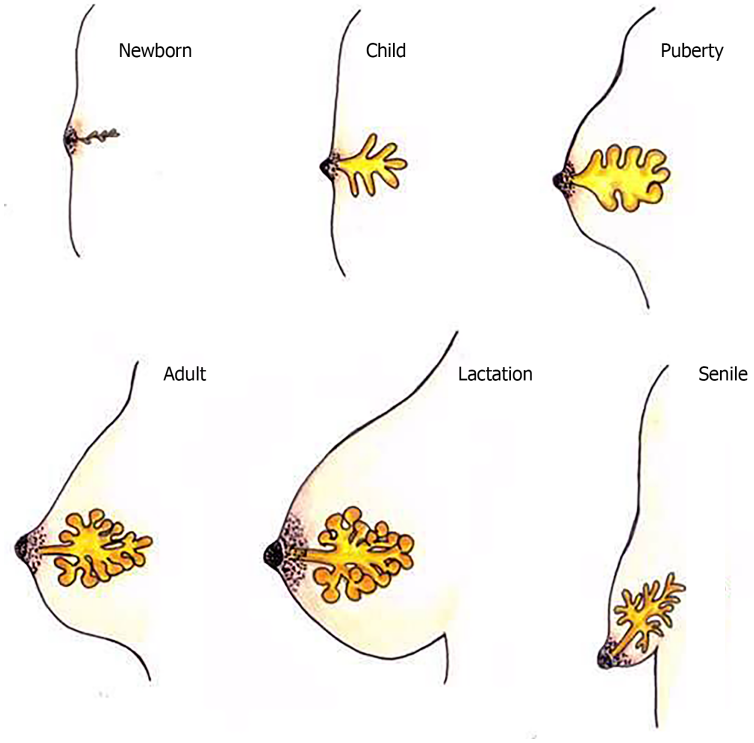

Figure 1 Transformation of the mammary gland throughout life.

Study selection and risk of bias in each study

Two independent reviewers (1 and 2) performed research and study selection. Data extraction was performed by reviewer 1 and fully reviewed by reviewer 2. A third investigator decided some conflicting points and made the final decision to choose the articles. Only studies reported in English were evaluated. The Cochrane instrument was adopted to assess the quality of the included studies[11].

Risk of bias

Considering the Cochrane tool for risk of bias, the overall evaluation resulted in five studies with a high risk of bias and three studies with uncertain risk. The domains that presented the highest risk of bias were related to a number of samples, volume of milk collected e income cells per milliliters (n = 3). Also, there was an absence of financial source in two studies and two studies did not disclose information on the conflict of interest statement.

RESULTS

In the review, 315 papers were initially retrieved (Figure 2), 21 of which were present in more than one database; therefore, they were excluded and considered only once, generating a total of 294 papers. Of these, only five papers met the eligibility criteria; their characteristics are summarized in Table 2.

Although there are few studies in the literature on hBSCs, it is evident that this cell population has mesenchymal characteristics. Positivity for smooth muscle actin (SMA), Vimentin, and Nestin markers, along with the expression of surface markers [cluster of differentiations (CDs)]: CD44, CD29, CD73, CD90, CD106, CD133, CD146, CD271, and spinocerebellar ataxia type 1 (SCA1) support this claim. Low expression of adhesion molecules markers CD146 and CD144 was also detected. In addition, negativity was found for CD33, CD34, CD45, CD105, CD123, CD133, stage-specific embryonic antigen-4 (SSEA-4), and SSEA-1/CD15, confirming that such cells have no hematopoietic origin (Table 3).

The expression of transcription factors considered embryonic stem cell (ESC) markers such as SEEA4, TRA 60-1, Oct4, sex-determining region Y (SRY)-box 2 (Sox2), and NANOG was also evaluated by immunofluorescence staining. A subpopulation of cells expressing CK18 was found, and the frequency of positive cells for that population was 65.33% ± 6.1%.

Table 1 Example of the research structure in PubMed

The same search strategy was used in the other databases	
PubMed (Title/Abstract)	Human Breastmilk OR Human Milk OR Breast Feeding OR Lactation
AND	
PubMed (Title/Abstract)	Stem Cells Or Progenitor Cells OR Mother Cells
NOT	
PubMed (Title/Abstract)	Cancer

The hBSCs differentiated into different cell types of mesodermal origin *in vitro* including adipocytes, chondrocytes, and osteocytes (Figure 3). Moreover, they differentiated into three neural lines: oligodendrocytes, astrocytes, and neurons. They also differentiated into tissues of endodermal origin, as hepatocytes, β-pancreatic cells, and cardiomyocytes. Therefore, their property of differentiation in multiple lines is explicit (Figure 4).

These findings provide evidence of the existence of a subpopulation of pluripotent hBSCs, suggesting that these cells, when isolated from breastmilk, can be stimulated to create several types of tissues and be used in regenerative medicine. If hBSCs have great potential to differentiate into neural cell lines or even into neural precursor cells spontaneously, they would have great applicability in the treatment of neurodegenerative diseases.

DISCUSSION

Considering the results obtained in this review, it was possible to observe the scarcity of studies seeking to verify the potential of cellular differentiation of hBSCs. BM-derived MSCs were the first to be described in 1966[36], and even though in recent years there has been an increase in reports on the isolation of MSCs from different sources, they are the most frequently studied cell type[5]. MSCs can be induced *in vitro* to differentiate into various cell types found at various stages of development, as well as at specific anatomical sites[22].

Although the International Society of Cell Therapy advocates that human MSCs contain the positive surface markers CD105, CD73 and CD90; and have negativity to CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leukocyte antigen-DR isotype, other surface antigens such as CD13, CD29, CD44, and CD10, which are often expressed in MSCs[37], should also be analyzed. Flow cytometry and immunocytochemistry analyses revealed that the hBSCs exhibited the positive surface markers CD29, CD44, CD90, CD146, CD271, and stem cell antigen-1. In addition, the markers CD33, CD34, CD45, CD73, CD123, and CD144 were considered negative by both techniques. Nevertheless, a discrepancy was observed concerning the CD105 marker, because it was negative (2.64% ± 0.55%) when analyzed by flow cytometry, and positive (68.3% ± 3.91%) when observed by immunocytochemistry[12,14,15]. Likewise, lack of expression of CD90 (7.7% ± 0.8%) and CD73 (2.1% ± 0.41%) surface markers was reported, but a positive marker was found for CD105 (47.7% ± 2.95%)[5].

CD146-positive cells have a greater potential for cell proliferation and differentiation[21]. The CD271 marker acts to maintain the clonogenicity of MSCs; however, most of these cells do not co-express CD90 and CD73, two general MSC markers. CD271 is not expressed in all MSCs, but is found at high levels in the BM, adipose tissue, and periodontal ligament. Moreover, it is expressed at low levels in MSCs derived from placenta and is not expressed in synovial membranes[22].

Regarding the transcription factors considered ESC markers that were evaluated by immunofluorescence, flow cytometry, and reverse transcription PCR (RT-PCR), the hBSCs did not express SSEA4 and SSEA1/CD15. Additionally, the markers *Oct4*, *Sox2*, *NANOG*, and *TRA-1-60/81* were reactive. The genes, *TRA-1-60/81* and *NANOG* were the most highly expressed, followed by *Oct4*. In fact, *NANOG* is considered a crucial regulator of self-renewal and maintenance of pluripotentiality[38].

SSEA-4 is a marker of ESCs initially used to isolate MSCs from the BM. It has been observed that when positively expressed, the cells in culture expand extensively and exhibit trilineage differentiation potency. Nevertheless, the use of SSEA-4 as a marker

Table 2 Details of selected articles

Ref.	Purpose	Number of samples	Vol. of milk collected	Income cellcells/ mL	Markers - FC	Markers - IF	PCR	WB	Differentiation cell	Conclusion
Patki *et al*[12]	To study the multipotent differentiation capacity of the long-term MSC culture.	35	15 mL	2.5 x 10^6-3.0 x 10^6/mL	CD29, CD33, CD34, CD44, CD45, CD73, SCA-1.	Nestin, Vimentin, Smooth Muscle Actin and E-Cadherin.	Not done	Not done	Adipogenic, chondrogenic, osteogenic lineages.	Mesenchymal stem cells isolated from human breast milk have cellular potency to differentiate into different cell types.
Hassiotou *et al*[13]	To examine regulators of self-renewal of hBSCs and their plasticity and potential to differentiate into cell types outside the mammary lineage.	> 70	5-200 mL	Not done	SSEA-4 e TRA-1-60 / TRA-1-81.	OCT4, SOX2 and Nanog.	*TRA-1-60 / TRA-1-81, Oct4, Sox2, Klf4* and *Nanog* genes.	*Oct4, Sox2* and *Nanog*.	Osteogenic, adipogenic, chondrogenic, neuronal, pancreatic, hepatocyte, cardiomyocytes, mammary differentiation and teratoma.	Stem cells in breast milk contain pluripotency properties.
Hosseini *et al*[14]	To differentiate the breast milk-derived stem cells toward neural stem cells and then into the neurons and neuroglia.	Not present	5-200 mL	4.0 x 10^4/mL	Not done	Nestin, Nanog, β-tubulin III, O4, GFAP, Oct4, Sox2, CD44, CD105, CD106, CD133.	Not done	Not done	Neurospheres, neurons, oligodendro-cytes, and astrocytes.	The breast milk-derived stem cells showed the capability to be differentiated into neural cell lineages and their similarity to both embryonic and mesenchymal stem cells makes them a good candidate for cell therapy in neurodegenerative diseases.
Sani *et al*[15]	To examine the pluripotency of the human breast milk-derived cells.	5	5-50 mL	Not done	CD90 CD44, CD144, CD 271, CD123, CD133, CD73, CD 106, CD146, CD45, CD 105,CD34, SSEA-4, TRA-60–1, CD 15/SSEA-1.	Oct4, Sox2, Nanog and CK18.	*SRY* gene	Not done	Osteogenic and adipogenic lineages.	Most of the cells found are mesenchymal stem cells, but, cells similar to embryonic stem cells are also present.

Sani *et al*[16]	To examine the differentiation potential of the hBSCs into functional hepatocytes *in vitro*.	15	5-50 mL	Not done	Not done	CK18, CK19, albumin, alpha-fetoprotein.	*AFP, CK19, CYP2B6, HNF4, G6P.*	Not done	Hepatocyte lineage.	The hBSCs demonstrate the ability to express many key factors that are important in liver functions.

hBSC: Human breastmilk stem cell.

is questionable since its positivity was suggested to be an artificial induction of *in vitro* culture using fetal bovine serum (FBS), which contains glycosphingolipids and globoserms that can be recognized by an SSEA-4 antibody. These findings raise the question of the physiological relevance and reliability of SSEA-4 as a marker for MSCs[22,39].

The transcription factors *Oct4* and NANOG are indispensable for maintaining the self-renewal of ESCs. In addition, *Oct4* directly inhibits the signaling pathway of bone morphogenetic protein-4, which activates the mesoderm and the extra-embryonic differentiation of the ectoderm and endoderm, while NANOG acts as a repressor of the neural crest and neuroectoderma lineage and is essential for maintaining the properties of MSCs[4,28].

Pluripotency genes can be strongly correlated with and are the main transcription factors controlling the differentiation and pluripotency of multiple lines of ESCs. The presence of these genes in hBSCs allows a strong association since they play an important role in lactation and the ability to differentiate multiple breastmilk cell lines[26].

Nestin is a well-known marker of SCs of various lineages, and its expression in hBSCs indicates an increase in the characteristics of mammary progenitor cells during lactation. It is also highly expressed in lactating mammary tissues[26].

Through the observation of positivity for SMA, Vimentin, E-cadherin, and Nestin markers by flow cytochemistry, it is possible to hypothesize that the origin of the hBSCs is myoepithelial. HBSCs are positive for CK5 and SMA, and such dual positivity indicates the precursor nature of the cells. In addition, when labeling for CK18 was analyzed, it was present at a frequency of 65.33% ± 6.1% of the cell pool, revealing that there is a subpopulation of cells in breastmilk that is derived from luminal epithelial cells[8].

Currently in the MSC population, only a subpopulation contemplates the criteria recommended by the International Society of Cell Therapy since more compromised or more immature cells can be observed. There is still no consensus on the use of only one marker for the identification of MSCs from different sources, because there are phenotypic and/or functional differences that need to be better understood. It is reinforced that MSC manipulation can generate phenotypic rearrangements, losing the expression of some markers and acquiring others[2]. This phenotype can be modulated in culture and does not reflect the phenotype *in vivo*.

By PCR in cells derived from fresh breastmilk, absence of the SRY gene in genomic DNA was shown, indicating that there are no cells originating from fetal tissues between the isolated cells; thus, there is no fetal microchemistry. Therefore, there is no exchange of stem cells between the mother and the embryo[40].

Among the criteria established for the characterization of MSCs, their ability to differentiate into osteogenic, chondrogenic and adipogenic lineage is included[13]. Several studies have shown that these cells can also differentiate into unrelated germ lines in a process called transdifferentiation. Thus, MSCs can differentiate into cells of mesodermal lineage, such as bone, fat and cartilage, as well as have the potential for endodermal and neuroectodermal differentiation[41].

The use of culture medium containing FBS supplemented with ascorbic acid, β-glycerophosphate, and dexamethasone is needed for osteogenic differentiation from MSCs. It induces an increase in alkaline phosphatase activity and calcium deposition. It was found that by subjecting hBSCs to the osteogenic differentiation environment, for 3 to 4 wk, the presence of osteocytes, the cell acquired cuboidal morphology, and produced mineralized material were observed. Calcium phosphate mineralization was verified by positive staining by Alizarin Red S, indicating that the calcium deposits as amorphous accumulation between the cells[12,15]. Nuclear expression of Runt-related transcription factor 2 *(RUNX2)*, an essential transcription factor for osteoblastic differentiation, was also detected. Some *RUNX2*-positive cells co-expressed osterix, an osteoblast-specific transcription factor required for bone

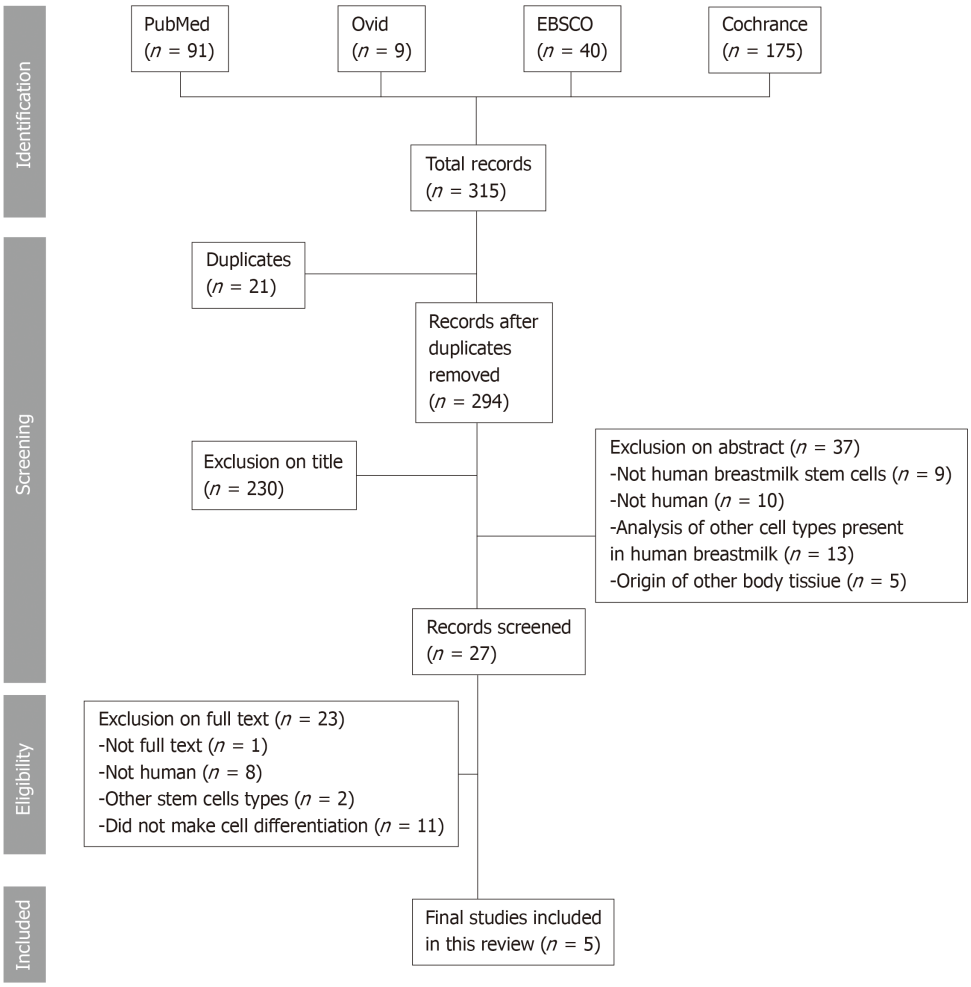

Figure 2 Flow chart demonstrating the search results with excluded and included studies.

formation[13].

Chondrogenic differentiation can be obtained by micromass culture utilizing an FBS-free culture medium containing transforming growth factor beta, which stimulates the production of highly sulfated proteoglycans, specific for cartilage and type II collagen. In contrast, cells synthesize extracellular matrix glycosaminoglycan, collagen type II, and aggrecan, which can be detected by immunohistological staining or by the expression of typical genes of the chondrogenic lineage *via* PCR. After chondrogenic differentiation environment exposure for 21 d, the hBSCs began to show changes in their cellular morphology including acquisition of the spindle shape, the presence of larger round cell aggregates, and the accumulation of cartilage characteristic sulfate proteoglycans, being ratified by staining of Safranin-O[12,15]. The transcription factors for chondrocytes, *RUNX2/Sox6*, were also identified in differentiated cells[13].

When exposed to culture medium containing FBS, dexamethasone, and isobutylmethylxanthine, MSCs were induced to differentiate into adipocytes. Adipogenic differentiation is characterized by the acquisition of an oval cellular morphology and the appearance of intracellular lipid droplets. Such changes can be verified in hBSCs stimulated to adipogenic differentiation for 21 d, which were confirmed by staining with Oil Red O and by positivity of the peroxisome proliferator-activated receptor gamma transcription factor, which is responsible for the regulation of the lipid metabolism[12,13,15].

Regarding the potential of hBSCs to generate different lineages, the presence of myoepithelial cells was observed in the adhered colonies after 2 wk of receiving induction medium for breast differentiation; by the 3rd wk, luminal cells were detected, some of which spontaneously synthesized milk proteins such as ß-casein, lactoferrin, and α-lactalbumin, which were detected in the culture supernatant[13].

It is interesting that many cells that constitute the liver are also the main progenitors for the formation of new hepatocytes. Moreover, when the tissue is compromised, SCs present in the bile ducts are stimulated, which leads to the

Table 3 CD and their functions

Marker	Function	Ref.
CD15/SSEA-1	Stage-specific embryonic antigen, also marker for primitive mesenchymal cells in human bone marrow.	Anjos-Afonso *et al*[17]
CD29	Expressed in myoepithelial cells including mammary gland cells.	Indumathi *et al*[5]
CD33	Present in immune cells of myeloid lineage.	
CD34	Marker of hematopoietic stem cells, vascular endothelium and fibroblasts of some tissues.	Indumathi *et al*[5]
CD44	Marker of myoepithelial cells.	Indumathi *et al*[5]
CD45	Membrane protein present in lymphocytes and absent in erythrocytes and platelets. This protein is lost during the maturation of red cells in the bone marrow. Because it is present in lymphocytes, it is called Common Leukocyte Antigen.	Notta *et al*[18]
CD73	Marker expressed in mesenchymal stem cells, possessing enzymatic activity and catalytic dephosphorylation of adenosine monophosphate converting it into adenosine.	Indumathi *et al*[5]
CD90	Marker of mesenchymal stem cells.	Indumathi *et al*[5]
CD105	Marker of mesenchymal stem cells.	Indumathi *et al*[5]
CD106	Acts on the cell cycle of T and B lymphocytes, in addition to being involved in tissue repair.	Indumathi *et al*[5]
CD123	The IL-3 receptor subunit plays an important role in the growth and differentiation of hematopoietic progenitor cells.	Testa *et al*[19]
CD133	Marker of hematopoietic stem cells.	Indumathi *et al*[5]
CD144	Also known as VE-cadherin and cadherin-5, it is a calcium-dependent transmembrane cell adhesion molecule, located in the intercellular borders of endothelial cells, hematopoietic stem cells and perineural cells.	Dejana *et al*[20]
CD146	Mediated cell-cell interactions, cytoskeletal remodeling, angiogenesis, and migration of endothelial cells.	Sorrentino *et al*[21]
CD271	Transmembrane protein found in neuronal axons, Schwann cells, and perineural cells of peripheral nerves. In addition, it can be found in some epithelial, mesenchymal, and lymphoid tissues.	Lv *et al*[22]
SSEA-4	Marker of mesenchymal stem cells derived from the BM, acting mainly on cell proliferation.	Lv *et al*[22]
TRA-60-1	Related to pluripotency of human embryonic stem cells, and lost in cell differentiation.	Schopperle *et al*[23]
NESTIN	Multipotent, neural, medullary, pancreatic, and epithelial stem cell marker.	Toma *et al*[24]
VIMENTIN	Cytoskeletal intermediate filament protein, associated with the nuclear and plasma membrane, maintains the position of the nucleus and the mitotic spindle during the life of the cell, and is found mainly in mesenchymal stem cells.	Mendez *et al*[25]
SMA	Marker of myoepithelial cells.	Twigger *et al*[26]
E-CADHERIN	Calcium-dependent cell adhesion molecule, with important function in the formation and maintenance of normal tissue architecture, present in epithelial cells of the human mammary gland and epithelial marker of mesenchymal transition in its first passages.	Klopp *et al*[27]
NANOG	Pluripotency marker.	Riekstina *et al*[28]
β-TUBULIN III	Considered an early neuronal marker because the tubulin protein is the main constituent of the microtubules, which are tubular structures that make up the cytoskeleton and they are involved in the transport of organelles and the elongation of axons and dendrites.	Kapitein *et al*[29]

O4	Specific marker of gangliosides expressed by pre-oligodendrocytes and premyelinated oligodendrocytes.	Girolamo *et al*[30]
GFAP	Glial fibrillary acidic protein forms subunits of the intermediate filaments of the cellular cytoskeleton, being present in the cytoplasm of the astrocytes.	Hol *et al*[31]
OCT4	Pluripotency marker.	Riekstina *et al*[28]
SOX2	Controls the undifferentiated and pluripotential state of the CTEs.	Young *et al*[32]
CK18	Expressed in the luminal cells of the breast, which synthesize proteins from breast milk.	Thomas *et al*[33]
KLF 4	Kruppel like factor 4 is a transcription factor related to pluripotency and cell proliferation potential.	Li *et al*[34]
SCA-1	The stem cell antigen 1 is found in stem cells.	Holmes *et al*[35]

differentiation of the hepatocytes. The differentiation of hepatocytes from non-hepatic ASCs has already been demonstrated by SCs originating from BM[42] in the umbilical cord blood[43] and adipose tissue[44]. The hBSCs were able to differentiate in hepatocytes using α-fetoprotein (AFP), pyruvate-kinase M2 isoenzyme, and the albumin functional marker in this study[13].

The markers CK18, CK19, and AFP were identified on differentiating hBSCs into hepatocytes on the 30th day of culture through immunocytochemistry analysis[16]. They also analyzed the expression of hepatocyte-specific genes, such as hepatocyte nuclear factor 4 (HNF4), albumin, CK19, cytochrome p450 family 2 subfamily B member 6 (CYP2B6), glucose 6-phosphate (G6P), and Claudin, which were detected in differentiated cells and may indicate the formation of bile canaliculi. Semi-quantitative RT-PCR showed that the amount of CK19, CYP2B6, and G6P was comparable to that of the positive control used. These cells showed the ability to store glycogen through indocyanine green clearance test, in which hepatocellular function was dynamically assessed, indicating that the differentiated hBSCs are functional *in vitro*. Therefore, these cells can differentiate into endoderm lineages.

Both MSCs obtained from BM[45] and adipose tissue[46] have the ability to differentiate into multilineage, including transdifferentiation in pancreatic beta cells. RT-PCR analysis revealed that hBSCs, when exposed to an environment that induces pancreatic differentiation, positively expressed the pancreatic and duodenal homeobox 1 marker and insulin marker[13]. These genes are associated with pancreatic development, as previously proven in the literature[47]. From these findings, the use of these cells could be considered an alternative in the treatment of type I diabetes, with the ultimate aim of restoring the patient's glycemic levels.

The hBSCs can also stimulate the formation of cardiospheres. These cells start to express troponin T after 4 wk of culture[13]. Remarkably, MSCs have been used for decades in cardiac regenerative therapy, with several sources of these cells such as the umbilical cord, placenta, and bone marrow. For example, MSC cord was seen to help in the recovery of motor function and increase strength of the cardiac muscle[48].

The ability of MSCs to differentiate into neural cells under specific culture conditions has also been reported. For instance, BM MSCs, when cultured in the presence of epidermal growth factor or brain-derived neurotrophic factor expressed glial fibrillary acidic protein (GFAP) and neuron-specific nuclear protein[49]. In a previous study[14], hBSCs were cultured in DMEM/F12 with B27 1% and N2 2% for 7-10 days. After this time, some bead-like cellular aggregations were formed, which were dissociated and conditioned on a different plate containing DMEM/F12, B27, N2, beta fibroblast growth factor, and epidermal growth factor. To analyze the differentiation induction process into neural cells, and in order to compare whether there were differences between the cells before and after this protocol, the authors performed immunocytochemistry techniques for the antibody Nestin and flow cytometry for the CD133 marker. They found that a subpopulation of MSCs derived from breastmilk expressed Nestin (7.4% ± 3.30%) and CD133 (2.76% ± 1.93%), but after exposure to neurogenic induction medium, the frequency of Nestin-positive cells (58.20% ± 6.71%) and those expressing positivity for CD133 (58.74% ± 3.36%) significantly increased. Since these are markers of neural stem cells, it can be inferred that hBSCs may exhibit behavior similar to that of neural stem cells. Recently, hBSCs were also observed to be prone to differentiate into the following three neural cell lines: neurons, astrocytes, and oligodendrocytes. The β-tubulin III marker was used to detect neurons, the O4 antibody for oligodendrocytes, and the GFAP antibody for astrocytes[13,14].

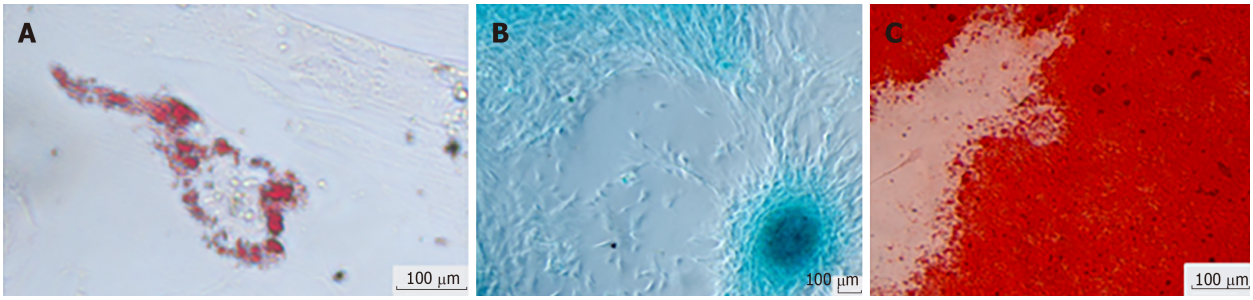

Figure 3 The hBSCs have the ability to differentiate into different cell types of mesodermal origin *in vitro*. A: Adipogenic; B: Chondrogenic; C: Osteogenic. Differentiation of hBSCs was stained with Oil Red O, Alcian Blue, and Alizarin Red, respectively (inversion optical microscope, 100x). Original figure from approved project No. 1.324.098 (11/16/2015) of the Human Ethical Committee of the Pequeno Príncipe Faculty. hBSC: Human breastmilk stem cell.

Mesenchymal breastmilk stem cells can differentiate *in vitro* into cell lines of all three germ layers. Some studies have already shown the *in vitro* differentiation of MSCs to cells with neuronal and glial morphology, which express neuronal and glial markers after stimulation with retinoic acid, nerve growth factor, beta fibroblast growth factor, valproic acid, forskolin, and glial growth factor[49-51], pointing out the transdifferentiation potential of primitive mesenchymal progenitors to cells derived from other embryonic leaflets.

A theoretical model of MSC differentiation was created, in which undifferentiated cells would go through two phases before acquiring a specific phenotype. In the first one, after asymmetric divisions, the MSCs originate a population of cells less undifferentiated and a population of precursor cells with restricted self-renewal and differentiation potential. The transition from the stem cell compartment to the compromised compartment would occur with the symmetric division of tri- or bi-potent progenitor cells, which would originate the unipotent progenitors. This process is well controlled and involves a change in the secretion profile of cytokines and growth factors, as well as a modification in the three-dimensional structure of the extracellular matrix, besides the activation and inactivation of transcriptional factors and consequent expression of genes related to this differentiation pathway[52].

Injecting MSCs into immunodeficient animals showed that they can respond to the different stimuli *in vivo*, differentiating themselves in a disorganized way and leading to the formation of cysts, teratomas, and tumors present in different types of tissues originating from each of the three embryonic leaflets[53]. It is noteworthy that one of the studies performed teratoma formation testing, where freshly isolated hBSCs and cultured sphered milk cells were injected subcutaneously into SCID mice (n = 15). After 9 wk of the procedure, the mice were examined, and no tumor formation was observed[13]. SCID lineage mice have great immunological compromises since they are deficient in T lymphocytes, B lymphocytes, and natural killer cells, and the use of these mice for teratoma formation tests is the most appropriate[54]. In fact, the existence of pluripotent ASCs that do not form teratomas was previously described in BM, considering that these cells have unique patterns of DNA methylation in some genes and it is suggested that the non-formation of teratomas would be associated with these epigenetic characteristics[55].

Even though ESCs originate from the internal mass of the blastocysts, it is also possible to find them in adult tissues in specialized niches. Recent evidence has suggested that certain subpopulations of cells within ASC compartments can differentiate into cell types outside their dermal origin under specific microenvironments *in vitro* and *in vivo*[32]. These results indicate that hBSCs possibly have pluripotency characteristics, with many characteristics to be still explored.

In conclusion, human breastmilk stem cells have high cellular plasticity, exhibiting the ability to generate cells from all three germ layers as demonstrated by their stemness. Thus, further studies are needed to elucidate the implication of these cells in regenerative medicine and bioengineering as well to explore these ASCs obtained from no invasive sources.

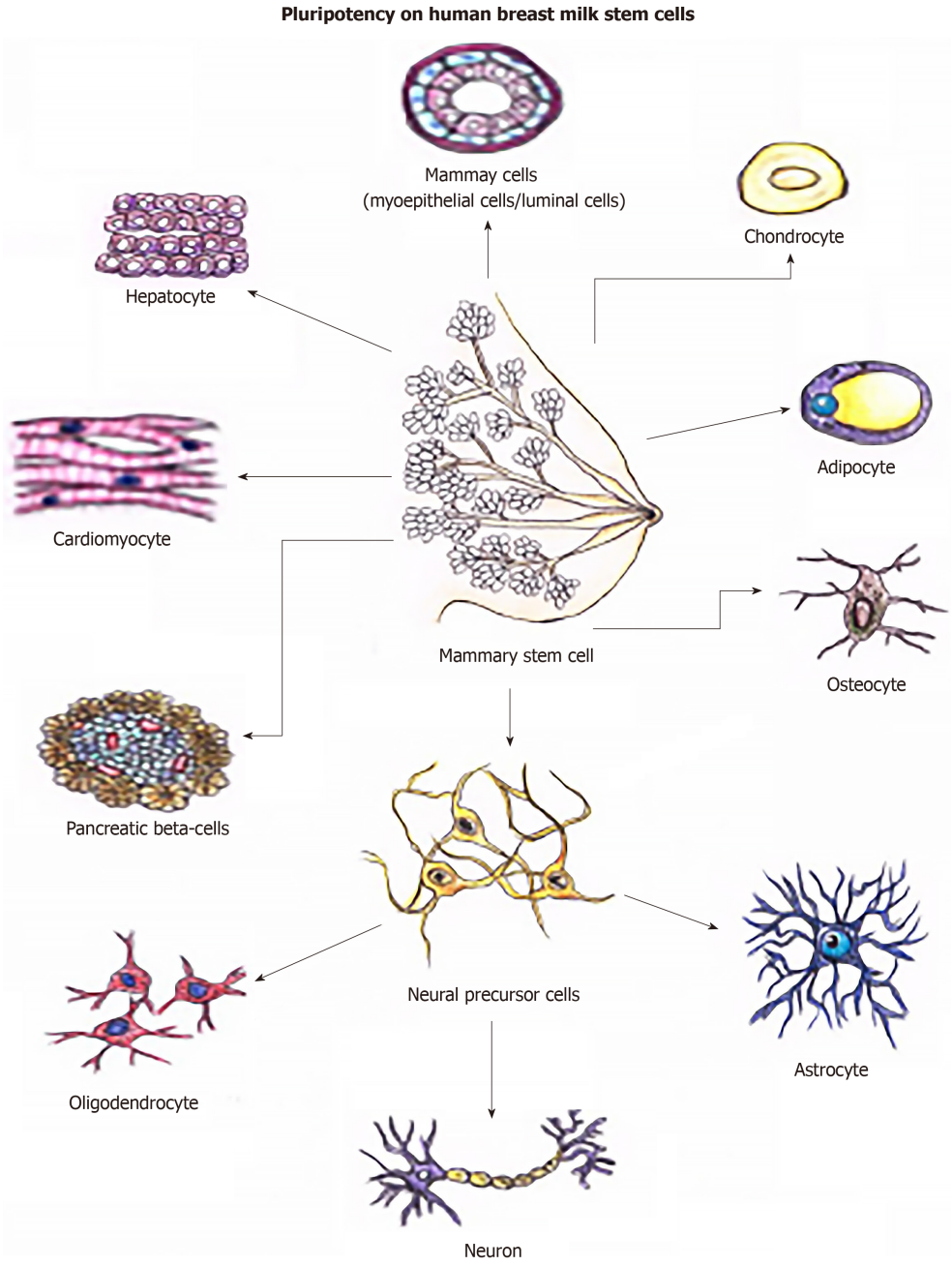

Figure 4 Multilineage potential of hBSCs. These cells can differentiate into mesodermal lineages, endodermal lineages, and the neuroectodermal lineage. hBSC: Human breastmilk stem cell.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells are pluripotent cells that have the ability to generate cells from a cell line or in other cell types from different tissues but from the same origin; although those cells have a more limited differentiation capacity than embryonic stem cells, they are easily obtained from the somatic tissue and can be grown in large quantities. This characteristic of undifferentiated stem cells differentiating into different cell lines arouses strategies in regenerative medicine for the treatment of different diseases, such as neurodegenerative diseases.

Research motivation

Mammary epithelium has been the focus of studies due to its remarkable population of human breastmilk stem cells (hBSCs). It will be important to evaluate the scientific literature as to the potential for differentiation of these cells for regenerative medicine.

Research objectives

The main objective was to evaluate the cell differentiation capacity of hBSCs for the three germ layers through a systematic review.

Research methods

The searched databases were PubMed, EMBASE, OVID, and COCHRANE LIBRARY; the inclusion criteria were all papers that: (1) addressed the plasticity of stem cells derived from human breastmilk, that is, the ability of the cell to differentiate into more than one cell line; (2) published between 2007 and 2018 in the English language; and (3) were *in vitro* studies for the analysis of the "cell differentiation potential" in the literature using the keywords "HUMAN BREASTMILK," "STEM CELLS," and keywords combined with the Boolean operator "NOT" used to exclude those articles that had the word "CANCER" and their respective synonyms, which were previously consulted according to the medical subject heading terms. PRISMA 2009 guidelines were followed in this study.

Research results

A total of 315 titles and abstracts of articles were examined. From these, 21 were in common with more than one database; remaining 294 articles. Out of that total, 5 publications met the inclusion criteria. When analyzing the publications, it was demonstrated that human breastmilk stem cells have a high cellular plasticity, exhibiting the ability to generate cells of all three germ layers: endoderm, mesoderm and ectoderm, demonstrating their stemness. Those cells expressed the genes, TRA-1-60/81, octamer-binding transcription factor 4, and NANOG; NANOG was the gene most highly expressed, which is a regulator for self-renewing and its maintenance. Those cells have the ability to differentiate *in vitro*: adipocytes, chondrocytes, osteocytes, oligodendrocytes, astrocytes, neurons as well hepatocytes, β-pancreatic cells, and cardiomyocytes.

Research conclusions

The hBSCs expressed the genes, TRA-1-60/81, NANOG, and Oct4. The pluripotentiality of hBSCs has been demonstrated by its NANOG expression, which is a regulator for self-renewal and its maintenance. This study opens the possibilities to use this source of adult stem cells for tissue engineering and cellular therapy. hBSCs demonstrated their stemness by high cellular plasticity and exhibiting the ability to generate cells of all three germ layers.

Research perspectives

There will be great potential of hBSCs for tissue engineering and cellular therapy, but more studies for a safe translation will be needed. The single cell analysis seems to be the best method for the next step to evaluate the genetic stability of hBSCs.

REFERENCES

1 **Bianco P**. "Mesenchymal" stem cells. *Annu Rev Cell Dev Biol* 2014; **30**: 677-704 [PMID: 25150008 DOI: 10.1146/ANNUREV-CELLBIO-100913-013132]
2 **Augello A**, De Bari C. The regulation of differentiation in mesenchymal stem cells. *Hum Gene Ther* 2010; **21**: 1226-1238 [PMID: 20804388 DOI: 10.1089/hum.2010.173]
3 **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]
4 **Tsai CC**, Hung SC. Functional roles of pluripotency transcription factors in mesenchymal stem cells. *Cell Cycle* 2012; **11**: 3711-3712 [PMID: 22951581 DOI: 10.4161/cc.22048]
5 **Indumathi S**, Dhanasekaran M, Rajkumar JS, Sudarsanam D. Exploring the stem cell and non-stem cell constituents of human breast milk. *Cytotechnology* 2013; **65**: 385-393 [PMID: 22940915 DOI: 10.1007/s10616-012-9492-8]
6 **Pang WW**, Hartmann PE. Initiation of human lactation: secretory differentiation and secretory activation. *J Mammary Gland Biol Neoplasia* 2007; **12**: 211-221 [PMID: 18027076 DOI: 10.1007/s10911-007-9054-4]
7 **Joshi PA**, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, Stingl J, Waterhouse PD, Khokha R. Progesterone induces adult mammary stem cell expansion. *Nature* 2010; **465**: 803-807 [PMID: 20445538 DOI: 10.1038/nature09091]
8 **Cregan MD**, Fan Y, Appelbee A, Brown ML, Klopcic B, Koppen J, Mitoulas LR, Piper KM, Choolani MA, Chong YS, Hartmann PE. Identification of nestin-positive putative mammary stem cells in human breastmilk. *Cell Tissue Res* 2007; **329**: 129-136 [PMID: 17440749 DOI: 10.1007/s00441-007-0390-x]
9 **Petersen OW**, Gudjonsson T, Villadsen R, Bissell MJ, Rønnov-Jessen L. Epithelial progenitor cell lines as models of normal breast morphogenesis and neoplasia. *Cell Prolif* 2003; **36** Suppl 1: 33-44 [PMID: 14521514]
10 **Liu S**, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. *Breast Cancer Res* 2005; **7**: 86-95 [PMID: 15987436 DOI: 10.1186/bcr1021]
11 **Higgins JPT**, Green S. Cochrane Handbook for Systematic Reviews of Interventions. Version 5.1.0 [updated March 2011]. The Cochrane Collaboration. 2011
12 **Patki S**, Kadam S, Chandra V, Bhonde R. Human breast milk is a rich source of multipotent mesenchymal stem cells. *Hum Cell* 2010; **23**: 35-40 [PMID: 20712706 DOI: 10.1111/j.1749-0774.2010.00083.x]
13 **Hassiotou F**, Beltran A, Chetwynd E, Stuebe AM, Twigger AJ, Metzger P, Trengove N, Lai CT, Filgueira L, Blancafort P, Hartmann PE. Breastmilk is a novel source of stem cells with multilineage differentiation potential. *Stem Cells* 2012; **30**: 2164-2174 [PMID: 22865647 DOI: 10.1002/stem.1188]
14 **Hosseini SM**, Talaei-Khozani T, Sani M, Owrangi B. Differentiation of human breast-milk stem cells to neural stem cells and neurons. *Neurol Res Int* 2014; **2014**: 807896 [PMID: 25506428 DOI: 10.1155/2014/807896]

15 **Sani M**, Hosseini SM, Salmannejad M, Aleahmad F, Ebrahimi S, Jahanshahi S, Talaei-Khozani T. Origins of the breast milk-derived cells; an endeavor to find the cell sources. *Cell Biol Int* 2015; **39**: 611-618 [PMID: 25572907 DOI: 10.1002/cbin.10432]

16 **Sani M**, Ebrahimi S, Aleahmad F, Salmannejad M, Hosseini SM, Mazarei G, Talaei-Khozani T. Differentiation Potential of Breast Milk-Derived Mesenchymal Stem Cells into Hepatocyte-Like Cells. *Tissue Eng Regen Med* 2017; **14**: 587-593 [PMID: 30603512 DOI: 10.1007/s13770-017-0066-x]

17 **Anjos-Afonso F**, Bonnet D. Flexible and dynamic organization of bone marrow stromal compartment. *Br J Haematol* 2007; **139**: 373-384 [PMID: 17910627 DOI: 10.1111/j.1365-2141.2007.06827.x]

18 **Notta F**, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 2011; **333**: 218-221 [PMID: 21737740 DOI: 10.1126/science.1201219]

19 **Testa U**, Pelosi E, Frankel A. CD 123 is a membrane biomarker and a therapeutic target in hematologic malignancies. *Biomark Res* 2014; **2**: 4 [PMID: 24513123 DOI: 10.1186/2050-7771-2-4]

20 **Dejana E**, Giampietro C. Vascular endothelial-cadherin and vascular stability. *Curr Opin Hematol* 2012; **19**: 218-223 [PMID: 22395663 DOI: 10.1097/MOH.0b013e3283523e1c]

21 **Sorrentino A**, Ferracin M, Castelli G, Biffoni M, Tomaselli G, Baiocchi M, Fatica A, Negrini M, Peschle C, Valtieri M. Isolation and characterization of CD146+ multipotent mesenchymal stromal cells. *Exp Hematol* 2008; **36**: 1035-1046 [PMID: 18504067 DOI: 10.1016/j.exphem.2008.03.004]

22 **Lv FJ**, Tuan RS, Cheung KM, Leung VY. Concise review: the surface markers and identity of human mesenchymal stem cells. *Stem Cells* 2014; **32**: 1408-1419 [PMID: 24578244 DOI: 10.1002/stem.1681]

23 **Schopperle WM**, DeWolf WC. The TRA-1-60 and TRA-1-81 human pluripotent stem cell markers are expressed on podocalyxin in embryonal carcinoma. *Stem Cells* 2007; **25**: 723-730 [PMID: 17124010 DOI: 10.1634/stemcells.2005-0597]

24 **Toma JG**, Akhavan M, Fernandes KJ, Barnabé-Heider F, Sadikot A, Kaplan DR, Miller FD. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001; **3**: 778-784 [PMID: 11533656 DOI: 10.1038/ncb0901-778]

25 **Mendez MG**, Kojima S, Goldman RD. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. *FASEB J* 2010; **24**: 1838-1851 [PMID: 20097873 DOI: 10.1096/fj.09-151639]

26 **Twigger AJ**, Hepworth AR, Lai CT, Chetwynd E, Stuebe AM, Blancafort P, Hartmann PE, Geddes DT, Kakulas F. Gene expression in breastmilk cells is associated with maternal and infant characteristics. *Sci Rep* 2015; **5**: 12933 [PMID: 26255679 DOI: 10.1038/srep12933]

27 **Klopp AH**, Lacerda L, Gupta A, Debeb BG, Solley T, Li L, Spaeth E, Xu W, Zhang X, Lewis MT, Reuben JM, Krishnamurthy S, Ferrari M, Gaspar R, Buchholz TA, Cristofanilli M, Marini F, Andreeff M, Woodward WA. Mesenchymal stem cells promote mammosphere formation and decrease E-cadherin in normal and malignant breast cells. *PLoS One* 2010; **5**: e12180 [PMID: 20808935 DOI: 10.1371/journal.pone.0012180]

28 **Riekstina U**, Cakstina I, Parfejevs V, Hoogduijn M, Jankovskis G, Muiznieks I, Muceniece R, Ancans J. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. *Stem Cell Rev* 2009; **5**: 378-386 [PMID: 20058201 DOI: 10.1007/s12015-009-9094-9]

29 **Kapitein LC**, Hoogenraad CC. Which way to go? Cytoskeletal organization and polarized transport in neurons. *Mol Cell Neurosci* 2011; **46**: 9-20 [PMID: 20817096 DOI: 10.1016/j.mcn.2010.08.015]

30 **Girolamo F**, Strippoli M, Errede M, Benagiano V, Roncali L, Ambrosi G, Virgintino D. Characterization of oligodendrocyte lineage precursor cells in the mouse cerebral cortex: a confocal microscopy approach to demyelinating diseases. *Ital J Anat Embryol* 2010; **115**: 95-102 [PMID: 21072997]

31 **Hol EM**, Pekny M. Glial fibrillary acidic protein (GFAP) and the astrocyte intermediate filament system in diseases of the central nervous system. *Curr Opin Cell Biol* 2015; **32**: 121-130 [PMID: 25726916 DOI: 10.1016/j.ceb.2015.02.004]

32 **Young RA**. Control of the embryonic stem cell state. *Cell* 2011; **144**: 940-954 [PMID: 21414485 DOI: 10.1016/j.cell.2011.01.032]

33 **Thomas E**, Lee-Pullen T, Rigby P, Hartmann P, Xu J, Zeps N. Receptor activator of NF-κB ligand promotes proliferation of a putative mammary stem cell unique to the lactating epithelium. *Stem Cells* 2012; **30**: 1255-1264 [PMID: 22593019 DOI: 10.1002/stem.1092]

34 **Li J**, Zheng H, Wang J, Yu F, Morris RJ, Wang TC, Huang S, Ai W. Expression of Kruppel-like factor KLF4 in mouse hair follicle stem cells contributes to cutaneous wound healing. *PLoS One* 2012; **7**: e39663 [PMID: 22745808 DOI: 10.1371/journal.pone.0039663]

35 **Holmes C**, Stanford WL. Concise review: stem cell antigen-1: expression, function, and enigma. *Stem Cells* 2007; **25**: 1339-1347 [PMID: 17379763 DOI: 10.1634/stemcells.2006-0644]

36 **Friedenstein AJ**, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966; **16**: 381-390 [PMID: 5336210]

37 **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]

38 **Ding J**, Xu H, Faiola F, Ma'ayan A, Wang J. Oct4 links multiple epigenetic pathways to the pluripotency network. *Cell Res* 2012; **22**: 155-167 [PMID: 22083510 DOI: 10.1038/cr.2011.179]

39 **Suila H**, Pitkänen V, Hirvonen T, Heiskanen A, Anderson H, Laitinen A, Natunen S, Miller-Podraza H, Satomaa T, Natunen J, Laitinen S, Valmu L. Are globoseries glycosphingolipids SSEA-3 and -4 markers for stem cells derived from human umbilical cord blood? *J Mol Cell Biol* 2011; **3**: 99-107 [PMID: 21149348 DOI: 10.1093/jmcb/mjq041]

40 **Barinaga M**. Cells exchanged during pregnancy live on. *Science* 2002; **296**: 2169-2172 [PMID: 12077398 DOI: 10.1126/science.296.5576.2169]

41 **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]

42 **Oh SH**, Miyazaki M, Kouchi H, Inoue Y, Sakaguchi M, Tsuji T, Shima N, Higashio K, Namba M. Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage *in vitro*. *Biochem Biophys Res Commun* 2000; **279**: 500-504 [PMID: 11118315 DOI: 10.1006/bbrc.2000.3985]

43 **Hong SH**, Gang EJ, Jeong JA, Ahn C, Hwang SH, Yang IH, Park HK, Han H, Kim H. *In vitro*

differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocyte-like cells. *Biochem Biophys Res Commun* 2005; **330**: 1153-1161 [PMID: 15823564 DOI: 10.1016/j.bbrc.2005.03.086]

44 **Seo MJ**, Suh SY, Bae YC, Jung JS. Differentiation of human adipose stromal cells into hepatic lineage *in vitro* and in vivo. *Biochem Biophys Res Commun* 2005; **328**: 258-264 [PMID: 15670778 DOI: 10.1016/j.bbrc.2004.12.158]

45 **Sun Y**, Chen L, Hou XG, Hou WK, Dong JJ, Sun L, Tang KX, Wang B, Song J, Li H, Wang KX. Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells *in vitro*. *Chin Med J (Engl)* 2007; **120**: 771-776 [PMID: 17531117]

46 **Amer MG**, Embaby AS, Karam RA, Amer MG. Role of adipose tissue derived stem cells differentiated into insulin producing cells in the treatment of type I diabetes mellitus. *Gene* 2018; **654**: 87-94 [PMID: 29452233 DOI: 10.1016/j.gene.2018.02.008]

47 **Marappagounder D**, Somasundaram I, Dorairaj S, Sankaran RJ. Differentiation of mesenchymal stem cells derived from human bone marrow and subcutaneous adipose tissue into pancreatic islet-like clusters *in vitro*. *Cell Mol Biol Lett* 2013; **18**: 75-88 [PMID: 23271432 DOI: 10.2478/s11658-012-0040-5]

48 **Singh A**, Singh A, Sen D. Mesenchymal stem cells in cardiac regeneration: a detailed progress report of the last 6 years (2010-2015). *Stem Cell Res Ther* 2016; **7**: 82 [PMID: 27259550 DOI: 10.1186/s13287-016-0341-0]

49 **Sanchez-Ramos J**, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp Neurol* 2000; **164**: 247-256 [PMID: 10915564 DOI: 10.1006/exnr.2000.7389]

50 **Qian L**, Saltzman WM. Improving the expansion and neuronal differentiation of mesenchymal stem cells through culture surface modification. *Biomaterials* 2004; **25**: 1331-1337 [PMID: 14643607 DOI: 10.1016/j.biomaterials.2003.08.013]

51 **Safford KM**, Safford SD, Gimble JM, Shetty AK, Rice HE. Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells. *Exp Neurol* 2004; **187**: 319-328 [PMID: 15144858 DOI: 10.1016/j.expneurol.2004.01.027]

52 **Baksh D**, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med* 2004; **8**: 301-316 [PMID: 15491506 DOI: 10.1111/j.1582-4934.2004.tb00320.x]

53 **Müller FJ**, Goldmann J, Löser P, Loring JF. A call to standardize teratoma assays used to define human pluripotent cell lines. *Cell Stem Cell* 2010; **6**: 412-414 [PMID: 20452314 DOI: 10.1016/j.stem.2010.04.009]

54 **Kuroda T**, Yasuda S, Sato Y. Tumorigenicity studies for human pluripotent stem cell-derived products. *Biol Pharm Bull* 2013; **36**: 189-192 [PMID: 23370350 DOI: 10.1248/bpb.b12-00970]

55 **Shin DM**, Zuba-Surma EK, Wu W, Ratajczak J, Wysoczynski M, Ratajczak MZ, Kucia M. Novel epigenetic mechanisms that control pluripotency and quiescence of adult bone marrow-derived Oct4(+) very small embryonic-like stem cells. *Leukemia* 2009; **23**: 2042-2051 [PMID: 19641521 DOI: 10.1038/leu.2009.153]

Published By Baishideng Publishing Group Inc
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-2238242
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com