ISSN 1948-0210 (online)

World Journal of *Stem Cells*

World J Stem Cells 2022 January 26; 14(1): 1-145

Published by Baishideng Publishing Group Inc

World Journal of Stem Cells

Contents

Monthly Volume 14 Number 1 January 26, 2022

World Journal of Stem Cells

Monthly Volume 14 Number 1 January 26, 2022

Contents

ABOUT COVER

Editorial Board Member of *World Journal of Stem Cells*, Venera Cardile, PhD, Professor, Department of Biomedical Sciences, University of Catania, Via Santa Sofia 97, Catania 95123, Italy. cardile@unict.it

AIMS AND SCOPE

The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJSC* publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, *etc*.

INDEXING/ABSTRACTING

The *WJSC* is now indexed in Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports/Science Edition, Biological Abstracts, BIOSIS Previews, Scopus, PubMed, and PubMed Central. The 2021 Edition of Journal Citation Reports® cites the 2020 impact factor (IF) for *WJSC* as 5.326; IF without journal self cites: 5.035; 5-year IF: 4.956; Journal Citation Indicator: 0.55; Ranking: 14 among 29 journals in cell and tissue engineering; Quartile category: Q2; Ranking: 72 among 195 journals in cell biology; and Quartile category: Q2. The *WJSC*'s CiteScore for 2020 is 3.1 and Scopus CiteScore rank 2020: Histology is 31/60; Genetics is 205/325; Genetics (clinical) is 64/87; Molecular Biology is 285/382; Cell Biology is 208/279.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Jia-Hui Li*; **Production Department Director:** *Xu Guo*; **Editorial Office Director:** *Ze-Mao Gong*.

NAME OF JOURNAL
World Journal of Stem Cells

ISSN
ISSN 1948-0210 (online)

LAUNCH DATE
December 31, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Shengwen Calvin Li, Carlo Ventura

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

PUBLICATION DATE
January 26, 2022

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 ETHICS
https://www.wjgnet.com/bpg/GerInfo/288

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 2022 January 26; 14(1): 1-40

DOI: 10.4252/wjsc.v14.i1.1

ISSN 1948-0210 (online)

REVIEW

Cardiac stem cells: Current knowledge and future prospects

Radwa A Mehanna, Marwa M Essawy, Mona A Barkat, Ashraf K Awaad, Eman H Thabet, Heba A Hamed, Hagar Elkafrawy, Nehal A Khalil, Abeer Sallam, Marwa A Kholief, Samar S Ibrahim, Ghada M Mourad

ORCID number: Radwa Ali Mehanna 0000-0001-9048-4464; Marwa M Essawy 0000-0002-4781-4293; Mona A Barkat 0000-0003-3850-0193; Ashraf K Awaad 0000-0001-8285-0188; Eman H Thabet 0000-0003-3490-3825; Heba A Hamed 0000-0001-8080-4620; Hagar Elkafrawy 0000-0002-1591-1689; Nehal A Khalil 0000-0001-9380-7425; Abeer Sallam 0000-0002-4690-6348; Marwa A Kholief 0000-0001-8776-5257; Samar S Ibrahim 0000-0002-5547-9667; Ghada M Mourad 0000-0002-6145-3076.

Author contributions: Mehanna RA designed, supervised and revised the manuscript, collected and analyzed the data, and shared in figures' creation; Mourad GM supervised the work and revised the paper; all other authors contributed to data collection, writing the manuscript, and creating figures and tables.

Conflict-of-interest statement: Dr. Mehanna has nothing to disclose.

Supported by Science and Technology Development Fund, No. 28932; and Cardiovascular Research, Education, Prevention Foundation, CVREP - Dr. Wael Al Mahmeed Grant.

Country/Territory of origin: Egypt

Specialty type: Cardiac and cardiovascular systems

Radwa A Mehanna, Eman H Thabet, Abeer Sallam, Medical Physiology Department/Center of Excellence for Research in Regenerative Medicine and Applications, Faculty of Medicine, Alexandria University, Alexandria 21500, Egypt

Marwa M Essawy, Oral Pathology Department, Faculty of Dentistry/Center of Excellence for Research in Regenerative Medicine and Applications, Faculty of Medicine, Alexandria University, Alexandria 21500, Egypt

Mona A Barkat, Human Anatomy and Embryology Department/Center of Excellence for Research in Regenerative Medicine and Applications, Faculty of Medicine, Alexandria University, Alexandria 21500, Egypt

Ashraf K Awaad, Samar S Ibrahim, Center of Excellence for Research in Regenerative Medicine and Applications, Faculty of Medicine, Alexandria University, Alexandria 21500, Egypt

Heba A Hamed, Ghada M Mourad, Histology and Cell Biology Department/Center of Excellence for Research in Regenerative Medicine and Applications, Faculty of Medicine, Alexandria University, Alexandria 21500, Egypt

Hagar Elkafrawy, Nehal A Khalil, Medical Biochemistry Department/Center of Excellence for Research in Regenerative Medicine and Applications, Faculty of Medicine, Alexandria University, Alexandria 21500, Egypt

Marwa A Kholief, Forensic Medicine and Clinical toxicology Department/Center of Excellence for Research in Regenerative Medicine and Applications, Faculty of Medicine, Alexandria University, Alexandria 21500, Egypt

Corresponding author: Radwa A Mehanna, MD, PhD, Academic Research, Professor, Executive President, Medical Physiology Department/Center of Excellence for Research in Regenerative Medicine and Applications, Faculty of Medicine, Alexandria University, Al Khartoum Square, Azareeta, Alexandria 21500, Egypt. radwa.mehanna@alexmed.edu.eg

Abstract

Regenerative medicine is the field concerned with the repair and restoration of the integrity of damaged human tissues as well as whole organs. Since the inception of the field several decades ago, regenerative medicine therapies, namely stem cells, have received significant attention in preclinical studies and clinical trials. Apart from their known potential for differentiation into the various body cells, stem cells enhance the organ's intrinsic regenerative capacity by altering its environment, whether by exogenous injection or introducing their products that

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 26, 2021
Peer-review started: February 26, 2021
First decision: June 24, 2021
Revised: July 2, 2021
Accepted: January 6, 2022
Article in press: January 6, 2022
Published online: January 26, 2022

P-Reviewer: Jin C, Matsushita S
S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM

modulate endogenous stem cell function and fate for the sake of regeneration. Recently, research in cardiology has highlighted the evidence for the existence of cardiac stem and progenitor cells (CSCs/CPCs). The global burden of cardiovascular diseases' morbidity and mortality has demanded an in-depth understanding of the biology of CSCs/CPCs aiming at improving the outcome for an innovative therapeutic strategy. This review will discuss the nature of each of the CSCs/CPCs, their environment, their interplay with other cells, and their metabolism. In addition, important issues are tackled concerning the potency of CSCs/CPCs in relation to their secretome for mediating the ability to influence other cells. Moreover, the review will throw the light on the clinical trials and the preclinical studies using CSCs/CPCs and combined therapy for cardiac regeneration. Finally, the novel role of nanotechnology in cardiac regeneration will be explored.

Key Words: Cardiac stem and progenitor cells; Cardiac stem cells' secretome; Cardiac stem cells' niche and metabolism; Nanotechnology; Clinical trials; Combined therapy

Core Tip: With the growing evidence for the existence of regenerating cardiac stem and progenitor cells, studies to evaluate their therapeutic potential have received increasing attention. Although pre-clinical research and clinical trials have demonstrated promising results, yet the latter were often inconsistent in many aspects thus imposing the need for deeper exploration of the molecular biology and relevant pathways regulating cardiogenesis and cardiac muscle repair. This review gives an insight into cardiac stem and progenitor cells regarding their embryological origin, populations, niche, secretome, and metabolism. It overviews the current preclinical research, including medical nanotechnology, and the clinical trials generally applied for cardiac regeneration.

Citation: Mehanna RA, Essawy MM, Barkat MA, Awaad AK, Thabet EH, Hamed HA, Elkafrawy H, Khalil NA, Sallam A, Kholief MA, Ibrahim SS, Mourad GM. Cardiac stem cells: Current knowledge and future prospects. *World J Stem Cells* 2022; 14(1): 1-40
URL: https://www.wjgnet.com/1948-0210/full/v14/i1/1.htm
DOI: https://dx.doi.org/10.4252/wjsc.v14.i1.1

INTRODUCTION

Cardiovascular diseases are the leading cause of death globally, as stated by the latest report 2019 for the World Health Organization, with 17.9 million deaths per year, accounting for 31% of all deaths worldwide.

The heart is one of the least proliferative organs in the human body, and its minimal regenerative capacity has been dogma for decades. Such dogma has been led by the belief that the heart cannot regenerate from ischemic damage. The absence of primary tumors in the heart has further supported the notion of low proliferation. In an alleged post-mitotic organ, it has been debatable whether cardiac cells repair through activation of resident cardiac stem cells (CSCs) and cardiac progenitor cells (CPCs) or by the proliferation of pre-existing cardiomyocytes (CMs). In 2009, Bergmann *et al*[1] were the first to refute that notion and have reported that the heart can in fact self-renew. Based on the results obtained from their carbon-14-labelled DNA study to track CMs, Bergmann *et al*[1] stated that about 50% of CMs renew over the lifespan of an adult. Hsieh *et al*[2] provided further evidence for the origin of newly generated CMs from progenitor cells in an alpha myosin heavy chain (MHC) transgenic model. They estimated that approximately 15% of CMs can regenerate in adult hearts following ischemic damage. With progression of research, lineage tracing of regenerated cardiac tissue confirmed that the newly regenerated CMs develop from a non-CM and possibly from stem cells (SCs)[2].

Further studies have revealed various CSC/CPC candidates that are morphologically and functionally distinct from each other yet act in a complementary fashion and contribute to the regeneration process. This complex cell aggregation is known as the CSC niche that has been a challenge to characterize and locate anatomically[3].

SC applications have been under intensive research interest since the early 20th century. Many types have been isolated, starting from the embryonic, amniotic, and cord blood mesenchymal stem cells (MSCs) and passing through the adult SCs till the induced pluripotent SCs (iPSCs). Adult MSCs are undifferentiated cells with the same potentials as progenitor cells regarding the ability to differentiate into all three germ layer cells[4]. Exogenous MSCs from various sources, including bone marrow, adipose tissue, umbilical cord, placenta, and amniotic fluid[5], have shown promising results in the treatment of cardiovascular diseases. However, the outcome of CSC therapy has shown superior results in experimental studies but to a lesser extent in human clinical trials[6]. The applications of SC therapy for cardiovascular regeneration still hold a plethora of queries to be answered as well as commandment of the molecular and signaling features for CSCs in order to standardize this therapy. Among the aspects that need optimization are the types of SCs and supporting cells to be used, the number of cells, the route of injection, the frequency, and best timing for transplantation. Standardization requires an advanced understanding of the full biological features of CSCs.

SC therapy in cardiac regeneration has dual beneficiary actions. Primarily, the transplanted exogenous SCs would directly differentiate into CMs. Concomitantly, SCs activate the endogenous progenitors through their rich secretome of extracellular vesicles, immunomodulatory and growth factors, protein, and nucleic acid families[7]. These paracrine factors act to activate resident SCs and enhance vascularization to potentiate cardiac repair.

This review aims to provide insight into CSCs/CPCs regarding their embryological origin, populations, niche, metabolism, secretome, and therapeutic potentials. Also discussed is the interplay of nanotechnology with SCs in several aspects, including differentiation, tracking, imaging, and assisted therapy, showing the prospects and limitations of nanoparticle (NP)-based cardiac therapy. Finally, preclinical trials and ongoing, completed, and future clinical trials using CSCs and combined therapy are shown to delineate the potential applications in treating cardiac disease.

EMBRYOLOGICAL ORIGIN OF CPCs

The heart is formed of a wide range of cell types originating from the mesodermal precursor cells. They include CMs and endocardial cells forming the inner layer, while epicardial-derived cells (EPDCs) and smooth muscle cells (SMCs) are found on the external layer. Differentiation of the mesodermal cells is initiated by the T-box transcriptional factors Brachyury (Bry) and Eomes. Bry$^+$ cells differentiate into insulin gene enhancer protein islet-1 (ISL1) and T-box transcription factor 5 (TBX5) expressing cells, while Eomes induce expression of mesoderm posterior 1 (MESP1). MESP1$^+$ cells are identified before the first heart field (FHF) and the second heart field (SHF) separations, so MESP1 serves as an indicator of early CPCs for both heart fields[8]. Chemokine receptor type 4 (CXCR4), fetal liver kinase 1 (FLK-1), and platelet derived growth factor receptor A are other surface markers that coincide with MESP1 and are used in combination to isolate CPCs[9,10].

In addition, a novel cell surface marker known as G protein-coupled receptor lysophosphatidic acid receptor 4 is specific to CPCs and determines its functional significance. Interestingly, its transient expression peaks in cardiac progenitors after 3 to 7 d of human (h)PSCs differentiation toward cardiac lineage, then it declines. *In vivo,* lysophosphatidic acid receptor 4 shows high expression in the initial stages of embryonic heart development and decreases throughout development[11].

The FHF cells are the firstly differentiated myocardial cells that are derived from cells in the anterior lateral plate mesoderm; they give rise to the left ventricle, partially some of the right ventricle population, sinoatrial node, atrioventricular node, and both atria[12]. Meanwhile, the SHF cells originate from the pharyngeal mesoderm to the posterior side of the heart and further divide into anterior and posterior SHF. They contribute to the right ventricle, atria, and the cardiac outflow tract (OFT) formation. Addition of the SHF-derived CMs to the ventricles depend on myocyte enhancer factor 2C (MEF2C). It has been found that MEF2C null mice die at 9.5-d post conception with severe heart defects due to failure of heart looping[13]. In OFT formation, two waves of SHF progenitors and their derivatives have been identified, making a differential

contribution to the aorta and pulmonary artery. The early wave of cells is favorably directed to the aorta, while the second wave of cells contributes to the pulmonary artery. Phosphoinositide-dependent kinase-1 critically regulates the second wave of cells, and its deletion results in pulmonary stenosis[14]. The epicardium of the heart is formed of a transient proepicardial organ. Proepicardium is formed from homeobox protein NKx2.5 (NKx2.5) and ISL1^{+} cells. After epicardial formation, subepicardial mesenchymal space is formed by epithelial to mesenchymal cell transformation of the epicardial cells[15] (Figure 1).

The differentiation in the posterior SHF is regulated by *Hoxb1* gene. Stimulation of *Hoxb1* in embryonic stem cells (ESCs) halts cardiac differentiation, while *Hoxb1*-deficiency shows premature cardiac differentiation in embryos. Moreover, an atrioventricular septal defect develops as a result of ectopic differentiation in the posterior SHF of embryos deficient in *Hoxb1* and its paralog *Hoxa1*[16].

Multiple signaling pathways have essential roles in cardiogenesis with a sequential arrangement. The transforming growth factor-β (TGF-β) superfamily, retinoic acid, Hedgehog, Notch, Wnt, and fibroblast growth factors (FGFs) pathways comprise the chief signaling pathways involved in cardiac development. These pathways, along with transcription factors and epigenetic regulators, regulate cardiac progenitors' specification, proliferation, and differentiation into the different cardiac cell lineages [17].

SIGNALING PATHWAYS DURING CARDIOGENESIS

TGF-β superfamily

The TGF-β superfamily members consist of over 30 structurally associated polypeptide growth factors including nodal and bone morphogenetic proteins (BMP)[18].

Nodal signaling is vital for the formation of sinoatrial node. Nodal inhibition during the cardiac mesoderm differentiation stage downregulates *PITX2c,* a transcription factor recognized to inhibit the formation of the sinoatrial in the left atrium during cardiac development[19]. Moreover, nodal signaling is dispensable for initiation of heart looping; however, it regulates asymmetries that result in a helical shape at the heart tube poles[20].

BMP signaling, as a member of TGF-β, has an important role in the different stages of heart development including the OFT formation, endocardium, and lastly the epicardium. The cardiac neural crest cells have a crucial role in normal cardiovascular development. They give rise to the vascular smooth muscle of the pharyngeal arch arteries, OFT septation, valvulogenesis, and development of the cardiac conduction system[21] (Figure 1). The role of BMP in OFT septation mainly depends on their gradient signaling, which arranges neural crest cell aggregation along the OFT; this Dullard-mediated tuning of BMP signaling ensures the fine timed zipper-like closure of the OFT by the neural crest cells[22]. Furthermore, the BMP signaling promotes the development of endocardial cells (ECs) from hPSC-derived cardiovascular progenitors [23]. It is also integrated with Notch signaling for influencing the proepicardium formation, where overexpression of Notch intracellular receptor in the endothelium enhances BMP expression and increases the number of phospho-Smad1/5^{+} cells for enhancing the formation of the proepicardium[24].

Retinoic acid, hedgehog, and Notch signaling pathways

Retinoic acid signaling plays a role in heart development. It is a key factor for efficient lateral mesoderm differentiation into atrial-like cells in a confined time frame. The structural, electrophysiological, and metabolic maturation of CMs are significantly influenced by retinoic acid[25]. However, it is reported that retinoic acid receptor agonists transiently enhance the proliferation of human CPCs at the expense of terminal cardiac differentiation[26].

The downregulation of the retinoic acid responsive gene, ripply transcriptional repressor 3 (*RIPPLY3)*, within the SHF progenitors by histone deacetylase 1 is required during OFT formation[27].

Hedgehog signaling has a role in OFT morphogenesis. Lipoprotein-related protein 2 (*LRP2*) is a member of the LDL receptor gene family, a class of multifunctional endocytic receptors that play crucial roles in embryonic development. LRP2 is expressed in the anterior SHF cardiac progenitor niche, which leads to the elongation of the OFT during separation into aorta and pulmonary trunk. Loss of LRP2 in mutant mice results in depleting a pool of sonic hedgehog-dependent progenitor cells in the anterior SHF as they migrate into the OFT myocardium due to premature differen-

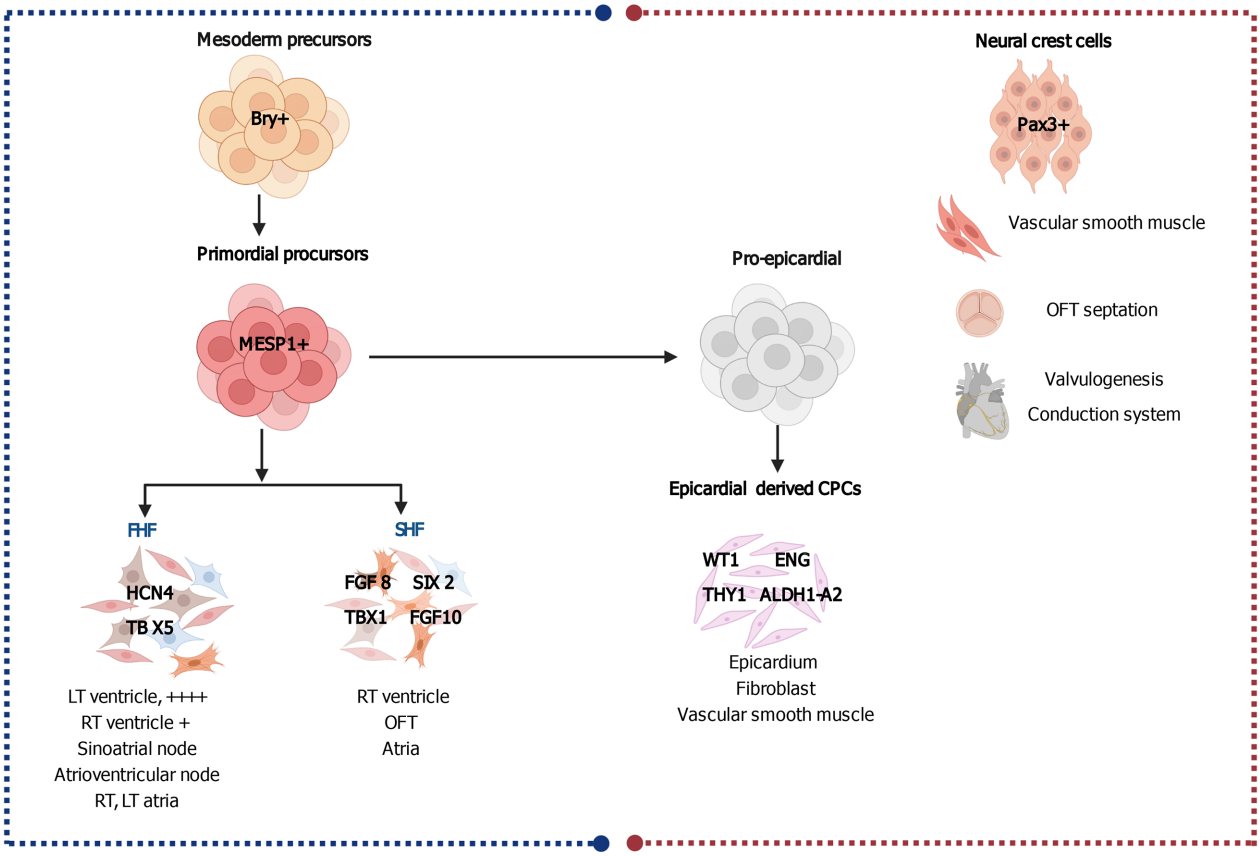

Figure 1 Embryonic cardiac progenitors, Brachyury-positive mesoderm precursors and Pax3$^+$ neural crest cells. Brachyury (Bry$^+$) mesoderm precursors give rise to the mesoderm posterior 1$^+$ primordial precursors, which are the origin of the first heart field, second heart field, and proepicardial progenitors, each population of which is responsible for the development of different parts in the heart. Pax3$^+$ neural crest cells are responsible for the development of vascular smooth muscle, outflow tract, valves and the conductive system. Progenitors are tagged with their specific markers. Created with BioRender.com. CPC: Cardiac progenitor cell; LT: Left; RT: Right; FHF: First heart field; SHF: Second heart field; OFT: Outflow tract.

tiation into CMs. This depletion results in aberrant shortening of the OFT[28].

Four Notch receptors (Notch1–Notch4) and five structurally similar Notch ligands [Delta-like (DLL) 1, DLL3, DLL4, Jagged1, and Jagged2] have been detected in mammals[29]. Activation of Notch signaling enhances CM differentiation from human PSCs. However, the CMs derived from Notch-induced cardiac mesoderm are developmentally immature[30]. *In vivo*, the Notch pathway plays a significant role in CPC biology. An arterial-specific Notch ligand known as DLL4 is expressed by SHF progenitors at critical time-points in SHF biology. The DLL4-mediated Notch signaling is a crucial requirement for maintaining an adequate SHF progenitor pool, in a way that *DLL4* knockout results in decreased proliferation and increased apoptosis. Reduced SHF progenitor pool leads to an underdeveloped OFT and right ventricle[31].

Wnt pathway

The Wnt signaling pathway has an essential role in many developmental stages of embryogenesis. The Wnt family consists of 19 distinct Wnt proteins and other 10 types of Frizzled receptors. On the basis of their primary functions, the Wnt and Frizzled receptors are divided into two major classes, which are the canonical and non-canonical Wnt pathways[32]. Accumulating evidence suggests a role for the dynamic balance between canonical and non-canonical Wnt signaling in cardiac formation and differentiation. Wnt/β-catenin signaling is required for proper mesoderm formation and proliferation of CMs but needs to be low for terminal differentiation and cardiac specification. In contrast, for cardiac specification in murine and human ESCs, non-canonical β-catenin independent Wnt signaling is essential, while the non-canonical Wnt signaling is necessary for terminal differentiation later in development[33].

The activation of non-canonical Wnt is non-catenin-independent, and the downstream proteins involve several kinases, including protein kinase C, calcium/calmodulin-dependent kinase, and Jun N terminal kinase (JNK). Wnt11 enhances angiogenesis and improves cardiac function through non-canonical Wnt-protein kinase C-Jun N terminal kinase dependent pathways in myocardial infarction (MI)

[34]. In hypoxia, Wnt11 expression preserves the integrity of mitochondrial membrane and facilitates the release of insulin growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF), thus protecting CMs against hypoxia[35]. Canonical dependent Wnt signaling, Wnt 3 Ligand, favors the pacemaker lineage, while its suppression promotes the chamber CM lineage[36].

TRANSCRIPTOME AND REGENERATIVE CAPACITY OF SUB-POPULATIONS

The regenerative capacity of most organs is contingent on the adult SC populations that exist in their niches and are activated by injury. Adult SC populations vary greatly in their molecular marker expression profile and hence in their possible role in regenerative medicine. The transcriptome is a representation of the gene read-outs, the cellular state, and is imperative for studying all genetic disease and biological processes. The genome-wide profiling using novel sequencing technology has made transcriptome research accessible.

c-KIT⁺ CPCs

Receptor tyrosine kinase (RTK) c-KIT (also referred to as SC factor receptor or CD117)-expressing CPCs are mainly located in the atria and the ventricular apex, comprising most of the ventricular and atrial myocardium[37]. c-KIT⁺ cells also express the cardiac transcription factors NKx2.5, GATA binding protein 4 (GATA4), and MEF2C but are negative for the hematopoietic markers CD45, CD3, CD34, CD19, CD16, CD20, CD14, and CD56[38,39]. SC factor ligand attaches to the c-KIT receptor and activates the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and p38 mitogen-activated protein kinase (MAPK) signaling pathways[40]. Both PI3K/AKT and MAPK pathways control various CPCs functions like self-renewal, proliferation, migration, and survival [41]. During embryonic development and the early post-natal time, c-KIT⁺ CPCs contribute to the generation of new CMs. Such capacity declines in the adult heart with only a few new CMs originating from CPCs[42]. In a rat MI model, the c-KIT⁺ CPCs have migrated through the collagen type I and type III matrices into the infarcted area. The transplanted CPCs have shown overexpressed matrix metalloproteinases (MMPs; MMP2, MMP9, and MMP14) that degrade extracellular matrix (ECM), concluding that c-KIT⁺ CPCs hold an invasive capacity[43]. Transplanted CPCs (c-KIT⁺ CPCs and cardiospheres) also show an endogenous proliferative potential *in vivo* and additionally activate endogenous CPCs[44].

SCA-1⁺ CPCs

Stem cell antigen 1 (SCA-1) expressing CPC population exists predominantly in the atrium, intra-atrial septum, and atrium-ventricular boundary and dispersed inside the epicardial layer of adult hearts[45]. SCA-1 is a cell surface protein of the lymphocyte antigen-6 *(Ly6)* gene family, which has roles in cell survival, proliferation, and differentiation[46]. A population of SCA-1⁺ cells from murine adult myocardium hold a telomerase activity comparable to that of a neonatal heart. This SCA-1⁺ population is different from hematopoietic SCs as they lack CD45, CD34, c-KIT, LIM domain only 2, GATA2, VEGF receptor 1, and T-cell acute lymphoblastic leukemia 1/SC leukemia proteins. SCA-1⁺ cells are also distinct from endothelial progenitor cells and express cardiac lineage transcriptional factors such as GATA4, MEF2C, and translation elongation factor 1 yet lack transcripts for cardiomyocytic structural genes such as *BMP1r1* and *α-, β-MHC*[47,48]. Although this population exhibits the endothelial marker CD31, it is suggested to be due to the contaminating endothelial CD31⁺/SCA-1⁺ cells. *In vitro* studies have revealed that 5-azacytidine (5-aza), a demethylating agent, pushed SCA-1⁺ cells to differentiate into CMs[48,49]. Further studies have isolated SCA-1⁺ cells that lack CD31 and CD45 markers, referring to them as lineage negative (Lin⁻). The SCA-1⁺/Lin⁻ cells display a mesenchymal cell-surface profile (CD34⁻, CD29⁺, CD90⁺, CD105⁺, and CD44⁺) and are able to differentiate, to a certain extent, into CMs and endothelial and smooth muscle-like cells[50,51].

Human SCA-1⁺-like cells also express early cardiac transcription factors (GATA4, MEF2C, insulin gene enhancer protein ISL-1, and Nkx-2.5) and can differentiate into contractile CMs[52]. Although a human ortholog of the SCA-1 protein has not been yet identified, an anti-mouse SCA-1 antibody is used to isolate SCA-1⁺-like cells from the adult human heart.

MESP1^{+} CPCs

MESP1 expressing cells mainly contribute to the mesoderm and to the myocardium of the heart tube during development[53]. Transient expression of MESP1 seems to accelerate and enhance the appearance of cardiac progenitor. However, homologous disruption of the *MESP1* gene has resulted in aberrant cardiac morphogenesis. *MESP1* interacts with the promoter area of main cardiac transcription factors, including heart and neural crest derivatives expressed 2, Nkx2-5, myocardin, and GATA4[54]. These factors induce fibroblasts to express a full battery of cardiac genes, form sarcomeres, develop CM-like electrical activity, and in a few cases elicit beating activity[55]. Several studies have shown that the addition of MESP1 could enhance the efficacy of direct reprogramming of fibroblasts into CMs[56,57]. The transdifferentiation of fibroblasts to CMs *via MESP1* suggests that *MESP1* chiefly modulates the gene regulatory network for cardiogenesis[52].

KDR^{+} CPCs

Kinase insert domain receptor (KDR), also known as Flk-1, is one of the earliest discovered cardiogenic progenitor cell markers acting during the early stages of cardiac development in human[58]. Nelson *et al*[59] have reported that Flk-1 has a distinctive transcriptome that has been evident at day 6, immediately after gastrulation but prior to the expression of the cardiac transcription factors. KDR^{+} population lack the pluripotent octamer-binding transcription factor 4, sex determining region Y-Box transcription factor (SOX) 2, and endoderm SOX17 markers. On the other hand, KDR^{+} CPCs have shown a noteworthy upregulation in SOX7, a vasculogenic transcription factor, overlapping with the emergence of primordial cardiac transcription factors GATA4, myocardin, and NKx2.5. Moreover, KDR subpopulations that overexpress SOX7 are associated with a vascular phenotype rather than a cardiogenic phenotype. These outcomes offer insights for refining the therapeutic regenerative interventions.

CPCs from the first and second heart fields

The FHF cells express hyperpolarization activated cyclic nucleotide gated potassium channel 4 and TBX5, while SHF progenitors express TBX1, FGF 8, FGF10, and sine oculis homeobox2 (Figure 1). Cells from the SHF exhibit high proliferative and migratory capacities and are mostly responsible for the elongation and winding of the heart tube. Moreover, SHF cells differentiate to CMs, SMCs, fibroblasts, and endothelial cells (ECs) along their journey in the heart tube to form the right ventricle, right ventricular OFT, and most of the atria[60,61]. However, FHF cells hold less proliferative and migratory potentials and differentiate predominantly to CMs that form the left ventricle and small parts of the atria[62]. The cells of the cardiac crescent, theoretically the progeny of FHF CPCs, are terminally differentiated cells expressing the markers of CMs, such as actin alpha cardiac muscle 1 and myosin light chain 7[63,64], hence they are unlikely to be multipotent progenitors. Therefore, it is difficult to identify FHF before Nkx2.5 and TBX5 expressions. Conversely, multipotent SHF CPCs were validated with a clonal tracing experiment and identified by ISL1 expression[65]. However, ISL1 expression is not specific for SHF and has been proposed to represent only the developmental stages[66]. Tampakakis *et al*[67] generated ESCs by using hyperpolarization activated cyclic nucleotide gated potassium channel 4-green fluorescent protein and TBX1-Cre; Rosa-red fluorescent protein reporters of the FHF and the SHF respectively, and also by using live immunostaining of the cell membrane CXCR4, a SHF marker and the reporters. The ESC-derived progenitor cells have shown functional properties and transcriptome similar to their *in vivo* equivalents. Thus, chamber-specific cardiac cells have been generated for modelling of heart diseases *in vitro*.

Epicardium-derived CPCs

The EPDCs are important as a signaling source for heart development, cardiac regeneration, and post-MI heart repair. Throughout the development of the heart in mice, EPDCs aid in the formation of various cardiac cell types and secrete paracrine factors for myocardial maturation[68]. In the adult heart, EPDCs are normally dormant and become stimulated following myocardial injury. Transcriptional analysis of the EPDCs derived from human (h)iPSCs cells have revealed several markers of EPDCs including Wilm's tumor protein 1, endoglin, thymus cell antigen 1, and aldehyde dehydrogenase 1 family member A2[69] (Figure 1). Following MI in mice, EPDCs undergo an epithelial-to-mesenchymal transition, with overexpression of Wilms tumor protein 1, and differentiate mainly into SMCs/fibroblasts[70,71]. EPDC-secreted paracrine factors include VEGF-A, FGF2, and PDGF-C, which support the growth of

blood vessels, protect the myocardium, and recover cardiac functions in an acute MI-mouse model[70].

Side population-derived CPCs

Side population (SP) cells have been detected in the heart and other various tissues and hold enhanced stem and progenitor cell activity[72]. SP cells, when stained *in vitro,* hold the ability to flush out the DNA Hoechst dye from their nuclei[73]. Gene expression profiling of SP cells after MI has revealed a downregulation of Wnt-related signals coupled with increased SP cell proliferation. This has been validated *in vitro* by treatment of isolated SP cells with canonical Wnt agonists or recombinant Wnt, where the proliferation of SP cells has been repressed with partial arresting the G1 cell cycle phase[74]. Consistent with this observation, delivery of secreted Frizzled-related proteins (SFRP; the Wnt antagonizer) improves post-MI remodeling[75,76].

SP cells can be identified by surface marker adenosine triphosphate (ATP) binding cassette subfamily G member 2 (ABCG2), also referred to as the breast cancer resistance protein1[77]. $ABCG2^+$ cells have been also observed in the adult heart and can differentiate *in vitro* into CMs[78]. When SP cells have been injected into the injured hearts of rats, they have been recruited to the injured regions, where they differentiate into CMs, ECs, and SMCs, suggesting that they may be endogenous SP cells[79]. However, ABCG2–CreER based genetic lineage tracing has demonstrated that $ABCG2^+$ cells could only differentiate into the multiple cardiac cell lineages during the embryonic stages but not in adulthood[80,81]. The combination of $ABCG2^+$ cells with pre-existing CMs is more likely to stimulate CM proliferation rather than differentiation into CMs directly[82]. Therefore, genetic fate mapping investigations have disproved the SP cells property of the adult endogenous $ABCG2^+$ SP and their *in vivo* renewing myogenic ability[83].

Cardiosphere-derived CPCs

Cardiospheres contain a combination of stromal, mesenchymal, and progenitor cells that are isolated from cultures of human heart biopsy[39,84]. They represent a niche-like environment, with cardiac-committed cells in the center and supporting cells in the periphery of the spherical cluster[85]. The cardiosphere-derived cells (CDCs) were originally isolated from mouse heart explants and human ventricular biopsies based on their ability to form three-dimensional (3D) spheroids in suspension cultures[86]. CDCs have grabbed much attention due to their proliferation and differentiation abilities by inherent stimulation of cardio-specific differentiation factors [GATA4, MEF2C, Nkx2.5, heart and neural crest derivatives expressed 2, and cardiac troponin T (TNNT2)] using a clustered regularly interspaced short palindromic repeat/dead Cas9 (CRISPR/dCas9) assisted transcriptional enhancement system[87,88]. Sano *et al*[89] have postulated that the CRISPR/dCas9 system may provide a proficient method of modifying *TNNT2* gene activation in SCs. Consequently, CRISPR/dCas9 can improve the therapeutic outcomes of patients with ischemic heart disease by enhancing the transplanted CDCs differentiation capacity within the ischemic myocardium. Heart tissue is usually obtained by endomyocardial biopsy or during open cardiac surgery and grown in explants to form CDCs. CDCs have shown a superior myogenic differentiation potential, angiogenesis, and paracrine factor secretion as compared to other cell types. In heart failure animal models, the injected CDCs potentially differentiated into CMs and vascular cells. Additionally, CDCs have diminished unfavorable remodeling and infarct size, and hence improve cardiac function[90]. Accordingly, cardiospheres and CDCs may be some of the most promising sources of CPCs for cardiac repair.

CSC niche

The niche in the heart integrates several heterogeneous cell types, including CSCs, progenitors, fibroblasts, SMCs, CMs, capillaries, and supporting telocytes (TCs)[91], together with the junctions and cementing ECM that hold the niche together. Such architectural arrangement is essential for protection against external damaging stimuli and for preserving the stemness of the CSCs (Figure 2). Without the niche microenvironment, CSCs lose their stemness and initiate differentiation eventually, leading to the exhaustion of the CSC pool. Similarly, *in vitro* studies require feeder layers and cytokines supplements in the culture media to ensure that SCs remain in their undifferentiated state[37].

In vitro studies have recapitulated the niche theory using cardiospheres, which are 20–150 μm spheres (Figure 2) of cells generated from the explant outgrowth of heart tissues[92,93]. Cardiospheres consist of CSCs in the core and cells committed to the cardiac lineage such as myofibroblasts, while vascular SMCs and ECs form the outer

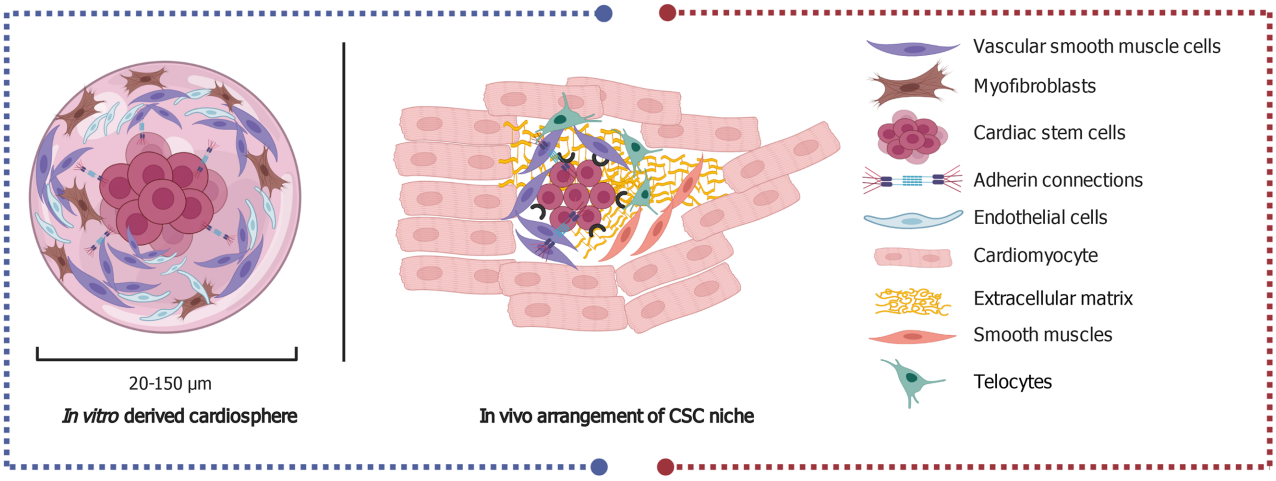

Figure 2 *In vivo* arrangement of the central cardiac stem cells and the surrounding cells that comprise the niche (right side) and the *in vitro* derived cardio spheres (left side). The key delineates the types of cells identified in the niche and cardio spheres. Created with BioRender. CSC: Cardiac stem cell.

layer of the spheres. The 3D structure of cardiospheres protects the interiorly located CSCs from oxidative stress as well as maintain their stemness and function[84].

Accurate anatomical identification of CSCs *in vivo* remains a challenge due to the lack of basal-apical anatomical orientation as seen in epithelial organs such as the intestines[94]. Moreover, the heart does not comprise a specific compartment, where cells form a well-defined lining as seen in the bone marrow osteoblasts[95]. The adult heart epicardial lining anatomically contains several classes of niches, which are not limited to the sub epicardium[96] but dispersed throughout the myocardium, more in the atria and apex away from hemodynamic stress[97]. Some niches have been described in the atrio-ventricular junction of adult mouse and rat hearts[98] and interestingly in the human hearts[99]. The young mouse heart has been studied morphometrically to identify the location of CSCs niche and has been defined as a randomly positioned ellipsoid structure consisting of cellular and extracellular components. Within the niches, undifferentiated CSCs are usually assembled together with early committed cells that express c-KIT on surface, Nkx2.5 in the nucleus, and the contractile protein α-sarcomeric actin in the cytoplasmic[97].

CSCs niche consists of clusters of c-kit$^+$, MDR1$^+$, and Sca-1$^+$ cells[98] but lack the expression of the transcription factors and cytoplasmic or membrane proteins of cardiac cells[99,100]. Cardiac c-kit$^+$/CD45$^-$ cells comprise about 1% of the CSC niche [97], are self-renewing clonogenic, and possess a cardiac multilineage differentiation potential comprise[101].

Within the niche, gap junctions (connexins) and (cadherins) connect SCs to their supporting cells, myocytes/fibroblasts. Conversely, ECs and SMCs do not act as supporting cells. Hence, the communication between CSCs with CMs and fibroblasts has been investigated by using *in vitro* assays[102]. The transmission of dyes *via* gap junctions between CSCs and CMs or fibroblasts was demonstrated previously and verified the functional coupling of these three cell populations[97]. In addition, micro ribonucleic acid (miRNA-499) translocates from CMs to CSCs comprising to the initiation of lineage specification and formation of myocytes[103].

Identification of SC niches is contingent upon the fulfillment of explicit criteria, including the recognition and determination of the affixing of SCs to their supporting cells as well as assuring the existence of an ancestor-progeny association[104]. Chemical and physical signals modulate the behavior of SCs within the niche. Amongst these signals are cytokines, cell surface adhesion molecules, shear forces, oxygen tension, innervation, and ions that serve as major determinants of SCs function [97]. Cell-to-cell signaling mediates the fate of SCs within the niches to promote self-renewal and favors their migration and differentiation. The fine-tuned crosstalk between SCs and their supporting cells regulates the state of the niche regarding quiescence or activity[105].

CSC niches, similar to the bone marrow, characteristically live in low oxygen tension, which favors a quiescent primitive state for SCs[106]. The longstanding perpetuation of the CSC niche requires a hypoxic environment, while physiological normoxia could be required for active cardiomyogenesis[107]. Hypoxic c-KIT$^+$ CSCs

within niches have been found throughout the myocardium, especially at the atria and apex. Throughout all ages, bundles of CSCs with low oxygen content coexist with normoxic CSCs niches. Hypoxic CSCs, especially in the atria, are quiescent cells undergoing cell cycle arrest and cannot divide. Normoxic CSCs are pushed into intense proliferation and differentiation with continuous telomere erosion, resulting finally in dysfunctional aged CMs[108]. Additionally, Nkx2.5 and GATA4 expressions are only restricted to the normoxic CSC niche. A balance between the hypoxic and normoxic niche is essential for the preservation of the CSC compartment and for the maintenance of myocardial homeostasis during the organ lifespan. Some factors such as aging cause an imbalance by expanding the hypoxic quiescent CSCs so that less pools of cycling CSCs maintain cell turnover[100]. Hypoxic cardiac niches are abundant in the epicardium and subepicardium in an adult mouse heart, which also fosters a metabolically distinctive population of glycolytic progenitor cells[109].

The pool of CSCs seems to be heterogeneous, incorporating quiescent and actively proliferating cells, migratory and adherent cells, uncommitted and early committed cells, with young and senescent cells. Additional surface epitopes remain to be disclosed to classify pools of CSCs holding specific properties. Surface Notch1 expression distinguishes multipotent CSCs that are poised for lineage commitment, while c-Met and ephrin type-A receptor 2 receptors reveal cells with particular migratory potential out of the niche area. A specific compartment of CSCs, expressing IGF-1 receptor, can be stimulated to regenerate damaged myocardium, while those expressing IGF-2 receptor hold higher probability for senescence and apoptosis. Although this arrangement of cells seems to equip properly the CSC with homeostasis regulation, it does not effectively protect against aging or ischemic injury of the heart [100].

CSCs RELATIONSHIP WITH OTHER CELLS

Circulatory angiogenic cells

Circulatory angiogenic cells (CACs) are endothelial progenitor cells involved in vasculogenesis, angiogenesis, and stimulating myocardial repair, mainly through paracrine action. Latham *et al*[110] demonstrated that conditioned medium from CAC–CSC co-cultures exhibited greatly mobilized CACs, with induction of tubule formation in human umbilical vein endothelial cells, mainly through the upregulation of the angiogenic factors angiogenin, stromal cell-derived factor 1 (SDF-1α), and VEGF. Moreover, administration of CACs and CSCs in infarcted hearts of non-obese/severe combined immunodeficient mice restored substantially the left ventricular ejection fraction (LVEF), with reduction of scar formation as revealed by echocardiography. Successful yet modest SMCs, ECs, and CM differentiation has been also reported.

Saphenous vein-derived pericytes

Pericytes (also called Rouget cells, mural cells, or perivascular mesenchymal precursor cells) are mesodermal cells that border the endothelial lining. They are highly proliferative cells and express neural/glial antigen 2, SOX-2, PDGFR-β, CD34, and several mesenchymal markers such as CD105, CD90, and CD44. It was previously reported that the transplantation of saphenous vein-derived pericytes (SVPs) into an ischemic limb of an immunodeficient mice restored the local circulatory network *via* angiogenesis[111]. Moreover, treatment with SVP reduced fibrotic scar, CM death, and vascular permeability in a mouse model of MI *via* miRNA-132 facilitated angiogenesis [112]. Avolio *et al*[113] were the first to describe the relationship between SVP and the endogenous CSCs. Combined CSC and SVP transplantation in the infarcted myocardium of severe combined immunodeficient/Beige-immunodeficient mice showed similar results to treatment with CSCs or SVP cells per se, regarding scar size and ventricular function, indicating that SVPs alone are as potent as CSCs.

TCs

TCs represent a recently described cell population in the stromal spaces located in many organs, including the heart. They are broadly dispersed throughout the heart and comprise a network in the three cardiac layers, heart valves, and in CSC niches. TCs have been documented also in primary culture from heart tissues[114,115]. The ratio of cardiac TCs (0.5%-1%) exceeds that of CSCs. Although they still represent a minute portion of human cardiac interstitial cells, their extremely long and extensive telopodes allow them to occupy more surface area, forming a 3D platform probably that extends to support other cells[116]. The telopodes act as tracks for the sliding of

precursor cells towards mature CMs and their integration into heart architecture[91]. TCs form a tandem with CSCs/CPCs in niches, where they communicate through direct physical contact by atypical junctions or indirect paracrine signaling[115].

TC-CSC co-culturing have suggested that TCs and CSCs act synergistically to control the level of secreted proteins, as shown by the increased levels of monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein1 α and 2 (MIP-1 α and MIP-2), and interleukin (IL)-13. Whereas, the level of IL-2 decreased compared to the monoculture of CSCs or TCs. IL-6 found in TC culture is behind the upregulation of these chemokines. Chemokines elucidated the role of TCs in directing the formation of CMs. Within the context, MIP-1α and MCP-1 play roles in the formation of SMCs in the airway. Additionally, MCP-1 is also involved in mouse skeletal muscle regeneration by recruiting macrophages. The enhancement of MCP-1 secretion serves as an activator of another cell population, primarily macrophages, which are generally involved in such processes[117].

IL-6 also activates downstream signaling pathways and contributes to cardioprotection and vessel formation in the heart through activation of gp130/signal transducer and activator of transcription 3. The Gp130/signal transducer and activator of transcription 3 is essential for the commitment of cardiac SCA-1$^+$ cells into endothelial lineage[118].

Furthermore, IL-6 targets *VEGF* and hepatocyte growth factor (*HGF*) genes. VEGF has a mitogenic effect on CMs[119]. It is known to mobilize bone marrow-derived mesenchymal stem cells (BM-MSCs) into the peripheral blood in MI patients[120]. HGF and its receptor (c-Met) are also involved in cardiogenesis, as it is expressed early during cardiac development[121]. The level of HGF mRNA is normally low in the heart, but it is upregulated for at least 14 d after ischemic insult in rats, enhancing CMs survival under ischemic conditions[122,123]. Moreover, it has the potential to generate an adhesive micro-environment for SCs, as demonstrated in a study of transplantation of HGF transfected BM-MSCs in the infarcted myocardium[124]. HGF is also a powerful angiogenic agent, conducting its mitogenic and morphogenic effects through the expression of its specific receptor in various types of cells, including myocytes. Moreover, HGF exerts antifibrotic and antiapoptotic effects on the myocardium[125, 126].

Transcriptomic analysis also has disclosed that TCs express pro-angiogenic miRNAs including let-7e, miRNA-21, miRNA-27b, miRNA-126, miRNA-130, miRNA-143, miRNA-503, and miRNA-100[127]. The TCs and CSCs interact *in vitro* forming atypical junctions, such as puncta adherentia and stromal synapses. The puncta adherentia consists of cadherin–catenin clusters. It controls the symmetry of division by facilitating the proper positioning of centrosomes. Therefore, an increased number of CSCs has been reported to be encountered in the presence of cardiac TCs[128,129].

CSCs SECRETOME

The paracrine potential of CSCs/CPCs has been recently under focus. CSC-derived cytokines and growth factors include epidermal growth factor (EGF), HGF, IGF-1, IGF-2, IL-6, IL-1α, and TGF-β1[130,131]. Exosomes appear to harbor relevant reparative signals, which mechanistically underlie the beneficial effects of CSCs transplantation [132].

Structurally, exosomes are lipid bilayer nano-sized organelles, 20-150 nm in diameter, secreted from all cell types, and function as intercellular communicators. Exosomes are highly heterogenic in content, and this stems from the unique packaging process that occurs inside progenitor and SCs. Exosomes carry lipids, proteins, and nucleic acids, with an abundance of miRNAs that hold profound post-transcriptional gene regulatory effects[133].

Protein content of exosomes

Amongst the distinctive protein content of cardiac exosomes are the chaperone proteins heat shock protein (HSP) 70 and HSP60. The HSP70 and HSP60, which under normal conditions assist in protein folding processes and deter misfolding and protein aggregation under pathological states induced by stress, also play major roles in apoptosis[134]. Circulating exosomes from healthy individuals have been found to activate cardioprotective pathways in CMs *via* HSP70 through extracellular signal-regulated kinase ½ and HSP27 phosphorylation[135].

The exosome protein cargo of CPCs is distinct from BM-MSCs, fibroblasts, and other sources as it contains ample amounts of the pregnancy-associated plasma protein-A

(PAPP-A). PAPP-A is present on the surface of human exosomes and interacts with IGF binding proteins (IGFBPs) to release IGF-1[136]. The cardioprotective role of CPCs-exosomes has been proven experimentally in *in vitro* ischemia/reperfusion and MI models and on CMs apoptosis to surpass that of BM-MSC-exosomes owing to their rich content of PAPP-A[137].

Exosomes' surface and intra-vesicular markers

Like all exosomes, mouse CPCs-derived exosomes are positive for the surface markers CD63, CD81, and CD9, TSG-101, and Alix, however, they express a high-level of GATA4-responsive-miRNA-451. MiRNA-451 has been shown to inhibit CM apoptosis in an acute mouse myocardial ischemia-reperfusion model through inhibition of the caspases 3/7. The expression of miRNA-21 in the mouse CPCs-exosomes additionally justifies their CM protection against oxidative stress and antiapoptotic effects *via* inhibition of programmed cell death protein 4 (PDCD4)[138]. Human CPCs-exosomes are enriched with miRNA-210, miRNA-132, and miRNA-146a-3p, which account for the diminished CM apoptosis, enhanced angiogenesis, and improved LVEF[139]. MiRNA-146a-5p is the most highly upregulated miRNA in human CPCs-exosomes and targets genes involved in inflammatory and cell death pathways[137].

The CDCs contain $CD34^+$ stromal cells of cardiac origin and are multipotent and clonogenic but not self-renewing[140]. CDCs secrete exosomes that induce cardiomyogenesis and angiogenesis, regulate the immune response, downgrade fibrosis, and improve the overall cardiac function[141,142]. Moreover, CDCs homogeneously express CD105 but not CD45 or other hematopoietic markers. They also exhibit a high expression of miRNA-126[143]. Circulating miRNA-126 may participate in cardiac repair during acute MI and has been demonstrated to be downregulated in heart damage[144].

Exosome secretion and function

While exosomes are constitutively secreted, changes in the surrounding microenvironment, such as hypoxia, can induce modifications in CPCs- and CM- derived extracellular vesicles. Hypoxic CMs secrete large extracellular vesicles containing long noncoding RNA neat 1 (LNCRNA NEAT1), which is transcriptionally regulated under basal conditions by p53, while during hypoxia it is regulated by the hypoxia inducible factor 2A. An uptake of the hypoxic CM-derived extracellular vesicles by fibroblasts can prompt the expression of profibrotic genes[145]. Oxidative stress may also induce the release of cardiac CPCs exosomes, which in turn inhibit apoptosis when taken up by H9C2 (rat cardiomyoblast cell line)[132]. Furthermore, oxidative stress stimulates secretion of miRNA-21 rich exosomes, which could inhibit H9C2 apoptosis by targeting PDCD4 and hence can be accounted as a new method to treat ischemia-reperfusion[138].

Intercellular communication *via* exosomes occurs as part of various biological processes, including immune modulation, vasculogenesis, transport of genetic materials, and pathological conditions such as inflammation, apoptosis, and fibrosis, which can lead to cardiovascular disease when altered[146]. Hence, isolation and analysis of cardiac exosomes contents, mainly miRNA and proteins, could offer diagnostic information for several cardiovascular diseases[147] (Figure 3).

Functionally, exosomes mediate several intra-cardiac inter-cellular communications such as:

CPC-CM crosstalk through factors, such as miRNA-146a and PAPP-A, which activate extracellular signal-regulated kinases 1/2 pathway and inhibit apoptosis[139].

CPC-macrophage (M1) crosstalk *via* miRNA-181b and Y-RNA fragment transforms M1 to M2 macrophages with attenuated proinflammatory cytokines and increased IL-10[148,149] (Figure 4).

CPC-fibroblast interaction *via* exosomes primes the fibroblasts and increases expression of VEGF and SDF-1. Experimental injection of fibroblasts primed with CPCs-exosomes into the myocardium of a MI model proved to reduce infarct size and improve cardiac function. In addition, cardiosphere-isolated exosomes have been used to prime inert fibroblasts, leading to an intensification of their angiogenic, cardiomyogenic, antifibrotic, and collective regenerative effects[150] (Figure 4).

CPC-self regulatory mechanisms: Exosomes derived from CPCs may play critical roles in maintaining the self-renewal state of CPCs themselves and balance their differentiation, *i.e.* preserve their stemness[151] (Figure 4). The CPC-derived exosomes activate the endogenous CPCs by transferring signal molecules directly within their niche[152].

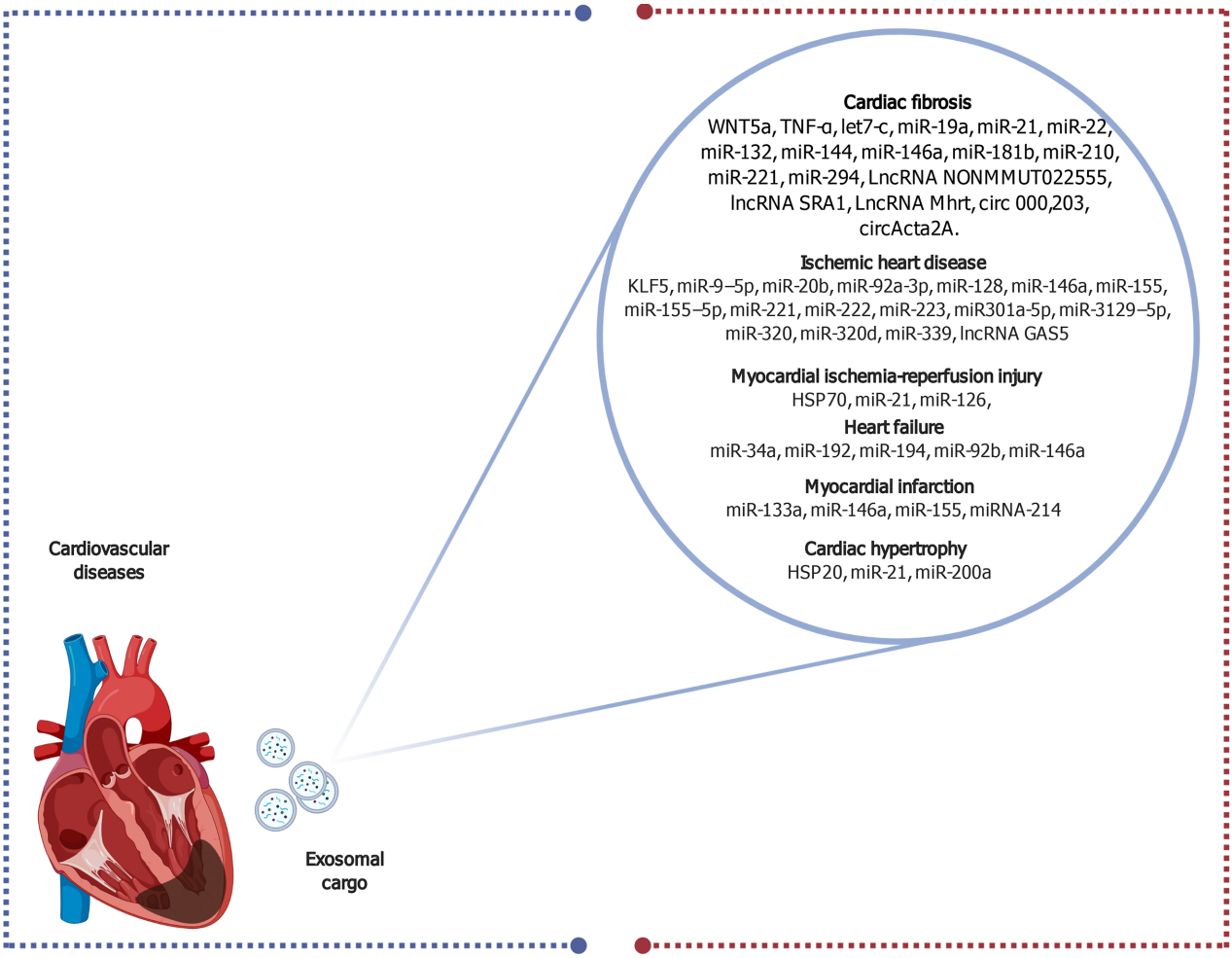

Figure 3 Schematic diagram elucidating the diverse exosomal contents that serve as biomarkers for several cardiovascular diseases.
Created with BioRender.com. HSP: Heat shock protein; lncRNA: Long non-coding RNA; miR: MicroRNA.

CPC-derived exosomes release various RNA species in the extracellular space, modulating endogenous SC plasticity and tissue regeneration through their cytoprotective, immunomodulatory, pro-angiogenic, and anti-apoptotic actions[153].

Fibroblasts and pericytes interact after transdifferentiating to myofibroblasts and deposit ECM causing cardiac fibrosis. These fibrotic changes are usually induced by cardiac damage and lead to scar formation. Exosomes serve as messengers for cell-to-cell communication during cardiac fibrosis[154]. Molecular mechanisms of cardiac fibrosis are primarily related to TGF-β pathways, IL-11 signaling pathway, nuclear factor-κβ pathway, and Wnt pathways[155]. Accordingly, the bioactive substances targeted at these pathways could hypothetically be applied in the treatment of cardiac fibrosis. Wnt3a, being highly expressed in exosomes, could activate the Wnt/β-catenin pathway in cardiac fibroblasts by restricting GSK3β activation[156]. Moreover, tumor necrosis factor α contained in exosomes can be transferred between cardiac myocytes. In general activation/inhibition of the exosomes conveying remodeling substance secretion or uptake can control the myocardial remodeling and repair following MI [154,157].

The highlighted complex cell-to-cell communication from endogenous or exogenous CSCs provides an optimal microenvironment for resident CPC proliferation and differentiation (Figure 4), rendering the environment receptive to transplanted CPCs. This adaptation is promoted through activation of pro-survival kinases, leading to the induction of a glycolytic switch in recipient CPCs[158].

Therapeutic efficiency of CPCs/CDCs exosomes

Data from experimental models suggest that the exosomal component of the CPC secretome can fully recapitulate the effects of cellular therapy on ischemic and non-ischemic heart models[140]. In an ischemia-reperfusion injury rat model, Ciullo and partners[159] have shown that the systemic injection of exosomes (genetically

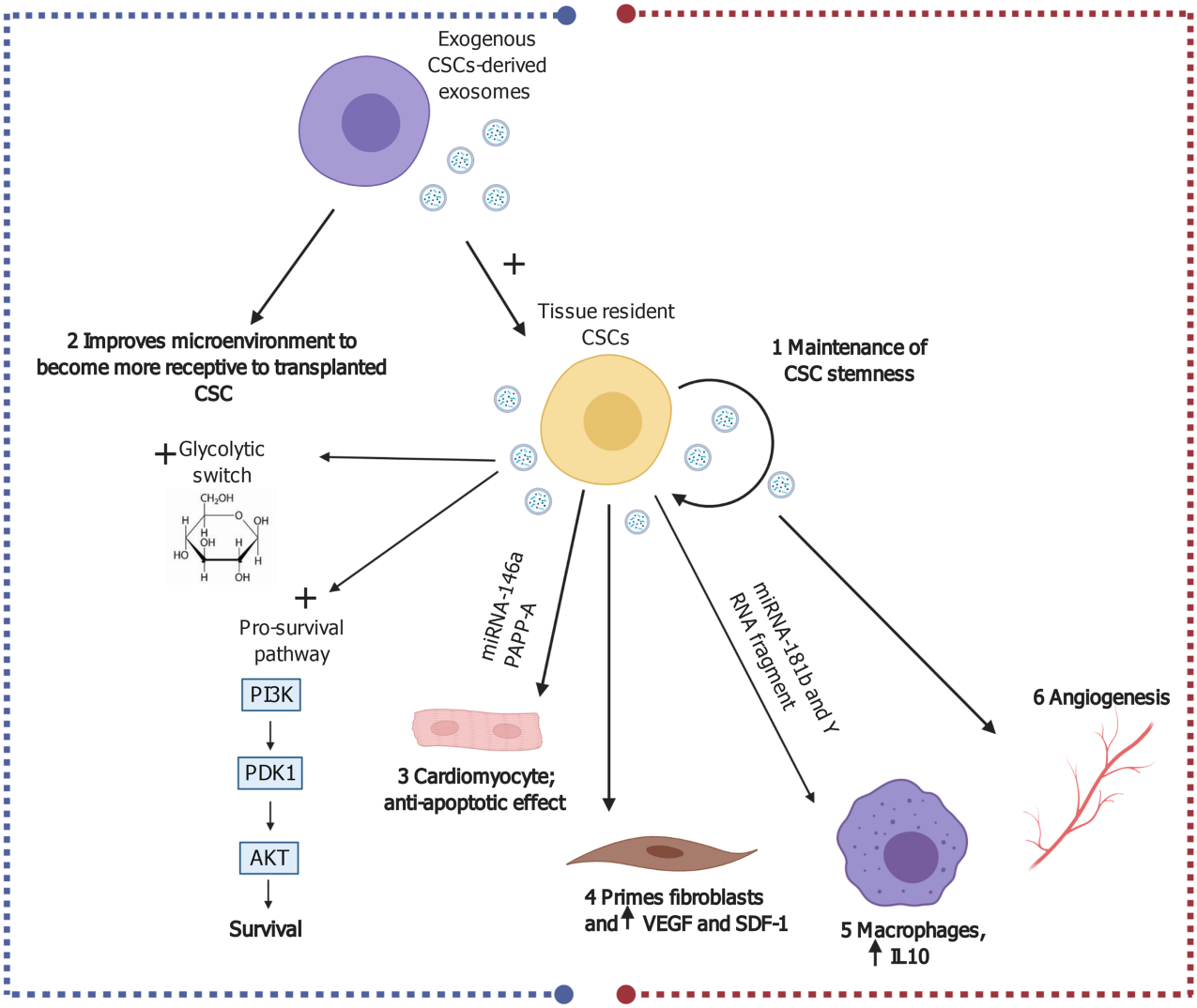

Figure 4 Possible cardiac reparative effects of cardiac stem cell/cardiosphere-derived cell-derived exosomes in myocardial ischemia and ischemia/reperfusion injury. Created with BioRender.com. CSC: Cardiac stem cell; IL: Interleukin; IR: Ischemia/reperfusion; miRNA: MicroRNA; PI3K: Phosphoinositide 3-kinase; SDF-1: Stromal cell-derived factor 1; VEGF: Vascular endothelial growth factor.

manipulated to overexpress CXCR4–ExoCXCR4) improve cardiac function. Additionally, expression of hypoxia-inducible factor 1 (HIF-1) in the infarcted myocardium is upregulated through the stimulation of SDF-1α. The latter is one of the CXC chemokine family overexpressed in heart post-MI that readily attaches to the CXCR4 receptor and acts as a potent chemoattractant for CXCR4 expressing circulating progenitor cells. The ExoCXCR4 are more bioactive in the infarcted zone than naturally occurring exosomes injected *via* tail-vein, confirming their superior homing and cardioprotective properties in the damaged heart.

Gallet *et al*[160] postulated the safety and efficiency of CDC-derived exosomes in acute and chronic myocardial injury animal models. Within the context of experimental research to validate the paracrine hypothesis for CDCs–derived exosomes, it has been proven that human CDC-exosomes can recapitulate CDC therapy and boost cardiac function post-MI in pig models. Intramyocardial injection of human CDC-exosomes has resulted in higher exosome retention and efficacy as compared to intracoronary injection, with great reduction of scar size and increased ejection fraction. This indicates that the route of administration is imperative for full functional capacity of the exosomes. Subsequently, the researchers have devised a randomized preclinical study by means of a NOGA-guided intramyocardial exosome injection. Decreased collagen content in the infarct and border zone and increased neovascularization and Ki67$^+$ CMs are indicative of the reparative functions of CDC-exosomes. Notably, human CDC-exosomes have shown a lack of an immune reaction, as seen by the lack of inflammatory reactions or CM necrosis in pig models. These observations strongly support the view that CDC-exosomes are ready to be tested in clinical trials.

Similar promising outcomes were observed in a Duchenne muscular dystrophy model (mdx), in which intramyocardial injection of CDC-exosomes efficiently recapitulated the effects of CDC injection on cardiac function, leading to recovery of movement. Administration of CPC-derived exosomes has resulted in transient restoration of partial expression of full-length dystrophin in mdx mice[161]. Further studies assessed the therapeutic potential of CPC-exosomes in a doxorubicin cardiotoxicity model and non-ischemic heart disease[162]. In addition, two concluded phase I clinical trials in patients with heart failure and revealed the capacity of CDCs to enhance cardiac function by reducing ventricular remodeling and scar formation. Despite receiving a single injection at the beginning of the study, the improvement in cardiac function was noted after the 1-year follow-up. This finding consequently leads to the proposition that transplanted CDCs mainly have imposed their actions at the site of injury by secreting paracrine factors including exosomes. In other words, CDC-exosomes achieved a biphasic beneficiary regenerative effect involving acute cardio protection coupled with long-term stimulation of endogenous cardiac repair[163].

METABOLISM OF CSCs

While the fetal heart obtains most of its ATP supply *via* glycolysis[164], the adult heart relies mainly on fatty acid oxidation to fulfill the contracting myocardium high energy demand[164,165]. The loss of the regenerative phenotype is related to the oxidative metabolism of glucose and fatty acids[166,167] and is mediated by various physiological changes including increased workload and the demand for growth, which cannot be solely met by glycolysis[168,169], as well as postnatal increase in both circulating levels of free fatty acids and blood oxygen levels[164,165]. Studies have shown the involvement of the HIF-1 signaling pathway[170], peroxisome proliferator-activated receptor α (PPARα)[171], and peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) in the switch toward oxidative metabolism[172], which is accompanied by dramatic increase in the number of mitochondria in CMs[173].

Notably, similar metabolic reprogramming occurs during differentiation from cardiac SCs to CMs[167]. Studies reported that after differentiation into CMs, there is an increase in the mitochondrial number and activity[174], increased oxidative metabolism[175], and increased respiratory capacity resulting in an increased adenosine diphosphate:ATP ratio[173] after differentiation into CMs.

The fact of the various metabolic changes that accompany the transition from glycolysis to fatty acids oxidation affect cardiac cell maturation[164,167] has mandated the consideration of substrate composition in cardiac differentiation protocols[167].

A study by Malandraki-Miller *et al*[176] investigated the effect of fatty acid supplementation, which mimics the metabolic switch from glucose to fatty acid oxidation, on adult cardiac progenitors. The study used radiolabeled substrate consumption for metabolic flux to investigate the role of the PPARα/PGC-1 axis during metabolic maturation. Oleic acid stimulated the PPARα pathway, enhanced the maturation of the cardiac progenitor, and increased the expression of MHC and connexin after differentiation. Moreover, total glycolytic metabolism, mitochondrial membrane potential, the expression of glucose, and fatty acid transporter increased. The recorded results contributed greatly in highlighting the role of fatty acids and PPARα in CPC differentiation.

Another study by Correia *et al*[177] has linked substrate utilization and functional maturation of CMs *via* studying the effect of the metabolic shift from glucose to galactose and fatty acid-containing medium in the maturation of hPSCs-derived CMs (hPSCs-CMs). The shift accelerated hPSC-CM maturation into adult-like CMs with higher oxidative metabolism, mature transcriptional signatures, higher myofibril density, improved calcium influx, and enhanced contractility. Galactose improved total oxidative capacity with reduction of fatty acid oxidation, thereby protecting the cells from lipotoxicity.

In CDCs, oxidative metabolism and cell differentiation reciprocally affect each other. *In vitro* cultures for CDCs revealed a PPARα agonist that triggers fatty acid oxidation. Metabolic changes have been characterized as the CDC differentiated towards a cardiac phenotype. Addition of a PPARα agonist at the onset of differentiation has induced a switch towards oxidative metabolism, as shown by changes in gene expression with decreasing glycolytic flux and increasing oxidation of glucose and palmitate. Undifferentiated CDCs have generated high levels of ATP from glycolysis and from oxidation of acetoacetate. Upon differentiation, oxidative metabolism of glucose and fatty acids is upregulated with decreased oxidation of

acetoacetate, a metabolic phenotype similar to that of the adult heart[178].

Taken together, the metabolic hallmarks of differentiated CMs vary from their undifferentiated SCs. Energy substrate metabolism during cardiac development and differentiation shows gradual decrease in the contribution of glycolysis to ATP synthesis with simultaneous increase in fatty acid–dependent mitochondrial respiration[179].

Common methods for the investigation of substrate metabolism include the measurement of metabolic fluxes using radio-labeled substrates, such as D-U-14C-glucose[180,181] as well as measurement of mitochondrial oxygen consumption rate and extracellular acidification rate using the XF Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, United States)[182,183].

Recently, a detailed protocol for metabolic characterization of hiPSCs-CMs has been developed. The hiPSCs are obtained from adult somatic cells *via* novel cell reprogramming approaches, followed by differentiation to CMs. The novel *in vitro* cardiac cellular model provided new insights into studying cardiac disease mechanisms and therapeutic potentials. The characterization protocol measures small metabolites and combines gas- and liquid-chromatography-mass spectrometry metabolic profiling, lactate/pyruvate, and glucose uptake assays as important tools[184]. Integration between the implemented assays has provided complementary metabolic characteristics besides the already established electrophysiological and imaging techniques, such as monitoring ion channel activities[185], measurement of action potentials, changes in Ca^{+2} fluxes[186], and mitochondria viability and apoptosis[187].

An alternative pathway for glucose metabolism in CMs involves the entry of glucose-6-phosphate (G6P) in the pentose phosphate pathway, with resultant generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH)[188]. Reduced NADPH helps to regenerate reduced glutathione and thus acts protectively against reactive oxygen species induced cell injury.

The cardioprotective role of the pentose/G6P/NADPH/glutathione pathway has been emphasized by Jain *et al*[189] who demonstrated that G6P dehydrogenase (G6PD) lacking mice have more severe heart damage induced by the myocardial ischemia reperfusion injury in Langendorff-perfused hearts as compared with wild-type mice.

Moreover, Katare *et al*[190] studied this pathway in CPCs isolated from hearts of diabetic mice. They reported that both G6PD and transketolase activities were markedly reduced in diabetes mellitus, which resulted in apoptosis of CMs. Interestingly, they have also reported that apoptosis was induced under high glucose conditions *via* inhibition of the pentose phosphate pathway, which mediates prosurvival signaling pathways.

Cellular metabolic transcriptome profile is an important determinant of many critical cell functions such as survival, growth, differentiation, and reprogramming. With the fast-track advancements in CSCs research, in-depth and thorough metabolic transcriptome analyses on CSCs are needed. It has been also suggested that metabolic genes can be targeted to manipulate the differentiation of ESCs into specific CM phenotypes or to modulate the maturation grade of CMs derived from ESCs[179].

As mentioned earlier in the review, the energy demand of the contracting myocardium of an adult heart is met mainly through fatty acid oxidation, which explains the fact that genes required for fatty acid metabolism are upregulated in the differentiated CMs. These genes include acetyl-CoA acyltransferase 2 (*ACAA2*), NADH dehydrogenase ubiquinone 1, α/β subcomplex 1 (*NDUFAB1*), protein kinase AMP-activated α-2 catalytic subunit (*PRKAA2*), and ECI1 enoyl-CoA delta isomerase 1 (*DCI*). In addition, other genes involved in glucose metabolism are also upregulated in α-MHC^{+} CMs, including protein phosphatase 1 regulatory subunit 3C (*PPP1R3C*), glycogen phosphorylase, muscle associated (*PYGM*), enolase 3 (*ENO3*), phosphoglycerate mutase 2 (*PGAM2*), amylo-α-1,6-glucosidase 4-α-glucanotransferase (*AGL*), 6-phosphofructokinase muscle (*PFKM*), and malate dehydrogenase 1 (*MDH1*)[191]. This is interpreted by the fact that adult cardiac cells are metabolically flexible, being capable of oxidizing other energy sources, such as glucose, lactate, amino acids, and ketone bodies for the production of ATP and non–ATP-producing intermediate metabolites with high biological significance[169]. Another example of CMs' metabolic plasticity is shown by *HIF-1* expression, which is important for their metabolic adaptation to hypoxic and ischemic conditions[192].

NANOTECHNOLOGY AND CSCs

Since Richard Feynman laid down the foundation of nanotechnology 1959[193],

remarkable developments have been witnessed attributed to the novel properties possessed by the materials at the nanoscale, which differ from their bulk forms. A panel of NPs, ranging from soft to inorganic, are used in nanomedicine, depending on their unique property matching the field of interest[194-197] (Figure 5).

The interplay of nanotechnology with SCs has gained increasing interest, whether for differentiating, tracking, imaging, or for therapeutic purposes. Accumulating evidence presents that the small size and bioactive characteristics of NPs could influence SC function. Several engineering techniques have been developed to obtain nano-fibrous scaffolds that facilitate controlling SC proliferation, migration, and differentiation. A diversity of SCs, including ESCs, skeletal myoblasts, BM-MSCs, and CSCs, have been tested to repair acutely or chronically damaged myocardium. However, the optimal cell type, the efficient cell number, the appropriate route for cell delivery, and the ideal time point for cell delivery after MI are still unanswered questions. The biodistribution of SCs and the specific mechanism by which therapeutic cells improve cardiac function remains under investigation[198]. Using NPs could solve some of these obstacles either by gene delivery to SCs, enhancing the retention of SCs, facilitating SCs' proangiogenic effect, or mimicking the ECM[199].

In view of this, we have summarized the impacts of NPs on SCs, especially CSCs, from differentiating, therapeutic, and tracking viewpoints (Figure 5, Table 1).

NP-assisted CSC differentiation

Nanotechnology has revolutionized the tissue engineering field and altered the landscape of scaffolds syntheses. In typical tissue engineering, a bio-mimicked scaffold provides adhesive surfaces for the seeded cells, where the SCs deposit their proteins to make the engineered-scaffold more biocompatible. However, improper vascularization, lack of functional cells, the low mechanical strength of engineered cells, immunological incompatibility with the host, and nutrient constraints are the main limitations encountered in tissue engineering. Therefore, synthesis of a biomimetic scaffold at the nanoscale, in a minimum of one dimension, would offer a more effective microenvironment needed for cell growth. Nano-tissue engineering provides the scaffold with a simple substrate for SC adhesion and active agents for their proliferation[200].

In consideration for CSCs, nano- and microstructured electrospun matrices have been used as non-woven scaffolds for the construction of cardiac tissue from primary CMs. Among different nanostructured poly (D, L-lactic-co-glycolic acid; PLGA) membranes, the poly (L-lactide; PLLA) scaffolds superiorly developed mature contractile machinery (sarcomeres). Functional studies (excitability) of CMs tested by optical imaging of electrical activity have confirmed the superior response on PLLA scaffolds compared with other ones[201].

An *in vitro* and *in vivo* study conducted by Liu *et al*[202] using porous ECM-mimicking nanofibrous PLLA scaffolds (porous NF PLLA) demonstrated cardiac tissue formation from CPCs. The scaffold has facilitated the *in vitro* differentiation of isolated mouse ESCs into CPCs. Thereafter, the transplanted NF PLLA/CPCs integrated successfully with the host tissue, with superior expression of cardiac committed markers cardiac troponin T, smooth muscle MHC, and CD31.

The inductive and therapeutic properties of biodegradable PLGA nanofibers have been tested *in vitro* and *in vivo*. Different hiPSCs-CMs have been seeded on aligned PLGA nanofibers to differentiate into high-quality cardiac tissue-like constructs, where cardiac biomarkers and cardiac functions have been upregulated. When utilized *in vivo* for treating MI, the cardiac tissue-like constructs have shown more robust results than the two-dimensional conventional control in improving the ejection fraction, the fractional shortening, and left ventricular end-systolic diameter[203].

Recently, an injectable ECM hydrogel loaded with gold (Au)/Laponite (Lap) nanocomposite has been tested on the biological activity of resident CSCs. The electroactive Au/Lap-ECM hydrogel improved cell biocompatibility and phenotypes maturation of cardiac-specific markers (SAC, cardiac troponin 1, and Cx43)[204].

NP-assisted CSC therapy

More than 90 years ago, the Nobel laureate German immunologist Paul Ehrlich proposed the term "magic bullets" to describe the artificial biochemical agents that would transport and release drugs at the desired sites only[205]. Since then, drug delivery research has witnessed notable growth due to NPs utilization as "controlled release reservoirs" for drug delivery in order to combat many diseases[206].

In cardiovascular diseases, NP-based drug delivery targeting CSCs would be a successful therapeutic regimen. In an *in vivo* study of induced MI, self-assembling peptide nanofibers tethered with NF-IGF-1 positively influenced CPCs in female

Table 1 Main outcomes of the studies investigated the impact of nanotechnology in cardiac stem cell-based studies

Nanotechnology field	Types of nanoparticles	Type of cardiac disease/stem cells	Type of research	Outcomes	Ref.
Tissue engineering	Semi-crystalline PLLA nanostructured membranes among several PLGA membranes	Non-diseased/primary CMs	*In vitro:* electrospun matrices were used as scaffolds for generating cardiac tissue constructs	Nanostructured non-woven PLLA scaffolds provide flexibility and guidance for CMs growth and can be successfully applied to obtain structurally and functionally competent cardiac tissue constructs.	[201]
Tissue engineering	ECM-mimicking nanofibrous PLLA scaffolds with porous structure (porous NF PLLA) of high interconnection for cardiac tissue formation	Non-diseased/mouse ESCs CPCs	*In vitro:* CPCs with porous NF PLLA *In vivo:* male athymic nude mice	*In vitro:* porous NF PLLA scaffolds facilitate cell attachment, extension, and differentiation. *In vivo:* subcutaneous implantation of cell/scaffold supports survival of grafted cells and differentiation to CMs, SMCs, ECs lineages.	[202]
Tissue engineering/therapeutic	Biodegradable ANF	MI/hiPSCs-CMs	*In vitro:* hiPSCs (253G1) *In vivo:* nude rat	*In vitro:* multilayered, elongated, organized CMs at high density along ANF, with up regulation of genes of sarcomere structures (*ACTN2, TNNT2, TNNI3*), cardiac maturation (*MYH7*), ventricular structures (*MYL2, HAND2*). *In vivo:* CTLCs improve MI functionally due to transplantation of organized functional CMs.	[203]
Tissue engineering	Electroactive Au-Lap NPs loaded myocardial ECM	Non diseased/Resident CSCs	*In vitro*: rat CMs from 2-d old neonatal rats	Combination of electrically active nano-formulations and biologically active ECM boost the expression of cardiac-specific proteins (SAC, cTnI, Cx43).	[204]
Therapeutic	Self-assembling peptide nanofibers tethered with insulin-like growth factor-1 (NF-IGF-1)	MI/CPCs	*In vitro*: clonogenic CPCs *In vivo*: female Fischer 344 rats	*In vitro*: NF-IGF-1 promote CPCs division (↑BrdU) and protect them from death signal (↓TdT). *In vivo*: CPCs-NF-IGF-1 enhance postinfarction ventricular remodeling, attenuate chamber dilation, and improve cardiac performance.	[207]
Therapeutic	Transplantation of self-assembling nanopeptides: Cell-PM complex	MI/cSCA-1$^+$ cardiac progenitors Other stem cells BM, SM, AMC	*In vivo:* Wild-type mice (C57Bl/6J); Adult GFP transgenic mice	cSCA-1/PM attenuates ventricular enlargement, restore cardiac function, with high capillary density (↑vWF) and conductive vessels (↑αSMA, ↑ VEGF). ↓TUNEL$^+$ CMs in the infarct area of cSCA-1/PM.	[208]
Therapeutic	CMMP contained control-released stem cell factors in its polymeric core and cloaked with hCSC membrane fragments on the surface	MI/Human CSCs	*In situ:* characterization *In vitro*: NRCM *In vivo*: male SCID Beige mice	*In situ*: CMMPs express hCSC surface markers. *In vitro*: CMMPs promote NRCM contractility and proliferation. *In vivo*: CMMPs preserve viable myocardium,	[209]

				augment cardiac functions, with safety profile.	
Therapeutic and drug delivery tool	Statin PLGA nanoparticles	MI/hAdSCs	*In vivo*: male nude mice (BALB/c nu/nu)	A small number of intravenously administered SimNP-loaded AdSCs improve cardiac function following MI, stimulating endogenous cardiac regeneration in the infarcted myocardium.	[244]
Tracking of treatment	Colloidal nanoparticles containing europium loaded on collagen matrix	MI/Lewis rat BM-MSCs	*In vivo*: female Fischer rat	Collagen matrix enhance transplanted MSC retention and reduce migration of the cells into remote organs as tracked by the radioactive NPs.	[211]
Tracking and magnetic targeting of treatment	Superparamagnetic iron microspheres	MI/Rat CDCs	*In vitro*: rat CDCs*In vivo*: female WKY rats	*In vitro*: ↓caspase 3^+, ↓$TUNEL^+$. *In vivo*: enhanced cell engraftment, with attenuated left ventricular remodeling and increased ejection fraction. ↑GFP^+, ↑$Ki67^+$ CMs, and ↑GFP^-/c-KIT^+ cells.	[212]
Imaging and therapeutic by magnetic targeting	Ferumoxytol (FDA-approved SPIONs) nanoparticles linked by heparin sulfate and protamine sulfate	MI/Human and ratCDCs	*In vitro*: hCDCs and rCDCs *In vivo*: female WKY rats	*In vitro*: ↓$TUNEL^+$, ↓ROS and ↑CCK-8, ↑Ki67. *In vivo*: augmentation of acute cell retention and attenuation of left ventricular remodeling, 3 wk after treatment by MRI, fluorescence imaging, qPCR.	[213]
Imaging and tracking for differentiation	Potassium niobate harmonic nanoparticles stabilized by polyethylene glycol	Non-diseased/ESC-derived CMs	*In vitro*: mouse ESC (CGR8 cell line)	Monitoring at high acquisition speed the rhythmic contractions of ESC-derived CMs beating within 3D cluster.	[245]
Therapeutic by magnetic guidance of NPs	Iron oxide nanoparticle-incorporated nanovesicles (exosome memetic nanovesicles); (IONP-NVs)	MI/MSCs	*In vitro*: rat CM, rat CFs, macrophage, HUVECs. *In vivo*: Fischer 344 rats	*In vitro*: under hypoxia IONP-MSCs exert Antiapoptotic effect on CMs: ↓caspase 3^+, ↑Cx43, ↑ PI3K. Antifibrotic effect on CFs: ↑Cx43, ↓TGFβ1, ↓αActa2, ↓MMP2, ↓MMP9. Anti-inflammatory effect on macrophage. Proangiogenic effect on HUVECs: ↑tube formation, ↑EC migration. *In vivo*: magnetic guidance increases IONP-MSCs retention within the infarcted heart, with early shift from inflammatory stage to reparative stage.	[217]

AMC: Adipose tissue-derived mesenchymal cell; ANF: Aligned PLGA nanofibers; Au-Lap NP: Gold and laponite nanoparticle; BrdU: Bromodeoxyuridine cell proliferation assay; CCK-8: Cell counting kit-8; CF: Cardiac fibroblast; CM: Cardiomyocyte; CMMP: Cell-mimicking microparticle; CPC: Cardiac progenitor cell; CTLC: Cardiac tissue-like construct; cTnI: Cardiac troponin 1; FDA: Food and Drug Administration; ECM: Extracellular matrix; ESC: Embryonic stem cell; hAdSC: Human adipose-derived stem cell; HUVEC: Human umbilical cord vein endothelial cell; IONP: Iron oxide nanoparticle; MRI: Magnetic resonance imaging; NF: Nanofibrous; MI: Myocardial infarction; MMP: Matrix metalloproteinase; NRCM: Neonatal rat cardiomyocyte; PLGA: Poly D,L-lactic-co-glycolic acid; PLLA: Poly (L-lactic acid); PM: Puramatrix™; qPCR: Quantitative polymerase chain reaction; SM: Skeletal myoblast; SPION: Superparamagnetic iron oxide NP; TdT: Terminal deoxynucleotidyl transferase apoptotic assay; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling apoptotic assay; vWF: von-Willebrand factor; WKY: Wistar-Kyoto; αSMA: Alpha smooth muscle actin; 3-D: Three-dimensional.

Fischer rats. The local injection of CPCs loaded on NF, with the prolonged release of

Other self-assembling nanopeptides loaded with cell-Puramatrix™ complex have revealed promising results in treating MI, especially when targeting clonal SCA-1$^+$ CPCs. The infarct area of SCA-1$^+$/PM became smaller than that of other tested SCs. Moreover, SCA-1$^+$/PM have secreted VEGF, enhancing their differentiation potentiality into CMs and vascular SMCs[208].

Interestingly, a synthetic cell-mimicking microparticle (CMMP) has recapitulated CSC function with a safe immunological profile. The core of the CMMP is a PLGA containing human-derived CSC (hCSC) secretome, while the surface has been cloaked with SC membrane fragments. These CD105$^+$ and CD90$^+$ CMMPs have shown synchronized movement with adjacent beating CMs *in vitro*. When injected in the MI mouse model, they have shown prolonged retention without eliciting the T-cell immunoreaction that transplanted hCSCs provoked[209].

NP-assisted CSC imaging and tracking

Combining the therapeutic effect of SC-based therapy with concomitant *in vitro* or *in vivo* visualization of the SCl is a strategy helped by the auto-luminescent NPs. Furthermore, delivery of the SCs to the infarcted cardiac area could be guided by a magnetic field depending on the unique magnetic properties of iron oxide NPs[210].

Isotopic colloidal NPs tagged with europium have successfully tracked the retention of BM-MSCs in the infarcted area. The NP-labeled MSCs loaded on a collagen matrix have shown reduced relocation of MSCs to remote organs. However, delivering the NP-MSCs *via* collagen has failed to improve cardiac function[211]. Further retention and magnetic targeting of CDCs have been retrieved using superparamagnetic microspheres (SPM). Quantitative polymerase chain reaction and optical imaging have confirmed the magnetic targeting and the increased cardiac retention of transplanted cells, with reduced lung migration in a rat model of ischemia/reperfusion injury. Moreover, the prolonged survival of SPM-labelled CDCs by cell counting kit-8 and Western blot has proved the safety profile of SPM[212].

The success of magnetic cell delivery in various preclinical studies potentiates the translation into clinical ones encouraged with the Food and Drug Administration-approved superparamagnetic iron oxide NP ferumoxytol. A thorough investigation of ferumoxytol-labeled (FHP) human and rat CDCs offered the potential for rapid clinical translation of the magnetically targeted cell delivery to an ischemic heart. The *in vitro* study proved that FHP nanocomplex is not toxic to hCDCs, where a panel of cytotoxicity assays have revealed prolonged survival, potentiated differentiation, and genetic stability of FHP-hCDCs. Furthermore, *in vivo* tracking of FHP-rCDCs by magnetic resonance imaging (MRI), fluorescence imaging, and quantitative polymerase chain reaction have shown that magnetic targeting increased cardiac retention without eliciting cardiac inflammation or causing iron overload. The histological assessment revealed enhancement of angiogenesis and cell engraftment in the hearts of the magnetic targeting group[213].

NP-based cardiac therapy limitations and prospects

Despite the promising results of NP-assisted SC interventions, the reported nanotoxicity is considered a major obstacle for clinical translation of these preclinical trials [214]. Besides, most of the *in vitro* and *in vivo* NP-based SC trials focused on the short-term effect of the NP interventions. The long-term safety profile of the injected NPs/CSCs or NPs/scaffolds with host interactions needs large scale investigations. These shortcomings have directed the search for natural cell-derived immune compatible nanostructures.

Exosomes attracted the attention as therapeutic cellular-derived NPs. However, the small quantity of these exosomes secreted from SCs is considered the main limitation to its therapeutic implementation. A novel exosome-mimetic extracellular nanovesicles (NVs) have bypassed this obstacle[215]. The large-scaled mechanically synthesized NVs from ESCs, by Jo and his colleagues[216], have conserved both RNA and protein profiles of the ESCs. Furthermore, treatment of MSCs with NVs has promoted cellular proliferation, which has been comparable with or even superior to the positive MSCs control treated with silica nanobeads that are well known for their ability to stimulate proliferation *via* activation of the MAPK pathway.

Recently, NVs derived from iron oxide NPs (IONPs) incorporated MSCs (IONP-MSCs) have co-cultured with different primary cell lines to investigate their physical and biological characteristics. IONPs-MSCs have revealed cardioprotective effects *via* PI3K/AKT activation. Under *in vitro* hypoxic conditions, IONPs-MSCs have upregulated Cx43, an electrical coupling molecule, whose reduction is responsible for arrhythmia and cardiac cell death in hypoxia. Furthermore, the NVs inhibited cardiac fibrosis by inhibiting the differentiation of cardiac fibroblasts into cardiac myofibro-

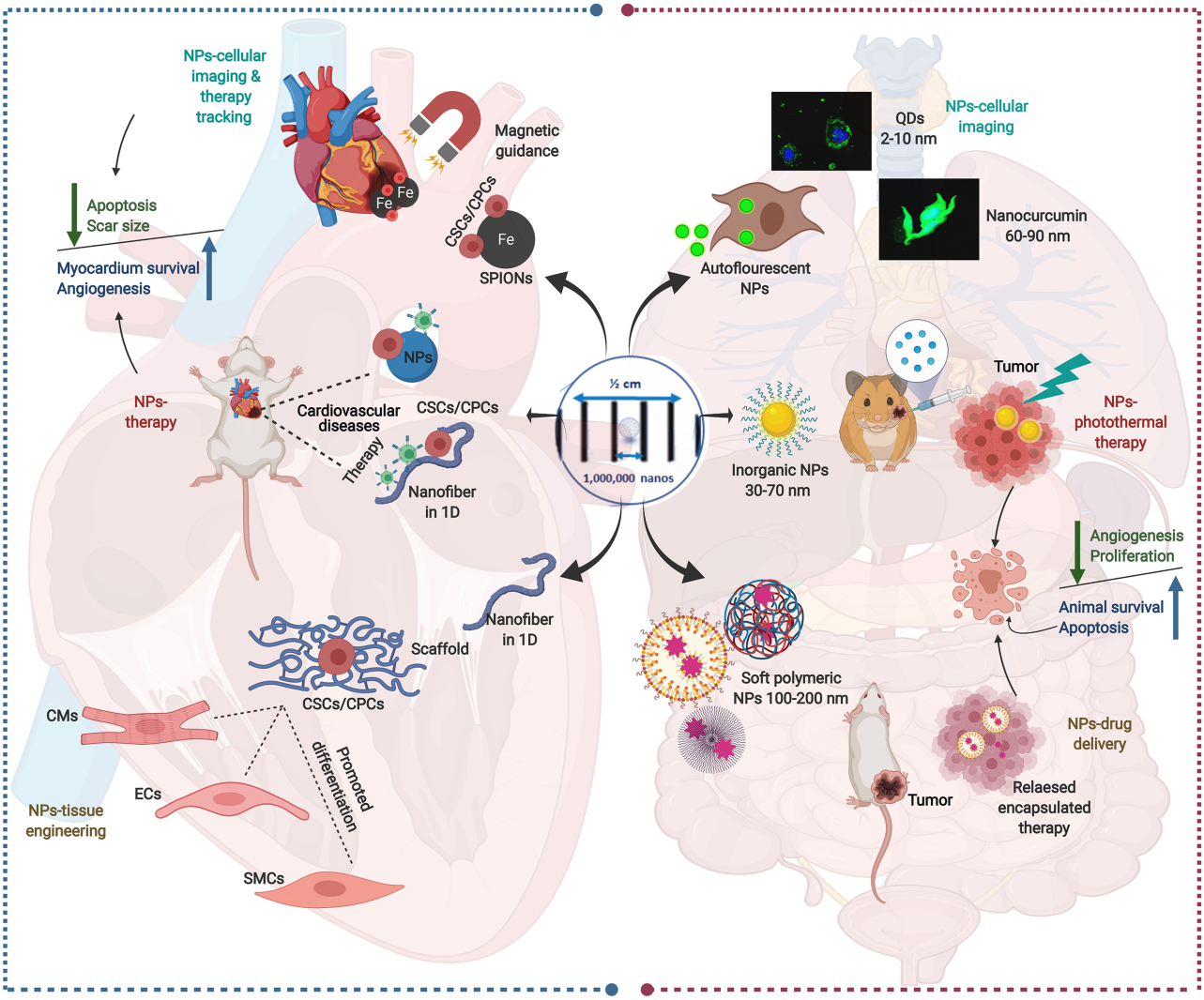

Figure 5 Schematic presentation of multiple nanoparticles-based paradigms in nanomedicine (right side) and their mirror images in nanoparticle-assisted cardiac stem cell interventions (left side). In the right side, the auto-luminescence of quantum dots and some polymeric natural nanoparticles (NPs) as nanocurcumin qualifies them to be used primarily in tracking and diagnostic imaging. Meanwhile, inorganic NPs have been extensively investigated in cancer treatment as photothermal therapy due to the plasmonic resonance of their outer electron. For drug delivery purposes, soft NPs, such as liposomes, polymicelles, and dendrimers, are used due to their flexibility and biophysical interaction with components of the cell membrane, which enable them to penetrate biological membranes. In the left side, the impact of different NPs on cardiac stem cell (CSC)-based therapy are shown. The superparamagnetic iron oxide NPs are used for tracking and imaging of the CSCs-therapy, while nanofibers are used either as a dual delivery system for CSCs and therapy. Moreover, nanofiber scaffold holding the CSCs is used in tissue engineering to assist CSCs' differentiation. The confocal images of NPs-cellular imaging are reproduced with permission [196]. Created with BioRender.com. NPs: Nanoparticles; SMCs: Smooth muscle cells; CMs: Cardiomyocytes; ECs: Endocardial cells; CSCs: Cardiac stem cells; CPCs: Cardiac progenitor cells; SPION: Superparamagnetic iron oxide NP.

blasts after hypoxia. Magnetically targeting an induced MI with IONPs-MSCs have attenuated apoptosis, reduced inflammation, and increased blood vessel density, with increased retention of IONPs-MSCs in the infarcted myocardium, improving left ventricular remodeling[217].

Designer exosomes is another hope, boosting the exosomal theragnostic potential independently of its low yielding. Kojima and coworkers[218] genetically engineered the producer cells with the three genes responsible for potentiating exosome production: Six-Transmembrane Epithelial Antigen of Prostate 3 for exosome biogenesis, syndecan-4 for budding of endosomal membranes, and the fragment of L-aspartate oxidase for cellular metabolism. These exosome production boosters have been further genetically upgraded to RNA packaging device and cytosolic delivery helper. Thereafter, they have used this collectively EXOtic device to deliver cargo messenger RNA to the mice brain, attenuating neurotoxicity in neuroinflammatory disease by enhancing cell-to-cell communication without the need for exosomes concentration. These results demonstrate the usefulness of designer exosomes for therapeutic RNA delivery that can be applied to CPC/CDC exosomes to enhance their efficacy.

CLINICAL TRIALS

The rationale of clinical studies testing CSC therapy on human subjects has been based on the conclusions from the promising results of experimental studies utilizing SCs [219,220]. Clinical trials using CSCs, CPCs, and CDCs have been and are still targeting variable cardiac diseases, namely ischemic heart diseases, non-ischemic cardiomyopathy, heart failure, congenital heart diseases, and pulmonary atrial hypertension (Figure 6, Tables 2 and 3).

Ischemic heart diseases

There have been numerous ischemic heart diseases (IHD) trials. Cardiosphere-derived autologous stem cells to reverse ventricular dysfunction (CAUDEUS) and allogeneic heart stem cells to achieve myocardial regeneration (ALLSTAR) are two complementary studies in which CAUDEUS is the phase I and ALLSTAR is the phase II of clinical trial of intracoronary delivery of autologous CDCs in patients with IHD and left ventricular dysfunction. Firstly, CAUDEUS, with 6 mo and 12-mo follow-up of patients has shown significant reduction in the scar size (-7.7%, -11.1% respectively), increased viable myocardium (+13 g, +22.6 g respectively), and improvement of the regional function of infarcted myocardium[221]. Then, ALLSTAR results showed attenuation of the post infarct cardiac remodeling and improvement in the left ventricular diastolic volume, but it has failed to achieve the results of CAUDEUS regarding the reduction of scar size; hence ALLSTAR was dismissed[222].

The clinical trial transplantation of human embryonic stem cell-derived progenitors in severe heart failure (ESCORT) was developed to assess the regenerative effects of human ESC-derived CPCs. At the beginning of coronary artery bypass grafting, patients received a fibrin gel implanted with the hESC derived CPCs. The main conclusion from this trial demonstrated the technical feasibility of producing a clinically operational product. This product is hESCs derived CPCs that can be safely transplanted to patients with severe ischemic left ventricle (LV) dysfunction. It also supports their short- and medium-term safety after transplantation in patients with severe post-infarction LV dysfunction[223]. Direct engraftment of CPCs into cardiac tissue along with the paracrine effects of CPCs are the main milieu of cardiac regeneration[224,225]. Additionally, fibrin gel has been used as a delivering synthetic biomaterial to improve the long term cell engraftment in the ischemic environment [226].

Safety and efficacy evaluation of intracoronary infusion of allogeneic human cardiac stem cells in patients with AMI (CAREMI), a phase I/II placebo-controlled clinical trial, has been designed to evaluate the safety, practicability, and efficiency of intracoronary transport of allogeneic adult CSC in patients with large ST segment elevation secondary to myocardial infarction, LV dysfunction at risk of developing heart failure. CAREMI has intended to interfere with allogeneic cells immediately after the initial ischemic insult (dodging the aggressive part of the first 5 d) and before myocardial scar starts to form (within 7 d after percutaneous infusion). CAREMI trial is documented proof that allogeneic CSCs intracoronary infusion early after acute MI is safe with rational efficacy outcomes and is promising hope for future clinical trials with Allo-CSCs[227].

Non-ischemic cardiomyopathy

Halt cardiomyopathy progression in Duchenne (HOPE) and dilated cardiomyopathy intervention with allogenic myocardially-regenerative cells (DYNAMIC) studies aim to assess safety and to discover the usefulness of CDCs in patients with non-ischemic cardiomyopathy. HOPE was targeted specifically to explore efficacy of CDCs in patients with advanced stages of Duchenne muscular dystrophy. The results have shown significant and sustained improvements in cardiac structure, function, and significant reduction in cardiac scarring as compared with the control group[228]. Meanwhile, the DYNAMIC study does not have any published data so far.

Heart failure

In autologous human cardiac-derived stem cell to treat ischemic cardiomyopathy (ALCADIA), intramyocardial delivery of CDCs with controlled releases of basic FGF in a biodegradable gelatin hydrogel sheet to patients with ischemic heart failure was done. This was at the time of coronary artery bypass grafting. After 6 mo, it was reported that small improvements in regional but not global function, *i.e.* LVEF (+ 9%-12%), had been noted as well as decreased scar sizes 3.3% at 6-mo follow-up *vs* baseline[229]. A randomized, double-blinded phase II clinical trial started in 2015 to

Table 2 Completed clinical trials reporting on the use of cardiac derived stem cells in cardiovascular disorders

Study name, NCT Number	Start year	Study phase	Cardio-vascular disease	Patients number	Type of cardiac stem cells/origin	Route of delivery /count of cells	Timing of cell delivery	Follow up times	Imagingtechniques	Outcomes	Ref.
CADUCEUS, 00893360	2009	1	IHD	17	Autologous CDCs/endomyocardial biopsies	IC/12.5-25 × 10^6	1.5-3 m post STEMI	6 mo	MRI	Significant reduction in infarct size Significant growth in viable mass global LVEF remains unchanged LVEF and volumes.	[221]
ALLSTAR, 01458405	2017	I/II	IHD	90	Allogenic CDCs/endomyocardial biopsies	IC/25 × 10^6	< 5 d post MI	12 mo	MRI	No effect on scar sizeAttenuation of post infarct cardiac remodeling Improvement in LV end diastolic volume	[222]
ALCADIA, 00981006	2010	I	CHFIHDVD	6	Autologous hCSCs/endomyocardial biopsies + bFGF on gelatin hydrogel sheet	IM/0.5 × 10^6 /kg and 200 μg of bFGF	At CABG	12 mo	Not mentioned	Decreased scar size	[229]
ESCORT, 020579000	2013	I	IHD	6	ESCs-derived ISL1$^+$ CSCs	Epicardial patch 5-10 × 10^6 CSCs embedded in a fibrin patch	At CABG	18 mo	CT PET scan Echo	Symptomatically improved patients with an increased systolic motion of the cell-treated segments. The protocol generated a highly purified population of cardiovascular progenitors. One patient died of heart failure after 22 mo	[223]
CAREMI, 02439398.	2014	I/II	AMI	55	Allogeneic hCSCs/right atrial appendage	IC/35 × 10^6 cells	5 to 7 d after successful reperfusion of AMI by PCI or 8 d from symptoms onset	1 wk, 1, 2, 3, 4, 5, 6, 9 and 12 mo	MRI ECG	Allogeneic CSCs intracoronary infusion early after AMI is safe and anticipates reasonable efficacy outcomes	[227]
CONSERT-HF, 02501811.	2015	II	HF	125	Autologous c-KIT$^+$ CPCs + MSCs/right ventricular endocardial biopsy + Bone marrow aspiration	Trans endocardial/150 × 10^6 MSCs and 5 × 10^6 CPCs	14 wk after cell harvest	6 and 12 mo	MRI Treadmill Questionnaire	Increased LVEF Decrease in infarct size with LV end systolic volume reduction. Strong safety profile	[230]
HOPE, 02485938	2015	I\II	CM secondary to DMD	25	Allogeneic CDCs	IC/75 × 10^6	Not specified	12 mo	MRI Questionnaire	Significant scar reduction improvement in inferior wall systolic thickening compared to the usual care group.	[228]

									CDCs are generally safe and well-tolerated	
DYNAMIC, 02293603	2014	I	Idiopathic dilated CM	42	Allogeneic CDC/not specified	IC/Stepwise dose escalation	Not specified	6, 12 mo	ECG Cardiac enzymes	Not published	_
TICAP, 01273857	2011	I	HLHS	7	Autologous CDCs/right atrial appendage	IC/0.3 × 10^6 cells/kg	1 m after cardiac surgery	36 mo	Echo	Safety of the procedure Increased right ventricle ejection fraction Improved somatic growth Reduced heart failure status	[233]
Perseus, 01829750	2013	II	HLHS	34	Autologous CDCs/not specified	IC/0.3 × 10^6 cells/kg	4 to 9 wk after surgery	3 and 12 mo follow-up	EchoMRIQuestionnaire	Significant improve of ventricular function Improved somatic growth, and quality of life Reduced heart failure status and cardiac fibrosis compared with baseline	[231]

AMI: Acute myocardial infarction; bFGF: Basic fibroblast growth factor; CABG: Coronary artery by bass graft; CHF: Congestive heart failure; CM: Cardiomyopathy; CSC: Cardiac stem cell; DMD: Duchenne muscle dystrophy; hCSC: Human-derived cardiac stem cell; HLHS: Hypoplastic left heart syndrome; IC: Intracoronary; IHD: Ischemic heart disease; IHF: Ischemic heart failure; IM: Intramyocardial; MRI: Magnetic resonance imaging; NCT: National clinical trial; PCI: Percutaneous infusion; STEMI: ST segment elevation after MI; VD: Ventricular dysfunction.

study the efficacy of combinatorial therapy in treatment of cardiac ischemic disorders. Combination of mesenchymal and c-KIT$^+$ CSCs as regenerative therapy for heart failure (CONCERT-HF) utilized autologous endomyocardial CSCs combined with MSCs for heart failure patients. After trans-endocardial injection of this combined therapy, the LVEF increased to 89%, with about 4.5% decrease in infarct size and LV end systolic volume. This is the first clinical trial to assess the benefits of cell combination therapy in humans, which addresses the therapeutic potential for cardiac ischemic patients[230]. In addition, according to the ClinicalTrials.gov Identifier (NCT02503280), there is an expected clinical study (TAC-HFT-II) that might start in 2025 to study the safety and effectiveness of trans-endocardial injection of combination of MSCs and CSCs in patients with post-MI HF. Moreover, another ongoing randomized, double blind, placebo-controlled phase II clinical trial for regression of fibrosis and reversal of diastolic dysfunction in heart failure with preserved ejection fraction patients treated with allogeneic CDCs (Regress-HFpEF, NCT02941705) was conducted to determine whether treatment with intracoronary allogeneic CDCs will affect clinical functional status of HFpEF patients, regarding regression of fibrosis and reversal of diastolic dysfunction.

Table 3 Ongoing and future expected clinical trials

Study name, NCT number	Year of study start	Study phase	Cardiovascular disease	Number of patients	Type of cardiac cells	Route of delivery
Regress-HFpEF, 02941705	2017	II	Symptomatic hypertensive heart disease induced HFpEF	40	Allogeneic CDCs	IC
APOLLON trial, 02781922	2016	III	HLHS	40	Autologous CSCs	IC
CHILD, 03406884	2019	I	HLHS	32	Autologous c-KIT$^+$ cells	IM
ALPHA, 03145298	2017	I	PAH	26	Allogeneic CDC	IV
TAC-HFT-II, 02503280	2025	I/II	IHF	0 enrollment until now	Autologous combination of MSCs and CSCs	Trans endocardial Injection

CSC: Cardiac stem cell; HFpEF: Heart failure with preserved ejection fraction; HLHS: Hypoplastic left heart syndrome; IC: Intracardiac; IHF: Ischemic heart failure; IM: Intramyocardial; IV: Intravenous; NCT: National clinical trial; PAH: Pulmonary atrial hypertension.

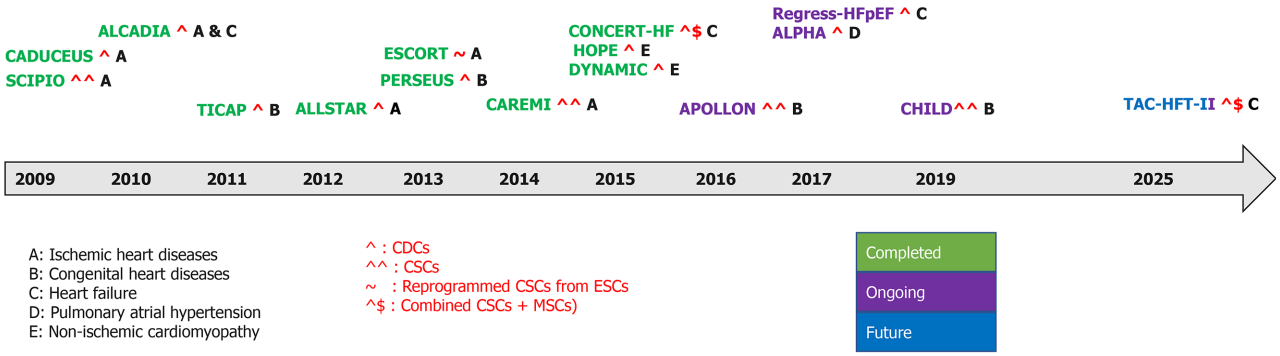

Figure 6 Timeline of completed, ongoing, and future clinical trials in cardiovascular diseases. CDCs: Cardiosphere-derived cells; CSCs: Cardiac stem cells; ESCs: Embryonic stem cells; MSCs: Mesenchymal stem cells.

Congenital heart diseases

There are reports on phase I, II, and III clinical trials assessing the use of autologous CDCs in pediatric patients. One of the early clinical trials in children is the transcoronary infusion of CPCs in patients with single ventricle physiology (TICAP) trial. It has confirmed the practicality of intracoronary delivery of CDCs in post palliative single ventricle physiology patients. In this study, autologous CDCs have been isolated, expanded, and then finally administered *via* intracoronary delivery 4-5 wk after the stage II/III palliative surgery. TICAP has proved that the methodology is safe and achievable for improving cardiac function after 18 mo. The safety of the CDC therapy has been also analyzed at 36 mo post-transplantation. Neither tumor formation nor arrhythmias have been reported, and the initial observed benefits have enhanced, with attenuation of ventricular stiffness and improvement of ventriculoarterial coupling[231].

Following the TICAP trial, the cardiac progenitor cell infusion to treat univentricular heart disease (PERSEUS) trial, a phase II randomized controlled trial has been planned to follow-up on the TICAP trial. CDCs have been given *via* the coronary arteries after stage II or III palliative operations[232]. The main result is the change in EF from baseline to 3 mo that has been measured by cardiac MRI and echocardiography. The CDC treated group showed greater improvement in right ventricle (RV) function at 3 mo than controls (+6.4% *vs* +1.3%) and at 1 year continued to exhibit augmented RV function. Follow-up analysis at 36 mo demonstrated an improvement in RVEF among patients receiving CDCs (+8.0% *vs* +2.2%) even at this late time point. Furthermore, the therapy is currently being tested in a phase III trial cardiac stem/progenitor cell infusion in univentricular physiology (APOLLON). The phase III trial is an extension of the TICAP and PERSEUS trials exploiting autologous CDCs administered by intracoronary injection in single ventricle patients, for which results are still awaiting[233]. In addition, there is another ongoing phase I clinical trial (CHILD, NCT03406884) for examining the safety and efficacy of intramyocardial

injection of c-KIT CSCs injection in patients with hypoplastic left heart syndrome.

Pulmonary atrial hypertension

Pulmonary arterial hypertension (PAH) is a progressive condition for which there is no cure. As reported in PAH archives and with the pharmacologic developments in the current treatment scope, survival remains poor. Preclinical studies suggest that the transplantation of allogeneic CDCs could reduce unfavorable PAH related arteriolar remodeling. Until now, there are no completed studies on PAH, though, there is an ongoing phase I clinical trial [(ALPHA), NCT03145298] exploring the potential beneficial effects of central intravenous delivery of allogeneic human CDCs.

THERAPEUTIC POTENTIAL OF COMBINED CELLULAR THERAPY

Despite the great differentiation potential of CSCs and their ability to restore the injured area, it is influenced by many factors, such as oxidative stress and the aging process, which diminish its function. Thus, the administration of mixtures of SCs can exhibit synergistic effects to get the maximum benefit from the transplanted cells in cardiac regeneration. Cell combination therapies are classified into various entities; the most commonly performed is combination of CSCs with MSCs either applied from autologous or allogeneic sources. The most recent combinatorial approaches are CardioChimerias (CCs) and CardioClusters. Merging several cell types harmonized efficiently cardiomyoplasty in preclinical cardiac ischemic models[234,235].

The mechanism behind the effect of MSCs when combined with CSCs has been clarified in a study conducted by Hatzistergos *et al*[236], where transendocardial injection of MSCs derived from allogenic male bone marrow in a reperfused MI swine model led to myocardial repair by stimulating and increasing the differentiation potential of endogenous CSCs to regenerate myocardium. Remarkably, 2 wk after MSC injection, the MSC-treated hearts showed chimeric collections containing both exogenous immature MSCs and endogenous CSCs with a 20-fold increase in c-KIT$^+$ cells, indicating cardiac lineage commitment. In addition, to confirm the origin of CSCs, porcine endomyocardial biopsies have been isolated and plated as explants with or without MSC feeder layers, signifying that the MSCs have stimulated the expansion of c-KIT$^+$ CSCs. The MSC co-culture derived CSCs have been more than 90% positive for Nkx2-5, which is considered an embryonic heart phenotype. MSCs have facilitated cardiac repair through stimulating a succession of secondary endogenous responses that triggered considerable amounts of adult CMs and immature CSCs to multiply and repair the injured areas with CMs, vascular SMCs, and ECs of host origin.

From this point of view, a study conducted by Williams *et al*[237] to assess the combination of CSCs and MSCs isolated from a human cardiac tissue and iliac crest, respectively, where 1 × 10^6 human CSCs and 200 × 10^6 human MSCs were injected intra-myocardially in the infarcted area in immunosuppressed pigs after 2 wk of MI induction. All cell-based cardiomyoplasty groups showed a decrease in the infarct size 4 wk following cell injection with a 2-fold greater reduction in scar mass with combined SCs in comparison with each therapy alone. The results have been evaluated by cardiac MRI and conductance catheterization hemodynamics, revealing the improvement of cardiac response to MI functionally and structurally with returning of EF values to baseline, where the combined cell therapy enhanced the LV performance during systole and diastole.

In addition to cardiomyogenic repair in acute MI, the combination therapy enhances cardiac performance, both functionally and structurally, in chronic ischemic cardiomyopathies. The hypothesis was adopted by Karantalis *et al*[238]. The autologous CSCs isolated from the septal wall of the right ventricle immediately after MI induction and the autologous MSCs obtained from the tibial cavity were injected trans-endocardially in the same non-immunosuppressed pigs that undergo ischemic injury for 3 mo. The favorable effects of combinational therapy occurred in chronic MI models, where the size of the infarcted area diminished with improvement of EF, diastolic strain, and cardiac output in comparison with single therapy with MSCs.

In agreement with previous studies to explore the effect of allogeneic combination therapy in non-immunosuppressed swine chronic ischemic cardiomyopathy model, Natsumeda *et al*[239] addressed the advantage of such therapy from allogeneic sources to reduce the evoked immune response to administrated CSCs alone. The MSCs inhibited the rejection of the immune system to allo-CSCs, improving the retention of the combined cell therapy in the affected area to maintain the regenerative capacity. Moreover, in comparison with the autologous combination approach, angiogenesis

increased with improvement of perfusion to the previously infarcted area. Furthermore, the added CSCs to allo-MSCs augmented the contractile function of cardiac muscle by enhancing CM proliferation. This denotes that allogeneic combination cell therapy exerts cardiomyogenic regeneration in chronic ischemic conditions without immune rejection from the host.

The enumerated promising findings demonstrate the privileges of combinational therapy to attain the desirable effects of the differentiation potentiality of CSCs with the supporting and paracrine effects of MSCs in cardiac regeneration. However, the effectiveness of this therapy has been compromised by certain factors such as the persistence and long-term cardiogenic repair. Additionally, optimization of the dose of each cell type in the combination mixture is undefined. Therefore, new *in vitro* cell engineering techniques[240-243] have emerged to unify the maximum favorable effects of this therapy and eliminate the limitations concerning cardiac repair.

A novel modality has been aroused by Zang *et al*[240] to enhance the engraftment and angiogenesis properties of c-KIT$^+$ CSCs to precondition the cells with exosomes-derived MSCs, resulting in increased proliferation, formation of angiotube, and enhanced migration *in vitro*. Moreover, in a rat MI model after 28 d, the CSCs combined with exosomes decreased the infarct size with neovascularization and increased the capillary density with higher EF relative to treated groups with CSCs only. The beneficial effect of exosomes on CSCs was evaluated by miRNA network analysis that exhibit influences on Wnt, VEGF, and PTEN/PI3K/Akt signaling pathways. These effects have enhanced CSCs potentials in cardiac repair by adding new properties to CSCs such as increased proliferation, differentiation, and angiogenesis to the pre infarcted area with defense against oxidative stress.

Quijada *et al*[241] is the first study conducted to administer a new cardiac hybrid murine MI model created by *in vitro* viral fusion between CPCs and MSCs with further clonal expansion known as CCs in order to improve and boost combinatorial cell delivery approaches. In this study, two variable clones were selected from 18 clones after 1 mo of fluorescent activated cell sorting of fused fluorescent-tagged CPCs (mcherry) and MSCs (enhanced green fluorescent protein) with an inactivated RNA Sendai virus to accomplish artificial cell fusion. It has been found that CCs exhibit augmented proliferation and survival properties in addition to opposing cell death (CC1 and CC2) compared with individual cell and combination therapies in a MI mouse model. The CCs injected into the acutely damaged heart have recurred structural integrity with functional improvement up to 18 wk with reduction of infarct size at 12 wk and earlier correction of EF at 6 wk. Moreover, they are not subjected to cell aging relative to combinational and single cell therapy. The tremendous effect of CCs was presented in enhanced engraftment in the border zone of infarcted area, which denotes the selectivity of this hybrid in addition to the already retained properties of CSCs and MSCs in cardiomyogenic repair. Notably, in comparison with mixed dual cell therapy the preservation of CM size and induction of c-KIT$^+$ cells have occurred at 12 wk, with enhanced capillary density maintaining a longstanding cardiac vasculature.

In agreement with reparative potentials of CCs, Firouzi *et al*[242] applied the principle of cell fusion between c-KIT$^+$ cardiac interstitial cells (cCICs) and MSCs in a 2:1 ratio with inactivated Sendai virus. The cells were isolated from human cardiac samples removed during left ventricular assisted device implantation, then were allowed for clonal expansion to study the phenotypic features and function as compared to the parent cells. It was been found that the survival of human CCs is prolonged more than parent cells in addition to the increased survival of CMs when co-cultured with serum deprived neonatal rat CMs. Compared to CCs originating from a mouse model, its ability to resist oxidative stress is increased, thus enhancing survival potential and inheriting the same karyotype of the parent cells with diploid DNA content. However, it has not yet been tested in a MI model to assess the degree of retention or the cardiomyogenic repair potentials.

The evolution of next generation applications to improve the efficacy of combinatorial cell therapy in heart failure have been accomplished by Monsanto *et al*[243] who have implemented a 3D structure composed of MSCs in the center surrounded by cCICs, while endothelial progenitor cells (EPCs) inhabited the outer layer. These isolated autologous SCs in a ratio 3:2:1 of EPCs, cCICs, and MSCs, respectively, have been co-cultured to enhance the interactions between the cells before injection intramyocardially in a murine MI model. Moreover, this scaffold has inherited the same efficiency of each cell type. The EPCs protected the cCICs from oxidative stress and cell death with neovascularization to the infarcted area. Thus, the power of cCIC differentiation was increased in addition to the supporting milieu exerted by MSCs. The beneficiary outcomes were evidenced by the improved cardiac function in the

murine model initiated from the first week post MI injection and sustained up to 20 wk, with greater improvement in EF, end systolic, and diastolic volume. Due to the administered distinct cell ratios and the controllable 3D structure size, which minimize cell loss, the CCs were retained for a long time and hence provided maximum effects of cell combination therapies in cardiomyogenic repair[240].

CONCLUSION

CSCs/CPCs carry a great therapeutic potential in different cardiac diseases owing to their numerous cell population, rich secretome and interplay with other cells. Conducted preclinical studies and clinical trials had demonstrated promising results in regards to the improvement of cardiac function. Studies using these cells targeting variable cardiac diseases are still ongoing aiming to discover more of their regenerative power and thus decrease the burden of morbidity and mortality due to cardiac tissue loss.

REFERENCES

1 **Bergmann O**, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. Evidence for cardiomyocyte renewal in humans. *Science* 2009; **324**: 98-102 [PMID: 19342590 DOI: 10.1126/science.1164680]

2 **Hsieh PC**, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nat Med* 2007; **13**: 970-974 [PMID: 17660827 DOI: 10.1038/nm1618]

3 **Wang X**. Stem cells in tissues, organoids, and cancers. *Cell Mol Life Sci* 2019; **76**: 4043-4070 [PMID: 31317205 DOI: 10.1007/s00018-019-03199-x]

4 **Krampera M**, Franchini M, Pizzolo G, Aprili G. Mesenchymal stem cells: from biology to clinical use. *Blood Transfus* 2007; **5**: 120-129 [PMID: 19204764 DOI: 10.2450/2007.0029-07]

5 **Henning RJ**. Current status of stem cells in cardiac repair. *Future Cardiol* 2018; **14**: 181-192 [PMID: 29405072 DOI: 10.2217/fca-2017-0072]

6 **Lee CY**, Kim R, Ham O, Lee J, Kim P, Lee S, Oh S, Lee H, Lee M, Kim J, Chang W. Therapeutic Potential of Stem Cells Strategy for Cardiovascular Diseases. *Stem Cells Int* 2016; **2016**: 4285938 [PMID: 27829839 DOI: 10.1155/2016/4285938]

7 **Madigan M**, Atoui R. Therapeutic Use of Stem Cells for Myocardial Infarction. *Bioengineering (Basel)* 2018; **5** [PMID: 29642402 DOI: 10.3390/bioengineering5020028]

8 **Swedlund B**, Lescroart F. Cardiopharyngeal Progenitor Specification: Multiple Roads to the Heart and Head Muscles. *Cold Spring Harb Perspect Biol* 2020; **12** [PMID: 31818856 DOI: 10.1101/cshperspect.a036731]

9 **Faustino RS**, Terzic A. Interactome of a cardiopoietic precursor. *J Cardiovasc Transl Res* 2008; **1**: 120-126 [PMID: 20559906 DOI: 10.1007/s12265-008-9019-z]

10 **Bondue A**, Tännler S, Chiapparo G, Chabab S, Ramialison M, Paulissen C, Beck B, Harvey R, Blanpain C. Defining the earliest step of cardiovascular progenitor specification during embryonic stem cell differentiation. *J Cell Biol* 2011; **192**: 751-765 [PMID: 21383076 DOI: 10.1083/jcb.201007063]

11 **Lee JW**, Lee CS, Ryu YR, Lee J, Son H, Cho HJ, Kim HS. Lysophosphatidic Acid Receptor 4 Is Transiently Expressed during Cardiac Differentiation and Critical for Repair of the Damaged Heart. *Mol Ther* 2021; **29**: 1151-1163 [PMID: 33160074 DOI: 10.1016/j.ymthe.2020.11.004]

12 **Meilhac SM**, Buckingham ME. The deployment of cell lineages that form the mammalian heart. *Nat Rev Cardiol* 2018; **15**: 705-724 [PMID: 30266935 DOI: 10.1038/s41569-018-0086-9]

13 **Kula-Alwar D**, Marber MS, Hughes SM, Hinits Y. Mef2c factors are required for early but not late addition of cardiomyocytes to the ventricle. *Dev Biol* 2021; **470**: 95-107 [PMID: 33245870 DOI: 10.1016/j.ydbio.2020.11.008]

14 **Antimisiaris SG**, Mourtas S, Marazioti A. Exosomes and Exosome-Inspired Vesicles for Targeted Drug Delivery. *Pharmaceutics* 2018; **10** [PMID: 30404188 DOI: 10.3390/pharmaceutics10040218]

15 **Lamouille S**, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* 2014; **15**: 178-196 [PMID: 24556840 DOI: 10.1038/nrm3758]

16 **Stefanovic S**, Laforest B, Desvignes JP, Lescroart F, Argiro L, Maurel-Zaffran C, Salgado D, Plaindoux E, De Bono C, Pazur K, Théveniau-Ruissy M, Béroud C, Puceat M, Gavalas A, Kelly RG, Zaffran S. *Hox*-dependent coordination of mouse cardiac progenitor cell patterning and differentiation. *Elife* 2020; **9** [PMID: 32804075 DOI: 10.7554/eLife.55124]

17 **Aval SF**, Lotfi H, Sheervalilou R, Zarghami N. Tuning of major signaling networks (TGF-β, Wnt, Notch and Hedgehog) by miRNAs in human stem cells commitment to different lineages: Possible clinical application. *Biomed Pharmacother* 2017; **91**: 849-860 [PMID: 28501774 DOI: 10.1016/j.biopha.2017.05.020]

18 **Tzavlaki K**, Moustakas A. TGF-β Signaling. *Biomolecules* 2020; **10** [PMID: 32210029 DOI:

10.3390/biom10030487]
19 **Li X**, Gao F, Wang X, Liang Q, Bai A, Liu Z, Chen X, Li E, Chen S, Lu C, Qian R, Sun N, Liang P, Xu C. E2A ablation enhances proportion of nodal-like cardiomyocytes in cardiac-specific differentiation of human embryonic stem cells. *EBioMedicine* 2021; **71**: 103575 [PMID: 34488017 DOI: 10.1016/j.ebiom.2021.103575]
20 **Desgrange A**, Le Garrec JF, Bernheim S, Bønnelykke TH, Meilhac SM. Transient Nodal Signaling in Left Precursors Coordinates Opposed Asymmetries Shaping the Heart Loop. *Dev Cell* 2020; **55**: 413-431.e6 [PMID: 33171097 DOI: 10.1016/j.devcel.2020.10.008]
21 **Phillips HM**, Mahendran P, Singh E, Anderson RH, Chaudhry B, Henderson DJ. Neural crest cells are required for correct positioning of the developing outflow cushions and pattern the arterial valve leaflets. *Cardiovasc Res* 2013; **99**: 452-460 [PMID: 23723064 DOI: 10.1093/cvr/cvt132]
22 **Darrigrand JF**, Valente M, Comai G, Martinez P, Petit M, Nishinakamura R, Osorio DS, Renault G, Marchiol C, Ribes V, Cadot B. Dullard-mediated Smad1/5/8 inhibition controls mouse cardiac neural crest cells condensation and outflow tract septation. *Elife* 2020; **9** [PMID: 32105214 DOI: 10.7554/eLife.50325]
23 **Morrell NW**, Bloch DB, ten Dijke P, Goumans MJ, Hata A, Smith J, Yu PB, Bloch KD. Targeting BMP signalling in cardiovascular disease and anaemia. *Nat Rev Cardiol* 2016; **13**: 106-120 [PMID: 26461965 DOI: 10.1038/nrcardio.2015.156]
24 **Andrés-Delgado L**, Galardi-Castilla M, Münch J, Peralta M, Ernst A, González-Rosa JM, Tessadori F, Santamaría L, Bakkers J, Vermot J, de la Pompa JL, Mercader N. Notch and Bmp signaling pathways act coordinately during the formation of the proepicardium. *Dev Dyn* 2020; **249**: 1455-1469 [PMID: 33103836 DOI: 10.1002/dvdy.229]
25 **Miao S**, Zhao D, Wang X, Ni X, Fang X, Yu M, Ye L, Yang J, Wu H, Han X, Qu L, Li L, Lan F, Shen Z, Lei W, Zhao ZA, Hu S. Retinoic acid promotes metabolic maturation of human Embryonic Stem Cell-derived Cardiomyocytes. *Theranostics* 2020; **10**: 9686-9701 [PMID: 32863954 DOI: 10.7150/thno.44146]
26 **Drowley L**, McPheat J, Nordqvist A, Peel S, Karlsson U, Martinsson S, Müllers E, Dellsén A, Knight S, Barrett I, Sánchez J, Magnusson B, Greber B, Wang QD, Plowright AT. Discovery of retinoic acid receptor agonists as proliferators of cardiac progenitor cells through a phenotypic screening approach. *Stem Cells Transl Med* 2020; **9**: 47-60 [PMID: 31508905 DOI: 10.1002/sctm.19-0069]
27 **Janesick A**, Shiotsugu J, Taketani M, Blumberg B. RIPPLY3 is a retinoic acid-inducible repressor required for setting the borders of the pre-placodal ectoderm. *Development* 2012; **139**: 1213-1224 [PMID: 22354841 DOI: 10.1242/dev.071456]
28 **Christ A**, Marczenke M, Willnow TE. LRP2 controls sonic hedgehog-dependent differentiation of cardiac progenitor cells during outflow tract formation. *Hum Mol Genet* 2020; **29**: 3183-3196 [PMID: 32901292 DOI: 10.1093/hmg/ddaa200]
29 **Badr AM**, Mahran YF. Notch pathway and its role in cardiovascular system: Review. *Sys Rev Pharm* 2020; **11**: 342-349 [DOI: 10.31838/srp.2020.10.56]
30 **Bardot ES**, Jadhav B, Wickramasinghe N, Rezza A, Rendl M, Sharp AJ, Dubois NC. Notch signaling commits mesoderm to the cardiac lineage. *BioRxiv* 2020; 2020.2002.2020.958348 [DOI: 10.1101/2020.02.20.958348]
31 **De Zoysa P**, Liu J, Toubat O, Choi J, Moon A, Gill PS, Duarte A, Sucov HM, Kumar SR. Delta-like ligand 4-mediated Notch signaling controls proliferation of second heart field progenitor cells by regulating Fgf8 expression. *Development* 2020; **147** [PMID: 32778568 DOI: 10.1242/dev.185249]
32 **Gordon MD**, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem* 2006; **281**: 22429-22433 [PMID: 16793760 DOI: 10.1074/jbc.R600015200]
33 **Guo Y**, Dorn T, Kühl SJ, Linnemann A, Rothe M, Pfister AS, Vainio S, Laugwitz KL, Moretti A, Kühl M. The Wnt inhibitor Dkk1 is required for maintaining the normal cardiac differentiation program in Xenopus laevis. *Dev Biol* 2019; **449**: 1-13 [PMID: 30797757 DOI: 10.1016/j.ydbio.2019.02.009]
34 **Wang J**, Gong M, Zuo S, Xu J, Paul C, Li H, Liu M, Wang YG, Ashraf M, Xu M. WNT11-Conditioned Medium Promotes Angiogenesis through the Activation of Non-Canonical WNT-PKC-JNK Signaling Pathway. *Genes (Basel)* 2020; **11** [PMID: 33137935 DOI: 10.3390/genes11111277]
35 **Li HX**, Lin J, Jiang B, Yang XJ. Wnt11 preserves mitochondrial membrane potential and protects cardiomyocytes against hypoxia through paracrine signaling. *J Cell Biochem* 2020; **121**: 1144-1155 [PMID: 31463993 DOI: 10.1002/jcb.29349]
36 **Gao Y**, Pu J. Differentiation and Application of Human Pluripotent Stem Cells Derived Cardiovascular Cells for Treatment of Heart Diseases: Promises and Challenges. *Front Cell Dev Biol* 2021; **9**: 658088 [PMID: 34055788 DOI: 10.3389/fcell.2021.658088]
37 **Marino F**, Scalise M, Cianflone E, Mancuso T, Aquila I, Agosti V, Torella M, Paolino D, Mollace V, Nadal-Ginard B, Torella D. Role of c-Kit in Myocardial Regeneration and Aging. *Front Endocrinol (Lausanne)* 2019; **10**: 371 [PMID: 31275242 DOI: 10.3389/fendo.2019.00371]
38 **Xu J**, Lian W, Li L, Huang Z. Generation of induced cardiac progenitor cells *via* somatic reprogramming. *Oncotarget* 2017; **8**: 29442-29457 [PMID: 28199972 DOI: 10.18632/oncotarget.15272]
39 **Le T**, Chong J. Cardiac progenitor cells for heart repair. *Cell Death Discov* 2016; **2**: 16052 [PMID: 27551540 DOI: 10.1038/cddiscovery.2016.52]

40 **Wandzioch E**, Edling CE, Palmer RH, Carlsson L, Hallberg B. Activation of the MAP kinase pathway by c-Kit is PI-3 kinase dependent in hematopoietic progenitor/stem cell lines. *Blood* 2004; **104**: 51-57 [PMID: 14996702 DOI: 10.1182/blood-2003-07-2554]

41 **Kuang D**, Zhao X, Xiao G, Ni J, Feng Y, Wu R, Wang G. Stem cell factor/c-kit signaling mediated cardiac stem cell migration *via* activation of p38 MAPK. *Basic Res Cardiol* 2008; **103**: 265-273 [PMID: 18087667 DOI: 10.1007/s00395-007-0690-z]

42 **van Berlo JH**, Kanisicak O, Maillet M, Vagnozzi RJ, Karch J, Lin SC, Middleton RC, Marbán E, Molkentin JD. c-kit+ cells minimally contribute cardiomyocytes to the heart. *Nature* 2014; **509**: 337-341 [PMID: 24805242 DOI: 10.1038/nature13309]

43 **Rota M**, Padin-Iruegas ME, Misao Y, De Angelis A, Maestroni S, Ferreira-Martins J, Fiumana E, Rastaldo R, Arcarese ML, Mitchell TS, Boni A, Bolli R, Urbanek K, Hosoda T, Anversa P, Leri A, Kajstura J. Local activation or implantation of cardiac progenitor cells rescues scarred infarcted myocardium improving cardiac function. *Circ Res* 2008; **103**: 107-116 [PMID: 18556576 DOI: 10.1161/CIRCRESAHA.108.178525]

44 **Malliaras K**, Zhang Y, Seinfeld J, Galang G, Tseliou E, Cheng K, Sun B, Aminzadeh M, Marbán E. Cardiomyocyte proliferation and progenitor cell recruitment underlie therapeutic regeneration after myocardial infarction in the adult mouse heart. *EMBO Mol Med* 2013; **5**: 191-209 [PMID: 23255322 DOI: 10.1002/emmm.201201737]

45 **van Vliet P**, Roccio M, Smits AM, van Oorschot AA, Metz CH, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ. Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J* 2008; **16**: 163-169 [PMID: 18566670 DOI: 10.1007/BF03086138]

46 **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]

47 **Välimäki MJ**, Leigh RS, Kinnunen SM, March AR, de Sande AH, Kinnunen M, Varjosalo M, Heinäniemi M, Kaynak BL, Ruskoaho H. GATA-targeted compounds modulate cardiac subtype cell differentiation in dual reporter stem cell line. *Stem Cell Res Ther* 2021; **12**: 190 [PMID: 33736688 DOI: 10.1186/s13287-021-02259-z]

48 **Valente M**, Nascimento DS, Cumano A, Pinto-do-Ó P. Sca-1+ cardiac progenitor cells and heart-making: a critical synopsis. *Stem Cells Dev* 2014; **23**: 2263-2273 [PMID: 24926741 DOI: 10.1089/scd.2014.0197]

49 **Tsuji-Takayama K**, Inoue T, Ijiri Y, Otani T, Motoda R, Nakamura S, Orita K. Demethylating agent, 5-azacytidine, reverses differentiation of embryonic stem cells. *Biochem Biophys Res Commun* 2004; **323**: 86-90 [PMID: 15351705 DOI: 10.1016/j.bbrc.2004.08.052]

50 **Barreto S**, Hamel L, Schiatti T, Yang Y, George V. Cardiac Progenitor Cells from Stem Cells: Learning from Genetics and Biomaterials. *Cells* 2019; **8**: 1536 [PMID: 31795206 DOI: 10.3390/cells8121536]

51 **Takamiya M**, Haider KH, Ashraf M. Identification and characterization of a novel multipotent sub-population of Sca-1^{+} cardiac progenitor cells for myocardial regeneration. *PLoS One* 2011; **6**: e25265 [PMID: 21980409 DOI: 10.1371/journal.pone.0025265]

52 **Smits AM**, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. *Nat Protoc* 2009; **4**: 232-243 [PMID: 19197267 DOI: 10.1038/nprot.2008.229]

53 **Saga Y**, Kitajima S, Miyagawa-Tomita S. Mesp1 expression is the earliest sign of cardiovascular development. *Trends Cardiovasc Med* 2000; **10**: 345-352 [PMID: 11369261 DOI: 10.1016/s1050-1738(01)00069-x]

54 **Bondue A**, Lapouge G, Paulissen C, Semeraro C, Iacovino M, Kyba M, Blanpain C. Mesp1 acts as a master regulator of multipotent cardiovascular progenitor specification. *Cell Stem Cell* 2008; **3**: 69-84 [PMID: 18593560 DOI: 10.1016/j.stem.2008.06.009]

55 **Rockey DC**, Bell PD, Hill JA. Fibrosis--a common pathway to organ injury and failure. *N Engl J Med* 2015; **372**: 1138-1149 [PMID: 25785971 DOI: 10.1056/NEJMra1300575]

56 **Yamakawa H**, Ieda M. Cardiac regeneration by direct reprogramming in this decade and beyond. *Inflamm Regen* 2021; **41**: 20 [PMID: 34193320 DOI: 10.1186/s41232-021-00168-5]

57 **Fu JD**, Stone NR, Liu L, Spencer CI, Qian L, Hayashi Y, Delgado-Olguin P, Ding S, Bruneau BG, Srivastava D. Direct reprogramming of human fibroblasts toward a cardiomyocyte-like state. *Stem Cell Reports* 2013; **1**: 235-247 [PMID: 24319660 DOI: 10.1016/j.stemcr.2013.07.005]

58 **Yang L**, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* 2008; **453**: 524-528 [PMID: 18432194 DOI: 10.1038/nature06894]

59 **Nelson TJ**, Chiriac A, Faustino RS, Crespo-Diaz RJ, Behfar A, Terzic A. Lineage specification of Flk-1+ progenitors is associated with divergent Sox7 expression in cardiopoiesis. *Differentiation* 2009; **77**: 248-255 [PMID: 19272523 DOI: 10.1016/j.diff.2008.10.012]

60 **Galdos FX**, Guo Y, Paige SL, VanDusen NJ, Wu SM, Pu WT. Cardiac Regeneration: Lessons From Development. *Circ Res* 2017; **120**: 941-959 [PMID: 28302741 DOI: 10.1161/CIRCRESAHA.116.309040]

61 **Kelly RG**, Buckingham ME, Moorman AF. Heart fields and cardiac morphogenesis. *Cold Spring Harb Perspect Med* 2014; **4** [PMID: 25274757 DOI: 10.1101/cshperspect.a015750]

62 **Zhou Z**, Wang J, Guo C, Chang W, Zhuang J, Zhu P, Li X. Temporally Distinct Six2-Positive Second Heart Field Progenitors Regulate Mammalian Heart Development and Disease. *Cell Rep* 2017; **18**: 1019-1032 [PMID: 28122228 DOI: 10.1016/j.celrep.2017.01.002]

63 **Rana MS**, Christoffels VM, Moorman AF. A molecular and genetic outline of cardiac morphogenesis. *Acta Physiol (Oxf)* 2013; **207**: 588-615 [PMID: 23297764 DOI: 10.1111/apha.12061]

64 **Xin M**, Olson EN, Bassel-Duby R. Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. *Nat Rev Mol Cell Biol* 2013; **14**: 529-541 [PMID: 23839576 DOI: 10.1038/nrm3619]

65 **Li Y**, Tian S, Lei I, Liu L, Ma P, Wang Z. Transplantation of multipotent Isl1+ cardiac progenitor cells preserves infarcted heart function in mice. *Am J Transl Res* 2017; **9**: 1530-1542 [PMID: 28386378]

66 **Zhou B**, von Gise A, Ma Q, Rivera-Feliciano J, Pu WT. Nkx2-5- and Isl1-expressing cardiac progenitors contribute to proepicardium. *Biochem Biophys Res Commun* 2008; **375**: 450-453 [PMID: 18722343 DOI: 10.1016/j.bbrc.2008.08.044]

67 **Tampakakis E**, Miyamoto M, Kwon C. In Vitro Generation of Heart Field-specific Cardiac Progenitor Cells. *J Vis Exp* 2019 [PMID: 31329174 DOI: 10.3791/59826]

68 **Streef TJ**, Smits AM. Epicardial Contribution to the Developing and Injured Heart: Exploring the Cellular Composition of the Epicardium. *Front Cardiovasc Med* 2021; **8**: 750243 [PMID: 34631842 DOI: 10.3389/fcvm.2021.750243]

69 **Synnergren J**, Drowley L, Plowright AT, Brolén G, Goumans MJ, Gittenberger-de Groot AC, Sartipy P, Wang QD. Comparative transcriptomic analysis identifies genes differentially expressed in human epicardial progenitors and hiPSC-derived cardiac progenitors. *Physiol Genomics* 2016; **48**: 771-784 [PMID: 27591124 DOI: 10.1152/physiolgenomics.00064.2016]

70 **Zhou B**, Honor LB, He H, Ma Q, Oh JH, Butterfield C, Lin RZ, Melero-Martin JM, Dolmatova E, Duffy HS, Gise Av, Zhou P, Hu YW, Wang G, Zhang B, Wang L, Hall JL, Moses MA, McGowan FX, Pu WT. Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J Clin Invest* 2011; **121**: 1894-1904 [PMID: 21505261 DOI: 10.1172/JCI45529]

71 **Zangi L**, Lui KO, von Gise A, Ma Q, Ebina W, Ptaszek LM, Später D, Xu H, Tabebordbar M, Gorbatov R, Sena B, Nahrendorf M, Briscoe DM, Li RA, Wagers AJ, Rossi DJ, Pu WT, Chien KR. Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. *Nat Biotechnol* 2013; **31**: 898-907 [PMID: 24013197 DOI: 10.1038/nbt.2682]

72 **Sandstedt J**, Jonsson M, Kajic K, Sandstedt M, Lindahl A, Dellgren G, Jeppsson A, Asp J. Left atrium of the human adult heart contains a population of side population cells. *Basic Res Cardiol* 2012; **107**: 255 [PMID: 22361742 DOI: 10.1007/s00395-012-0255-7]

73 **Hierlihy AM**, Seale P, Lobe CG, Rudnicki MA, Megeney LA. The post-natal heart contains a myocardial stem cell population. *FEBS Lett* 2002; **530**: 239-243 [PMID: 12387899 DOI: 10.1016/s0014-5793(02)03477-4]

74 **Oikonomopoulos A**, Sereti KI, Conyers F, Bauer M, Liao A, Guan J, Crapps D, Han JK, Dong H, Bayomy AF, Fine GC, Westerman K, Biechele TL, Moon RT, Force T, Liao R. Wnt signaling exerts an antiproliferative effect on adult cardiac progenitor cells through IGFBP3. *Circ Res* 2011; **109**: 1363-1374 [PMID: 22034491 DOI: 10.1161/CIRCRESAHA.111.250282]

75 **Barandon L**, Casassus F, Leroux L, Moreau C, Allières C, Lamazière JM, Dufourcq P, Couffinhal T, Duplàa C. Secreted frizzled-related protein-1 improves postinfarction scar formation through a modulation of inflammatory response. *Arterioscler Thromb Vasc Biol* 2011; **31**: e80-e87 [PMID: 21836067 DOI: 10.1161/ATVBAHA.111.232280]

76 **Fu WB**, Wang WE, Zeng CY. Wnt signaling pathways in myocardial infarction and the therapeutic effects of Wnt pathway inhibitors. *Acta Pharmacol Sin* 2019; **40**: 9-12 [PMID: 30002488 DOI: 10.1038/s41401-018-0060-4]

77 **Zhou S**, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001; **7**: 1028-1034 [PMID: 11533706 DOI: 10.1038/nm0901-1028]

78 **Higashikuni Y**, Sainz J, Nakamura K, Takaoka M, Enomoto S, Iwata H, Sahara M, Tanaka K, Koibuchi N, Ito S, Kusuhara H, Sugiyama Y, Hirata Y, Nagai R, Sata M. The ATP-binding cassette transporter BCRP1/ABCG2 plays a pivotal role in cardiac repair after myocardial infarction *via* modulation of microvascular endothelial cell survival and function. *Arterioscler Thromb Vasc Biol* 2010; **30**: 2128-2135 [PMID: 20829509 DOI: 10.1161/ATVBAHA.110.211755]

79 **Oyama T**, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K, Takahashi T, Goto M, Mikami Y, Yasuda N, Akazawa H, Uezumi A, Takeda S, Komuro I. Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. *J Cell Biol* 2007; **176**: 329-341 [PMID: 17261849 DOI: 10.1083/jcb.200603014]

80 **Maher TJ**, Ren Y, Li Q, Braunlin E, Garry MG, Sorrentino BP, Martin CM. ATP-binding cassette transporter Abcg2 lineage contributes to the cardiac vasculature after oxidative stress. *Am J Physiol Heart Circ Physiol* 2014; **306**: H1610-H1618 [PMID: 24727496 DOI: 10.1152/ajpheart.00638.2013]

81 **Doyle MJ**, Maher TJ, Li Q, Garry MG, Sorrentino BP, Martin CM. Abcg2-Labeled Cells Contribute to Different Cell Populations in the Embryonic and Adult Heart. *Stem Cells Dev* 2016; **25**: 277-284 [PMID: 26573225 DOI: 10.1089/scd.2015.0272]

82 **Yellamilli A**, Ren Y, McElmurry RT, Lambert JP, Gross P, Mohsin S, Houser SR, Elrod JW, Tolar

J, Garry DJ, van Berlo JH. Abcg2-expressing side population cells contribute to cardiomyocyte renewal through fusion. *FASEB J* 2020; **34**: 5642-5657 [PMID: 32100368 DOI: 10.1096/fj.201902105R]

83 **Yellamilli A**, van Berlo JH. The Role of Cardiac Side Population Cells in Cardiac Regeneration. *Front Cell Dev Biol* 2016; **4**: 102 [PMID: 27679798 DOI: 10.3389/fcell.2016.00102]

84 **Cho HJ**, Lee HJ, Chung YJ, Kim JY, Cho HJ, Yang HM, Kwon YW, Lee HY, Oh BH, Park YB, Kim HS. Generation of human secondary cardiospheres as a potent cell processing strategy for cell-based cardiac repair. *Biomaterials* 2013; **34**: 651-661 [PMID: 23103158 DOI: 10.1016/j.biomaterials.2012.10.011]

85 **Li TS**, Cheng K, Lee ST, Matsushita S, Davis D, Malliaras K, Zhang Y, Matsushita N, Smith RR, Marbán E. Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions, rationalizing their enhanced functional potency for myocardial repair. *Stem Cells* 2010; **28**: 2088-2098 [PMID: 20882531 DOI: 10.1002/stem.532]

86 **Cheng K**, Malliaras K, Smith RR, Shen D, Sun B, Blusztajn A, Xie Y, Ibrahim A, Aminzadeh MA, Liu W, Li TS, De Robertis MA, Marbán L, Czer LSC, Trento A, Marbán E. Human cardiosphere-derived cells from advanced heart failure patients exhibit augmented functional potency in myocardial repair. *JACC Heart Fail* 2014; **2**: 49-61 [PMID: 24511463 DOI: 10.1016/j.jchf.2013.08.008]

87 **Vincentz JW**, Barnes RM, Firulli BA, Conway SJ, Firulli AB. Cooperative interaction of Nkx2.5 and Mef2c transcription factors during heart development. *Dev Dyn* 2008; **237**: 3809-3819 [PMID: 19035347 DOI: 10.1002/dvdy.21803]

88 **Carroll KJ**, Makarewich CA, McAnally J, Anderson DM, Zentilin L, Liu N, Giacca M, Bassel-Duby R, Olson EN. A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9. *Proc Natl Acad Sci U S A* 2016; **113**: 338-343 [PMID: 26719419 DOI: 10.1073/pnas.1523918113]

89 **Sano T**, Ito T, Ishigami S, Bandaru S, Sano S. Intrinsic activation of cardiosphere-derived cells enhances myocardial repair. *J Thorac Cardiovasc Surg* 2020 [PMID: 32682583 DOI: 10.1016/j.jtcvs.2020.05.040]

90 **Hare JM**, Fishman JE, Gerstenblith G, DiFede Velazquez DL, Zambrano JP, Suncion VY, Tracy M, Ghersin E, Johnston PV, Brinker JA, Breton E, Davis-Sproul J, Schulman IH, Byrnes J, Mendizabal AM, Lowery MH, Rouy D, Altman P, Wong Po Foo C, Ruiz P, Amador A, Da Silva J, McNiece IK, Heldman AW, George R, Lardo A. Comparison of allogeneic vs autologous bone marrow–derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. *JAMA* 2012; **308**: 2369-2379 [PMID: 23117550 DOI: 10.1001/jama.2012.25321]

91 **Sukhacheva TV**, Nizyaeva NV, Samsonova MV, Chernyaev AL, Shchegolev AI, Serov RA. Telocytes in the Myocardium of Children with Congenital Heart Disease Tetralogy of Fallot. *Bull Exp Biol Med* 2020; **169**: 137-146 [PMID: 32488790 DOI: 10.1007/s10517-020-04840-7]

92 **Tan JJ**, Carr CA, Stuckey DJ, Ellison GM, Messina E, Giacomello A, Clarke K. Isolation and expansion of cardiosphere-derived stem cells. *Curr Protoc Stem Cell Biol* 2011; **16**: 2C.-3.1 [DOI: 10.1002/9780470151808.sc02c03s16]

93 **Chen L**, Pan Y, Zhang L, Wang Y, Weintraub N, Tang Y. Two-step protocol for isolation and culture of cardiospheres. *Methods Mol Biol* 2013; **1036**: 75-80 [PMID: 23807787 DOI: 10.1007/978-1-62703-511-8_6]

94 **Barker N**. Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nat Rev Mol Cell Biol* 2014; **15**: 19-33 [PMID: 24326621 DOI: 10.1038/nrm3721]

95 **Bianco P**. Bone and the hematopoietic niche: a tale of two stem cells. *Blood* 2011; **117**: 5281-5288 [PMID: 21406722 DOI: 10.1182/blood-2011-01-315069]

96 **Huson DH**, Bryant D. Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* 2006; **23**: 254-267 [PMID: 16221896 DOI: 10.1093/molbev/msj030]

97 **Urbanek K**, Cesselli D, Rota M, Nascimbene A, De Angelis A, Hosoda T, Bearzi C, Boni A, Bolli R, Kajstura J, Anversa P, Leri A. Stem cell niches in the adult mouse heart. *Proc Natl Acad Sci USA* 2006; **103**: 9226-9231 [PMID: 16754876 DOI: 10.1073/pnas.0600635103]

98 **Vukusic K**, Asp J, Henriksson HB, Brisby H, Lindahl A, Sandstedt J. Physical exercise affects slow cycling cells in the rat heart and reveals a new potential niche area in the atrioventricular junction. *J Mol Histol* 2015; **46**: 387-398 [PMID: 26047663 DOI: 10.1007/s10735-015-9626-3]

99 **Bearzi C**, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. Human cardiac stem cells. *Proc Natl Acad Sci USA* 2007; **104**: 14068-14073 [PMID: 17709737 DOI: 10.1073/pnas.0706760104]

100 **Leri A**, Rota M, Hosoda T, Goichberg P, Anversa P. Cardiac stem cell niches. *Stem Cell Res* 2014; **13**: 631-646 [PMID: 25267073 DOI: 10.1016/j.scr.2014.09.001]

101 **Vicinanza C**, Aquila I, Scalise M, Cristiano F, Marino F, Cianflone E, Mancuso T, Marotta P, Sacco W, Lewis FC, Couch L, Shone V, Gritti G, Torella A, Smith AJ, Terracciano CM, Britti D, Veltri P, Indolfi C, Nadal-Ginard B, Ellison-Hughes GM, Torella D. Adult cardiac stem cells are multipotent and robustly myogenic: c-kit expression is necessary but not sufficient for their identification. *Cell Death Differ* 2017; **24**: 2101-2116 [PMID: 28800128 DOI: 10.1038/cdd.2017.130]

102 **Leri A**, Kajstura J, Anversa P. Cardiac Stem Cells and Mechanisms of Myocardial Regeneration. *Physiol Rev* 2005; **85**: 1373-1416 [PMID: 16183916 DOI: 10.1152/physrev.00013.2005]

103 **Scalise M**, Marino F, Salerno L, Mancuso T, Cappetta D, Barone A, Parrotta EI, Torella A, Palumbo D, Veltri P, De Angelis A, Berrino L, Rossi F, Weisz A, Rota M, Urbanek K, Nadal-Ginard B, Torella D, Cianflone E. In vitro CSC-derived cardiomyocytes exhibit the typical microRNA-mRNA blueprint of endogenous cardiomyocytes. *Commun Biol* 2021; **4**: 1146 [PMID: 34593953 DOI: 10.1038/s42003-021-02677-y]

104 **Scadden DT**. Nice neighborhood: emerging concepts of the stem cell niche. *Cell* 2014; **157**: 41-50 [PMID: 24679525 DOI: 10.1016/j.cell.2014.02.013]

105 **Donnelly H**, Salmeron-Sanchez M, Dalby MJ. Designing stem cell niches for differentiation and self-renewal. *J R Soc Interface* 2018; **15** [PMID: 30158185 DOI: 10.1098/rsif.2018.0388]

106 **Simsek T**, Kocabas F, Zheng J, Deberardinis RJ, Mahmoud AI, Olson EN, Schneider JW, Zhang CC, Sadek HA. The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell Stem Cell* 2010; **7**: 380-390 [PMID: 20804973 DOI: 10.1016/j.stem.2010.07.011]

107 **Kimura W**, Sadek HA. The cardiac hypoxic niche: emerging role of hypoxic microenvironment in cardiac progenitors. *Cardiovasc Diagn Ther* 2012; **2**: 278-289 [PMID: 24282728 DOI: 10.3978/j.issn.2223-3652.2012.12.02]

108 **Mas-Bargues C**, Sanz-Ros J, Román-Domínguez A, Inglés M, Gimeno-Mallench L, El Alami M, Viña-Almunia J, Gambini J, Viña J, Borrás C. Relevance of Oxygen Concentration in Stem Cell Culture for Regenerative Medicine. *Int J Mol Sci* 2019; **20**: 1195 [PMID: 30857245 DOI: 10.3390/ijms20051195]

109 **Kocabas F**, Mahmoud AI, Sosic D, Porrello ER, Chen R, Garcia JA, DeBerardinis RJ, Sadek HA. The hypoxic epicardial and subepicardial microenvironment. *J Cardiovasc Transl Res* 2012; **5**: 654-665 [PMID: 22566269 DOI: 10.1007/s12265-012-9366-7]

110 **Latham N**, Ye B, Jackson R, Lam BK, Kuraitis D, Ruel M, Suuronen EJ, Stewart DJ, Davis DR. Human blood and cardiac stem cells synergize to enhance cardiac repair when cotransplanted into ischemic myocardium. *Circulation* 2013; **128**: S105-S112 [PMID: 24030393 DOI: 10.1161/CIRCULATIONAHA.112.000374]

111 **Teng YC**, Porfírio-Sousa AL, Ribeiro GM, Arend MC, da Silva Meirelles L, Chen ES, Rosa DS, Han SW. Analyses of the pericyte transcriptome in ischemic skeletal muscles. *Stem Cell Res Ther* 2021; **12**: 183 [PMID: 33726849 DOI: 10.1186/s13287-021-02247-3]

112 **Silvestre JS**, Smadja DM, Lévy BI. Postischemic revascularization: from cellular and molecular mechanisms to clinical applications. *Physiol Rev* 2013; **93**: 1743-1802 [PMID: 24137021 DOI: 10.1152/physrev.00006.2013]

113 **Avolio E**, Meloni M, Spencer HL, Riu F, Katare R, Mangialardi G, Oikawa A, Rodriguez-Arabaolaza I, Dang Z, Mitchell K, Reni C, Alvino VV, Rowlinson J, Livi U, Cesselli D, Angelini G, Emanueli C, Beltrami AP, Madeddu P. Combined intramyocardial delivery of human pericytes and cardiac stem cells additively improves the healing of mouse infarcted hearts through stimulation of vascular and muscular repair. *Circ Res* 2015; **116**: e81-e94 [PMID: 25801898 DOI: 10.1161/CIRCRESAHA.115.306146]

114 **Li YY**, Zhang S, Li YG, Wang Y. Isolation, culture, purification and ultrastructural investigation of cardiac telocytes. *Mol Med Rep* 2016; **14**: 1194-1200 [PMID: 27314536 DOI: 10.3892/mmr.2016.5386]

115 **Li H**, Zhang Y, Zhang W, Zhong X, Zhao G, Ge J. Paracrine Endocrinology of cardiac telocytes in culture. *Cardiol Plus* 2017; **2**: 8 [DOI: 10.4103/2470-7511.248218]

116 **Sukhacheva TV**, Nizyaeva NV, Samsonova MV, Cherniaev AL, Burov AA, Iurova MV, Shchegolev AI, Serov RA, Sukhikh GT. Morpho-functional changes of cardiac telocytes in isolated atrial amyloidosis in patients with atrial fibrillation. *Sci Rep* 2021; **11**: 3563 [PMID: 33574429 DOI: 10.1038/s41598-021-82554-0]

117 **Albulescu R**, Tanase C, Codrici E, Popescu DI, Cretoiu SM, Popescu LM. The secretome of myocardial telocytes modulates the activity of cardiac stem cells. *J Cell Mol Med* 2015; **19**: 1783-1794 [PMID: 26176909 DOI: 10.1111/jcmm.12624]

118 **Mohri T**, Fujio Y, Obana M, Iwakura T, Matsuda K, Maeda M, Azuma J. Signals through glycoprotein 130 regulate the endothelial differentiation of cardiac stem cells. *Arterioscler Thromb Vasc Biol* 2009; **29**: 754-760 [PMID: 19213943 DOI: 10.1161/ATVBAHA.108.174870]

119 **Laguens R**, Cabeza Meckert P, Vera Janavel G, Del Valle H, Lascano E, Negroni J, Werba P, Cuniberti L, Martínez V, Melo C, Papouchado M, Ojeda R, Criscuolo M, Crottogini A. Entrance in mitosis of adult cardiomyocytes in ischemic pig hearts after plasmid-mediated rhVEGF165 gene transfer. *Gene Ther* 2002; **9**: 1676-1681 [PMID: 12457281 DOI: 10.1038/sj.gt.3301844]

120 **Sid-Otmane C**, Perrault LP, Ly HQ. Mesenchymal stem cell mediates cardiac repair through autocrine, paracrine and endocrine axes. *J Transl Med* 2020; **18**: 336 [PMID: 32873307 DOI: 10.1186/s12967-020-02504-8]

121 **Gatti S**, Leo C, Gallo S, Sala V, Bucci E, Natale M, Cantarella D, Medico E, Crepaldi T. Gene expression profiling of HGF/Met activation in neonatal mouse heart. *Transgenic Res* 2013; **22**: 579-593 [PMID: 23224784 DOI: 10.1007/s11248-012-9667-2]

122 **Funatsu T**, Sawa Y, Ohtake S, Takahashi T, Matsumiya G, Matsuura N, Nakamura T, Matsuda H. Therapeutic angiogenesis in the ischemic canine heart induced by myocardial injection of naked complementary DNA plasmid encoding hepatocyte growth factor. *J Thorac Cardiovasc Surg* 2002; **124**: 1099-1105 [PMID: 12447174 DOI: 10.1067/mtc.2002.123809]

123 **Zhang J**, Wang LL, Du W, Yu YC, Ju WZ, Man YL, Li XR, Chen Y, Wang ZD, Gu WJ, Zhang FX,

Wang H, Wu CT, Cao KJ. Hepatocyte growth factor modification enhances the anti-arrhythmic properties of human bone marrow-derived mesenchymal stem cells. *PLoS One* 2014; **9**: e111246 [PMID: 25360679 DOI: 10.1371/journal.pone.0111246]

124 **Duan HF**, Wu CT, Wu DL, Lu Y, Liu HJ, Ha XQ, Zhang QW, Wang H, Jia XX, Wang LS. Treatment of myocardial ischemia with bone marrow-derived mesenchymal stem cells overexpressing hepatocyte growth factor. *Mol Ther* 2003; **8**: 467-474 [PMID: 12946320 DOI: 10.1016/s1525-0016(03)00186-2]

125 **Nakamura T**, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. *J Clin Invest* 2000; **106**: 1511-1519 [PMID: 11120758 DOI: 10.1172/jci10226]

126 **Shirakawa Y**, Sawa Y, Takewa Y, Tatsumi E, Kaneda Y, Taenaka Y, Matsuda H. Gene transfection with human hepatocyte growth factor complementary DNA plasmids attenuates cardiac remodeling after acute myocardial infarction in goat hearts implanted with ventricular assist devices. *J Thorac Cardiovasc Surg* 2005; **130**: 624-632 [PMID: 16153905 DOI: 10.1016/j.jtcvs.2004.02.045]

127 **Liao Z**, Chen Y, Duan C, Zhu K, Huang R, Zhao H, Hintze M, Pu Q, Yuan Z, Lv L, Chen H, Lai B, Feng S, Qi X, Cai D. Cardiac telocytes inhibit cardiac microvascular endothelial cell apoptosis through exosomal miRNA-21-5p-targeted *cdip1* silencing to improve angiogenesis following myocardial infarction. *Theranostics* 2021; **11**: 268-291 [PMID: 33391474 DOI: 10.7150/thno.47021]

128 **Gherghiceanu M**, Popescu LM. Heterocellular communication in the heart: electron tomography of telocyte-myocyte junctions. *J Cell Mol Med* 2011; **15**: 1005-1011 [PMID: 21426485 DOI: 10.1111/j.1582-4934.2011.01299.x]

129 **Ceausu Z**, Socea B, Dimitriu MCT, Predescu D, Constantin VD, Bacalbaşa N, Cîrstoveanu C, Costache M, Ceausu M. Dormant cardiac stem cells: A promising tool in cardiac regeneration. *Exp Ther Med* 2020; **20**: 3452-3457 [PMID: 32905130 DOI: 10.3892/etm.2020.9015]

130 **Wang L**, Gu H, Turrentine M, Wang M. Estradiol treatment promotes cardiac stem cell (CSC)-derived growth factors, thus improving CSC-mediated cardioprotection after acute ischemia/reperfusion. *Surgery* 2014; **156**: 243-252 [PMID: 24957669 DOI: 10.1016/j.surg.2014.04.002]

131 **Sadat S**, Gehmert S, Song YH, Yen Y, Bai X, Gaiser S, Klein H, Alt E. The cardioprotective effect of mesenchymal stem cells is mediated by IGF-I and VEGF. *Biochem Biophys Res Commun* 2007; **363**: 674-679 [PMID: 17904522 DOI: 10.1016/j.bbrc.2007.09.058]

132 **Liu Q**, Piao H, Wang Y, Zheng D, Wang W. Circulating exosomes in cardiovascular disease: Novel carriers of biological information. *Biomed Pharmacother* 2021; **135**: 111148 [PMID: 33412387 DOI: 10.1016/j.biopha.2020.111148]

133 Gould SJ. Exosomes and Microvesicles. In: Encyclopedia of Biological Chemistry: Second Edition. Elsevier Inc. 2013: 262-264

134 **Hartl FU**. Molecular chaperones in cellular protein folding. *Nature* 1996; **381**: 571-579 [PMID: 8637592 DOI: 10.1038/381571a0]

135 **Boulanger CM**, Loyer X, Rautou PE, Amabile N. Extracellular vesicles in coronary artery disease. *Nat Rev Cardiol* 2017; **14**: 259-272 [PMID: 28150804 DOI: 10.1038/nrcardio.2017.7]

136 **Xiao J**, Pan Y, Li XH, Yang XY, Feng YL, Tan HH, Jiang L, Feng J, Yu XY. Cardiac progenitor cell-derived exosomes prevent cardiomyocytes apoptosis through exosomal miR-21 by targeting PDCD4. *Cell Death Dis* 2016; **7**: e2277 [PMID: 27336721 DOI: 10.1038/cddis.2016.181]

137 **Wu Q**, Wang J, Tan WLW, Jiang Y, Wang S, Li Q, Yu X, Tan J, Liu S, Zhang P, Tiang Z, Chen Z, Foo RS, Yang HT. Extracellular vesicles from human embryonic stem cell-derived cardiovascular progenitor cells promote cardiac infarct healing through reducing cardiomyocyte death and promoting angiogenesis. *Cell Death Dis* 2020; **11**: 354 [PMID: 32393784 DOI: 10.1038/s41419-020-2508-y]

138 **Oxvig C**. The role of PAPP-A in the IGF system: location, location, location. *J Cell Commun Signal* 2015; **9**: 177-187 [PMID: 25617049 DOI: 10.1007/s12079-015-0259-9]

139 **Andriolo G**, Provasi E, Lo Cicero V, Brambilla A, Soncin S, Torre T, Milano G, Biemmi V, Vassalli G, Turchetto L, Barile L, Radrizzani M. Exosomes From Human Cardiac Progenitor Cells for Therapeutic Applications: Development of a GMP-Grade Manufacturing Method. *Front Physiol* 2018; **9**: 1169 [PMID: 30197601 DOI: 10.3389/fphys.2018.01169]

140 **White AJ**, Smith RR, Matsushita S, Chakravarty T, Czer LS, Burton K, Schwarz ER, Davis DR, Wang Q, Reinsmoen NL, Forrester JS, Marbán E, Makkar R. Intrinsic cardiac origin of human cardiosphere-derived cells. *Eur Heart J* 2013; **34**: 68-75 [PMID: 21659438 DOI: 10.1093/eurheartj/ehr172]

141 **Davis DR**, Zhang Y, Smith RR, Cheng K, Terrovitis J, Malliaras K, Li TS, White A, Makkar R, Marbán E. Validation of the cardiosphere method to culture cardiac progenitor cells from myocardial tissue. *PLoS One* 2009; **4**: e7195 [PMID: 19779618 DOI: 10.1371/journal.pone.0007195]

142 **Marbán E**. The Secret Life of Exosomes: What Bees Can Teach Us About Next-Generation Therapeutics. *J Am Coll Cardiol* 2018; **71**: 193-200 [PMID: 29325643 DOI: 10.1016/j.jacc.2017.11.013]

143 **Yu H**, Lu K, Zhu J, Wang J. Stem cell therapy for ischemic heart diseases. *Br Med Bull* 2017; **121**: 135-154 [PMID: 28164211 DOI: 10.1093/bmb/ldw059]

144 **Zhang L**, Ding H, Zhang Y, Wang Y, Zhu W, Li P. Circulating MicroRNAs: Biogenesis and Clinical Significance in Acute Myocardial Infarction. *Front Physiol* 2020; **11**: 1088 [PMID: 33013463 DOI: 10.3389/fphys.2020.01088]

145 **Kenneweg F**, Bang C, Xiao K, Boulanger CM, Loyer X, Mazlan S, Schroen B, Hermans-Beijnsberger S, Foinquinos A, Hirt MN, Eschenhagen T, Funcke S, Stojanovic S, Genschel C, Schimmel K, Just A, Pfanne A, Scherf K, Dehmel S, Raemon-Buettner SM, Fiedler J, Thum T. Long Noncoding RNA-Enriched Vesicles Secreted by Hypoxic Cardiomyocytes Drive Cardiac Fibrosis. *Mol Ther Nucleic Acids* 2019; **18**: 363-374 [PMID: 31634682 DOI: 10.1016/j.omtn.2019.09.003]

146 **Rezaie J**, Rahbarghazi R, Pezeshki M, Mazhar M, Yekani F, Khaksar M, Shokrollahi E, Amini H, Hashemzadeh S, Sokullu SE, Tokac M. Cardioprotective role of extracellular vesicles: A highlight on exosome beneficial effects in cardiovascular diseases. *J Cell Physiol* 2019; **234**: 21732-21745 [PMID: 31140622 DOI: 10.1002/jcp.28894]

147 **Soda N**, Rehm BHA, Sonar P, Nguyen NT, Shiddiky MJA. Advanced liquid biopsy technologies for circulating biomarker detection. *J Mater Chem B* 2019; **7**: 6670-6704 [PMID: 31646316 DOI: 10.1039/c9tb01490j]

148 **Viola M**, de Jager SCA, Sluijter JPG. Targeting Inflammation after Myocardial Infarction: A Therapeutic Opportunity for Extracellular Vesicles? *Int J Mol Sci* 2021; **22**: 7831 [PMID: 34360595 DOI: 10.3390/ijms22157831]

149 **Cambier L**, de Couto G, Ibrahim A, Echavez AK, Valle J, Liu W, Kreke M, Smith RR, Marbán L, Marbán E. Y RNA fragment in extracellular vesicles confers cardioprotection *via* modulation of IL-10 expression and secretion. *EMBO Mol Med* 2017; **9**: 337-352 [PMID: 28167565 DOI: 10.15252/emmm.201606924]

150 **Tseliou E**, Fouad J, Reich H, Slipczuk L, de Couto G, Aminzadeh M, Middleton R, Valle J, Weixin L, Marbán E. Fibroblasts Rendered Antifibrotic, Antiapoptotic, and Angiogenic by Priming With Cardiosphere-Derived Extracellular Membrane Vesicles. *J Am Coll Cardiol* 2015; **66**: 599-611 [PMID: 26248985 DOI: 10.1016/j.jacc.2015.05.068]

151 **Balbi C**, Costa A, Barile L, Bollini S. Message in a Bottle: Upgrading Cardiac Repair into Rejuvenation. *Cells* 2020; **9** [PMID: 32183455 DOI: 10.3390/cells9030724]

152 **Wu R**, Hu X, Wang J. Concise Review: Optimized Strategies for Stem Cell-Based Therapy in Myocardial Repair: Clinical Translatability and Potential Limitation. *Stem Cells* 2018; **36**: 482-500 [PMID: 29330880 DOI: 10.1002/stem.2778]

153 **Barile L**, Milano G, Vassalli G. Beneficial effects of exosomes secreted by cardiac-derived progenitor cells and other cell types in myocardial ischemia. *Stem Cell Investig* 2017; **4**: 93 [PMID: 29270419 DOI: 10.21037/sci.2017.11.06]

154 **Rogers RG**, Ciullo A, Marbán E, Ibrahim AG. Extracellular Vesicles as Therapeutic Agents for Cardiac Fibrosis. *Front Physiol* 2020; **11**: 479 [PMID: 32528309 DOI: 10.3389/fphys.2020.00479]

155 **Tikhomirov R**, Donnell BR, Catapano F, Faggian G, Gorelik J, Martelli F, Emanueli C. Exosomes: From Potential Culprits to New Therapeutic Promise in the Setting of Cardiac Fibrosis. *Cells* 2020; **9** [PMID: 32131460 DOI: 10.3390/cells9030592]

156 **Yuan Y**, Du W, Liu J, Ma W, Zhang L, Du Z, Cai B. Stem Cell-Derived Exosome in Cardiovascular Diseases: Macro Roles of Micro Particles. *Front Pharmacol* 2018; **9**: 547 [PMID: 29904347 DOI: 10.3389/fphar.2018.00547]

157 **Prathipati P**, Nandi SS, Mishra PK. Stem Cell-Derived Exosomes, Autophagy, Extracellular Matrix Turnover, and miRNAs in Cardiac Regeneration during Stem Cell Therapy. *Stem Cell Rev Rep* 2017; **13**: 79-91 [PMID: 27807762 DOI: 10.1007/s12015-016-9696-y]

158 **Chimenti I**, Forte E, Angelini F, Giacomello A, Messina E. From ontogenesis to regeneration: learning how to instruct adult cardiac progenitor cells. *Prog Mol Biol Transl Sci* 2012; **111**: 109-137 [PMID: 22917228 DOI: 10.1016/B978-0-12-398459-3.00005-8]

159 **Ciullo A**, Biemmi V, Milano G, Bolis S, Cervio E, Fertig ET, Gherghiceanu M, Moccetti T, Camici GG, Vassalli G, Barile L. Exosomal Expression of CXCR4 Targets Cardioprotective Vesicles to Myocardial Infarction and Improves Outcome after Systemic Administration. *Int J Mol Sci* 2019; **20** [PMID: 30678240 DOI: 10.3390/ijms20030468]

160 **Gallet R**, Dawkins J, Valle J, Simsolo E, de Couto G, Middleton R, Tseliou E, Luthringer D, Kreke M, Smith RR, Marbán L, Ghaleb B, Marbán E. Exosomes secreted by cardiosphere-derived cells reduce scarring, attenuate adverse remodelling, and improve function in acute and chronic porcine myocardial infarction. *Eur Heart J* 2017; **38**: 201-211 [PMID: 28158410 DOI: 10.1093/eurheartj/ehw240]

161 **Makkar RR**, Smith RR, Cheng K, Malliaras K, Thomson LE, Berman D, Czer LS, Marbán L, Mendizabal A, Johnston PV, Russell SD, Schuleri KH, Lardo AC, Gerstenblith G, Marbán E. Intracoronary cardiosphere-derived cells for heart regeneration after myocardial infarction (CADUCEUS): a prospective, randomised phase 1 trial. *Lancet* 2012; **379**: 895-904 [PMID: 22336189 DOI: 10.1016/S0140-6736(12)60195-0]

162 **Ghafouri-Fard S**, Niazi V, Hussen BM, Omrani MD, Taheri M, Basiri A. The Emerging Role of Exosomes in the Treatment of Human Disorders With a Special Focus on Mesenchymal Stem Cells-Derived Exosomes. *Front Cell Dev Biol* 2021; **9**: 653296 [PMID: 34307345 DOI: 10.3389/fcell.2021.653296]

163 **Milano G**, Biemmi V, Lazzarini E, Balbi C, Ciullo A, Bolis S, Ameri P, Di Silvestre D, Mauri P, Barile L, Vassalli G. Intravenous administration of cardiac progenitor cell-derived exosomes protects against doxorubicin/trastuzumab-induced cardiac toxicity. *Cardiovasc Res* 2020; **116**: 383-392 [PMID: 31098627 DOI: 10.1093/cvr/cvz108]

164 **Lopaschuk GD**, Jaswal JS. Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. *J Cardiovasc Pharmacol* 2010; **56**: 130-140 [PMID:

20505524 DOI: 10.1097/FJC.0b013e3181e74a14]

165 **Breckenridge RA**, Piotrowska I, Ng KE, Ragan TJ, West JA, Kotecha S, Towers N, Bennett M, Kienesberger PC, Smolenski RT, Siddall HK, Offer JL, Mocanu MM, Yelon DM, Dyck JR, Griffin JL, Abramov AY, Gould AP, Mohun TJ. Hypoxic regulation of hand1 controls the fetal-neonatal switch in cardiac metabolism. *PLoS Biol* 2013; **11**: e1001666 [PMID: 24086110 DOI: 10.1371/journal.pbio.1001666]

166 **Puente BN**, Kimura W, Muralidhar SA, Moon J, Amatruda JF, Phelps KL, Grinsfelder D, Rothermel BA, Chen R, Garcia JA, Santos CX, Thet S, Mori E, Kinter MT, Rindler PM, Zacchigna S, Mukherjee S, Chen DJ, Mahmoud AI, Giacca M, Rabinovitch PS, Aroumougame A, Shah AM, Szweda LI, Sadek HA. The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell* 2014; **157**: 565-579 [PMID: 24766806 DOI: 10.1016/j.cell.2014.03.032]

167 **Malandraki-Miller S**, Lopez CA, Al-Siddiqi H, Carr CA. Changing Metabolism in Differentiating Cardiac Progenitor Cells-Can Stem Cells Become Metabolically Flexible Cardiomyocytes? *Front Cardiovasc Med* 2018; **5**: 119 [PMID: 30283788 DOI: 10.3389/fcvm.2018.00119]

168 **Lai L**, Leone TC, Zechner C, Schaeffer PJ, Kelly SM, Flanagan DP, Medeiros DM, Kovacs A, Kelly DP. Transcriptional coactivators PGC-1alpha and PGC-lbeta control overlapping programs required for perinatal maturation of the heart. *Genes Dev* 2008; **22**: 1948-1961 [PMID: 18628400 DOI: 10.1101/gad.1661708]

169 **Karwi QG**, Uddin GM, Ho KL, Lopaschuk GD. Loss of Metabolic Flexibility in the Failing Heart. *Front Cardiovasc Med* 2018; **5**: 68 [PMID: 29928647 DOI: 10.3389/fcvm.2018.00068]

170 **Menendez-Montes I**, Escobar B, Palacios B, Gómez MJ, Izquierdo-Garcia JL, Flores L, Jiménez-Borreguero LJ, Aragones J, Ruiz-Cabello J, Torres M, Martin-Puig S. Myocardial VHL-HIF Signaling Controls an Embryonic Metabolic Switch Essential for Cardiac Maturation. *Dev Cell* 2016; **39**: 724-739 [PMID: 27997827 DOI: 10.1016/j.devcel.2016.11.012]

171 **Steinmetz M**, Quentin T, Poppe A, Paul T, Jux C. Changes in expression levels of genes involved in fatty acid metabolism: upregulation of all three members of the PPAR family (alpha, gamma, delta) and the newly described adiponectin receptor 2, but not adiponectin receptor 1 during neonatal cardiac development of the rat. *Basic Res Cardiol* 2005; **100**: 263-269 [PMID: 15754086 DOI: 10.1007/s00395-005-0520-0]

172 **Lehman JJ**, Barger PM, Kovacs A, Saffitz JE, Medeiros DM, Kelly DP. Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. *J Clin Invest* 2000; **106**: 847-856 [PMID: 11018072 DOI: 10.1172/JCI10268]

173 **Chung S**, Dzeja PP, Faustino RS, Perez-Terzic C, Behfar A, Terzic A. Mitochondrial oxidative metabolism is required for the cardiac differentiation of stem cells. *Nat Clin Pract Cardiovasc Med* 2007; **4** Suppl 1: S60-S67 [PMID: 17230217 DOI: 10.1038/ncpcardio0766]

174 **Dai DF**, Danoviz ME, Wiczer B, Laflamme MA, Tian R. Mitochondrial Maturation in Human Pluripotent Stem Cell Derived Cardiomyocytes. *Stem Cells Int* 2017; **2017**: 5153625 [PMID: 28421116 DOI: 10.1155/2017/5153625]

175 **Birket MJ**, Casini S, Kosmidis G, Elliott DA, Gerencser AA, Baartscheer A, Schumacher C, Mastroberardino PG, Elefanty AG, Stanley EG, Mummery CL. PGC-1α and reactive oxygen species regulate human embryonic stem cell-derived cardiomyocyte function. *Stem Cell Reports* 2013; **1**: 560-574 [PMID: 24371810 DOI: 10.1016/j.stemcr.2013.11.008]

176 **Malandraki-Miller S**, Lopez CA, Alonaizan R, Purnama U, Perbellini F, Pakzad K, Carr CA. Metabolic flux analyses to assess the differentiation of adult cardiac progenitors after fatty acid supplementation. *Stem Cell Res* 2019; **38**: 101458 [PMID: 31102832 DOI: 10.1016/j.scr.2019.101458]

177 **Correia C**, Koshkin A, Duarte P, Hu D, Teixeira A, Domian I, Serra M, Alves PM. Distinct carbon sources affect structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Sci Rep* 2017; **7**: 8590 [PMID: 28819274 DOI: 10.1038/s41598-017-08713-4]

178 **Pakzad KK**, Tan JJ, Anderson S, Board M, Clarke K, Carr CA. Metabolic maturation of differentiating cardiosphere-derived cells. *Stem Cell Res* 2021; 54: 102422 [PMID: 34118565 DOI: 10.1016/j.scr.2021.102422]

179 **Lopaschuk GD**, Ussher JR, Folmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. *Physiol Rev* 2010; **90**: 207-258 [PMID: 20086077 DOI: 10.1152/physrev.00015.2009]

180 **Schnelle M**, Chong M, Zoccarato A, Elkenani M, Sawyer GJ, Hasenfuss G, Ludwig C, Shah AM. In vivo [U-^{13}C]glucose labeling to assess heart metabolism in murine models of pressure and volume overload. *Am J Physiol Heart Circ Physiol* 2020; **319**: H422-H431 [PMID: 32648823 DOI: 10.1152/ajpheart.00219.2020]

181 **Allen DK**, Young JD. Tracing metabolic flux through time and space with isotope labeling experiments. *Curr Opin Biotechnol* 2020; **64**: 92-100 [PMID: 31864070 DOI: 10.1016/j.copbio.2019.11.003]

182 **Mookerjee SA**, Goncalves RLS, Gerencser AA, Nicholls DG, Brand MD. The contributions of respiration and glycolysis to extracellular acid production. *Biochim Biophys Acta* 2015; **1847**: 171-181 [DOI: 10.1016/j.bbabio.2014.10.005]

183 **Plitzko B**, Loesgen S. Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in culture cells for assessment of the energy metabolism. *Bio Protoc* 2018; **8**: e2850 [PMID: 34285967 DOI: 10.21769/BioProtoc.2850]

184 **House A**, Fatica E, Shah R, Stergar J, Pearce R, Sandlers Y. A protocol for metabolic characterization of human induced pluripotent stem cell-derived cardiomyocytes (iPS-CM). *MethodsX* 2020; **7**: 100572 [PMID: 32140435 DOI: 10.1016/j.mex.2019.05.028]

185 **Rajamohan D**, Kalra S, Duc Hoang M, George V, Staniforth A, Russell H, Yang X, Denning C. Automated Electrophysiological and Pharmacological Evaluation of Human Pluripotent Stem Cell-Derived Cardiomyocytes. *Stem Cells Dev* 2016; **25**: 439-452 [PMID: 26906236 DOI: 10.1089/scd.2015.0253]

186 **Sirenko O**, Crittenden C, Callamaras N, Hesley J, Chen YW, Funes C, Rusyn I, Anson B, Cromwell EF. Multiparameter in vitro assessment of compound effects on cardiomyocyte physiology using iPSC cells. *J Biomol Screen* 2013; **18**: 39-53 [PMID: 22972846 DOI: 10.1177/1087057112457590]

187 **Mioulane M**, Foldes G, Ali NN, Schneider MD, Harding SE. Development of high content imaging methods for cell death detection in human pluripotent stem cell-derived cardiomyocytes. *J Cardiovasc Transl Res* 2012; **5**: 593-604 [PMID: 22896035 DOI: 10.1007/s12265-012-9396-1]

188 **Gupte SA**. Glucose-6-phosphate dehydrogenase: a novel therapeutic target in cardiovascular diseases. *Curr Opin Investig Drugs* 2008; **9**: 993-1000 [PMID: 18729006]

189 **Jain M**, Cui L, Brenner DA, Wang B, Handy DE, Leopold JA, Loscalzo J, Apstein CS, Liao R. Increased myocardial dysfunction after ischemia-reperfusion in mice lacking glucose-6-phosphate dehydrogenase. *Circulation* 2004; **109**: 898-903 [PMID: 14757696 DOI: 10.1161/01.CIR.0000112605.43318.CA]

190 **Katare R**, Oikawa A, Cesselli D, Beltrami AP, Avolio E, Muthukrishnan D, Munasinghe PE, Angelini G, Emanueli C, Madeddu P. Boosting the pentose phosphate pathway restores cardiac progenitor cell availability in diabetes. *Cardiovasc Res* 2013; **97**: 55-65 [PMID: 22997160 DOI: 10.1093/cvr/cvs291]

191 **Doss MX**, Gaspar JA, Winkler J, Hescheler J, Schulz H, Sachinidis A. Specific gene signatures and pathways in mesodermal cells and their derivatives derived from embryonic stem cells. *Stem Cell Rev Rep* 2012; **8**: 43-54 [PMID: 21519850 DOI: 10.1007/s12015-011-9263-5]

192 **Semenza GL**. Hypoxia-inducible factor 1: regulator of mitochondrial metabolism and mediator of ischemic preconditioning. *Biochim Biophys Acta* 2011; **1813**: 1263-1268 [PMID: 20732359 DOI: 10.1016/j.bbamcr.2010.08.006]

193 **Bayda S**, Adeel M, Tuccinardi T, Cordani M, Rizzolio F. The History of Nanoscience and Nanotechnology: From Chemical-Physical Applications to Nanomedicine. *Molecules* 2019; **25** [PMID: 31892180 DOI: 10.3390/molecules25010112]

194 **Wang M**, Thanou M. Targeting nanoparticles to cancer. *Pharmacol Res* 2010; **62**: 90-99 [PMID: 20380880 DOI: 10.1016/j.phrs.2010.03.005]

195 **Afifi MM**, El Sheikh SM, Abdelsalam MM, Ramadan H, Omar TA, El Tantawi M, Abdel-Razek KM, Mohamed M. Therapeutic efficacy of plasmonic photothermal nanoparticles in hamster buccal pouch carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2013; **115**: 743-751 [PMID: 23454046 DOI: 10.1016/j.oooo.2012.11.020]

196 **Mohamed MM**, Raslan HS, Ramadan OR, Rafik ST, Awaad AK, Essawy MM. Biocompatible Luminescent Nanosized Curcumin: Verified Parameters Affecting Stability and Bioavailability. *Int J Dentistry Oral Sci* 2020; **7**: 1000-1006 [DOI: 10.19070/2377-8075-20000238]

197 **Essawy MM**, El-Sheikh SM, Raslan HS, Ramadan HS, Kang B, Talaat IM, Afifi MM. Function of gold nanoparticles in oral cancer beyond drug delivery: Implications in cell apoptosis. *Oral Dis* 2021; **27**: 251-265 [PMID: 32657515 DOI: 10.1111/odi.13551]

198 **Chavakis E**, Urbich C, Dimmeler S. Homing and engraftment of progenitor cells: a prerequisite for cell therapy. *J Mol Cell Cardiol* 2008; **45**: 514-522 [PMID: 18304573 DOI: 10.1016/j.yjmcc.2008.01.004]

199 **Sun Y**, Lu Y, Yin L, Liu Z. The Roles of Nanoparticles in Stem Cell-Based Therapy for Cardiovascular Disease. *Front Bioeng Biotechnol* 2020; **8**: 947 [PMID: 32923434 DOI: 10.3389/fbioe.2020.00947]

200 **Kingsley JD**, Ranjan S, Dasgupta N, Saha P. Nanotechnology for tissue engineering: need, techniques and applications. *J Pharm Res* 2013; **7**: 200-204 [DOI: 10.1016/j.jopr.2013.02.021]

201 **Zong X**, Bien H, Chung CY, Yin L, Fang D, Hsiao BS, Chu B, Entcheva E. Electrospun fine-textured scaffolds for heart tissue constructs. *Biomaterials* 2005; **26**: 5330-5338 [PMID: 15814131 DOI: 10.1016/j.biomaterials.2005.01.052]

202 **Liu Q**, Tian S, Zhao C, Chen X, Lei I, Wang Z, Ma PX. Porous nanofibrous poly(L-lactic acid) scaffolds supporting cardiovascular progenitor cells for cardiac tissue engineering. *Acta Biomater* 2015; **26**: 105-114 [PMID: 26283164 DOI: 10.1016/j.actbio.2015.08.017]

203 **Li J**, Minami I, Shiozaki M, Yu L, Yajima S, Miyagawa S, Shiba Y, Morone N, Fukushima S, Yoshioka M, Li S, Qiao J, Li X, Wang L, Kotera H, Nakatsuji N, Sawa Y, Chen Y, Liu L. Human Pluripotent Stem Cell-Derived Cardiac Tissue-like Constructs for Repairing the Infarcted Myocardium. *Stem Cell Reports* 2017; **9**: 1546-1559 [PMID: 29107590 DOI: 10.1016/j.stemcr.2017.09.007]

204 **Zhang Y**, Fan W, Wang K, Wei H, Zhang R, Wu Y. Novel preparation of Au nanoparticles loaded Laponite nanoparticles/ECM injectable hydrogel on cardiac differentiation of resident cardiac stem cells to cardiomyocytes. *J Photochem Photobiol B* 2019; **192**: 49-54 [PMID: 30682654 DOI: 10.1016/j.jphotobiol.2018.12.022]

205 **Strebhardt K**, Ullrich A. Paul Ehrlich's magic bullet concept: 100 years of progress. *Nat Rev Cancer* 2008; **8**: 473-480 [PMID: 18469827 DOI: 10.1038/nrc2394]

206 **Naahidi S**, Jafari M, Edalat F, Raymond K, Khademhosseini A, Chen P. Biocompatibility of engineered nanoparticles for drug delivery. *J Control Release* 2013; **166**: 182-194 [PMID: 23262199 DOI: 10.1016/j.jconrel.2012.12.013]

207 **Padin-Iruegas ME**, Misao Y, Davis ME, Segers VF, Esposito G, Tokunou T, Urbanek K, Hosoda T, Rota M, Anversa P, Leri A, Lee RT, Kajstura J. Cardiac progenitor cells and biotinylated insulin-like growth factor-1 nanofibers improve endogenous and exogenous myocardial regeneration after infarction. *Circulation* 2009; **120**: 876-887 [PMID: 19704095 DOI: 10.1161/CIRCULATIONAHA.109.852285]

208 **Tokunaga M**, Liu ML, Nagai T, Iwanaga K, Matsuura K, Takahashi T, Kanda M, Kondo N, Wang P, Naito AT, Komuro I. Implantation of cardiac progenitor cells using self-assembling peptide improves cardiac function after myocardial infarction. *J Mol Cell Cardiol* 2010; **49**: 972-983 [PMID: 20869968 DOI: 10.1016/j.yjmcc.2010.09.015]

209 **Tang J**, Shen D, Caranasos TG, Wang Z, Vandergriff AC, Allen TA, Hensley MT, Dinh PU, Cores J, Li TS, Zhang J, Kan Q, Cheng K. Therapeutic microparticles functionalized with biomimetic cardiac stem cell membranes and secretome. *Nat Commun* 2017; **8**: 13724 [PMID: 28045024 DOI: 10.1038/ncomms13724]

210 **Mazrouaa AM**, Mohamed MG, Fekry M. Physical and magnetic properties of iron oxide nanoparticles with a different molar ratio of ferrous and ferric. *Egypt J Petrol* 2019; **28**: 165-171 [DOI: 10.1016/j.ejpe.2019.02.002]

211 **Dai W**, Hale SL, Kay GL, Jyrala AJ, Kloner RA. Delivering stem cells to the heart in a collagen matrix reduces relocation of cells to other organs as assessed by nanoparticle technology. *Regen Med* 2009; **4**: 387-395 [DOI: 10.2217/rme.09.2]

212 **Cheng K**, Malliaras K, Li TS, Sun B, Houde C, Galang G, Smith J, Matsushita N, Marbán E. Magnetic enhancement of cell retention, engraftment, and functional benefit after intracoronary delivery of cardiac-derived stem cells in a rat model of ischemia/reperfusion. *Cell Transplant* 2012; **21**: 1121-1135 [PMID: 22405128 DOI: 10.3727/096368911X627381]

213 **Vandergriff AC**, Hensley TM, Henry ET, Shen D, Anthony S, Zhang J, Cheng K. Magnetic targeting of cardiosphere-derived stem cells with ferumoxytol nanoparticles for treating rats with myocardial infarction. *Biomaterials* 2014; **35**: 8528-8539 [PMID: 25043570 DOI: 10.1016/j.biomaterials.2014.06.031]

214 **Sukhanova A**, Bozrova S, Sokolov P, Berestovoy M, Karaulov A, Nabiev I. Dependence of Nanoparticle Toxicity on Their Physical and Chemical Properties. *Nanoscale Res Lett* 2018; **13**: 44 [PMID: 29417375 DOI: 10.1186/s11671-018-2457-x]

215 **Oh K**, Kim SR, Kim DK, Seo MW, Lee C, Lee HM, Oh JE, Choi EY, Lee DS, Gho YS, Park KS. In Vivo Differentiation of Therapeutic Insulin-Producing Cells from Bone Marrow Cells *via* Extracellular Vesicle-Mimetic Nanovesicles. *ACS Nano* 2015; **9**: 11718-11727 [PMID: 26513554 DOI: 10.1021/acsnano.5b02997]

216 **Jo W**, Jeong D, Kim J, Park J. Self-Renewal of Bone Marrow Stem Cells by Nanovesicles Engineered from Embryonic Stem Cells. *Adv Healthc Mater* 2016; **5**: 3148-3156 [PMID: 27860451 DOI: 10.1002/adhm.201600810]

217 **Lee JR**, Park BW, Kim J, Choo YW, Kim HY, Yoon JK, Kim H, Hwang JW, Kang M, Kwon SP, Song SY, Ko IO, Park JA, Ban K, Hyeon T, Park HJ, Kim BS. Nanovesicles derived from iron oxide nanoparticles-incorporated mesenchymal stem cells for cardiac repair. *Sci Adv* 2020; **6**: eaaz0952 [PMID: 32494669 DOI: 10.1126/sciadv.aaz0952]

218 **Kojima R**, Bojar D, Rizzi G, Hamri GC, El-Baba MD, Saxena P, Ausländer S, Tan KR, Fussenegger M. Designer exosomes produced by implanted cells intracerebrally deliver therapeutic cargo for Parkinson's disease treatment. *Nat Commun* 2018; **9**: 1305 [PMID: 29610454 DOI: 10.1038/s41467-018-03733-8]

219 **von Harsdorf R**, Poole-Wilson PA, Dietz R. Regenerative capacity of the myocardium: implications for treatment of heart failure. *Lancet* 2004; **363**: 1306-1313 [PMID: 15094278 DOI: 10.1016/S0140-6736(04)16006-6]

220 **Laflamme MA**, Zbinden S, Epstein SE, Murry CE. Cell-based therapy for myocardial ischemia and infarction: pathophysiological mechanisms. *Annu Rev Pathol* 2007; **2**: 307-339 [PMID: 18039102 DOI: 10.1146/annurev.pathol.2.010506.092038]

221 **Malliaras K**, Makkar RR, Smith RR, Cheng K, Wu E, Bonow RO, Marbán L, Mendizabal A, Cingolani E, Johnston PV, Gerstenblith G, Schuleri KH, Lardo AC, Marbán E. Intracoronary cardiosphere-derived cells after myocardial infarction: evidence of therapeutic regeneration in the final 1-year results of the CADUCEUS trial (CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction). *J Am Coll Cardiol* 2014; **63**: 110-122 [PMID: 24036024 DOI: 10.1016/j.jacc.2013.08.724]

222 **Makkar RR**, Kereiakes DJ, Aguirre F, Kowalchuk G, Chakravarty T, Malliaras K, Francis GS, Povsic TJ, Schatz R, Traverse JH, Pogoda JM, Smith RR, Marbán L, Ascheim DD, Ostovaneh MR, Lima JAC, DeMaria A, Marbán E, Henry TD. Intracoronary ALLogeneic heart STem cells to Achieve myocardial Regeneration (ALLSTAR): a randomized, placebo-controlled, double-blinded trial. *Eur Heart J* 2020; **41**: 3451-3458 [PMID: 32749459 DOI: 10.1093/eurheartj/ehaa541]

223 **Menasché P**, Vanneaux V, Hagège A, Bel A, Cholley B, Parouchev A, Cacciapuoti I, Al-Daccak R, Benhamouda N, Blons H, Agbulut O, Tosca L, Trouvin JH, Fabreguettes JR, Bellamy V, Charron D, Tartour E, Tachdjian G, Desnos M, Larghero J. Transplantation of Human Embryonic Stem Cell-Derived Cardiovascular Progenitors for Severe Ischemic Left Ventricular Dysfunction. *J Am Coll*

Cardiol 2018; **71**: 429-438 [PMID: 29389360 DOI: 10.1016/j.jacc.2017.11.047]

224 **Mirotsou M**, Jayawardena TM, Schmeckpeper J, Gnecchi M, Dzau VJ. Paracrine mechanisms of stem cell reparative and regenerative actions in the heart. *J Mol Cell Cardiol* 2011; **50**: 280-289 [PMID: 20727900 DOI: 10.1016/j.yjmcc.2010.08.005]

225 **Lalit PA**, Salick MR, Nelson DO, Squirrell JM, Shafer CM, Patel NG, Saeed I, Schmuck EG, Markandeya YS, Wong R, Lea MR, Eliceiri KW, Hacker TA, Crone WC, Kyba M, Garry DJ, Stewart R, Thomson JA, Downs KM, Lyons GE, Kamp TJ. Lineage Reprogramming of Fibroblasts into Proliferative Induced Cardiac Progenitor Cells by Defined Factors. *Cell Stem Cell* 2016; **18**: 354-367 [PMID: 26877223 DOI: 10.1016/j.stem.2015.12.001]

226 **Witman N**, Sahara M. Cardiac Progenitor Cells in Basic Biology and Regenerative Medicine. *Stem Cells Int* 2018; **2018**: 8283648 [PMID: 29535783 DOI: 10.1155/2018/8283648]

227 **Sanz-Ruiz R**, Casado Plasencia A, Borlado LR, Fernández-Santos ME, Al-Daccak R, Claus P, Palacios I, Sádaba R, Charron D, Bogaert J, Mulet M, Yotti R, Gilaberte I, Bernad A, Bermejo J, Janssens S, Fernández-Avilés F. Rationale and Design of a Clinical Trial to Evaluate the Safety and Efficacy of Intracoronary Infusion of Allogeneic Human Cardiac Stem Cells in Patients With Acute Myocardial Infarction and Left Ventricular Dysfunction: The Randomized Multicenter Double-Blind Controlled CAREMI Trial (Cardiac Stem Cells in Patients With Acute Myocardial Infarction). *Circ Res* 2017; **121**: 71-80 [PMID: 28533209 DOI: 10.1161/CIRCRESAHA.117.310651]

228 **Victor RG**. Significant Improvements Reported in Duchenne Muscular Dystrophy Patients Treated with Capricor's Investigational Cell Therapy. Capricor Therapeutics, Inc. 2017. [cited 26 February 2021] Available from: http://www.prnewswire.com/news-releases/significant-improvements-reported-in-duchenne-muscular-dystrophy-patients-treated-with-capricors-investigational-cell-therapy-300556679.html

229 **Takehara N**, Tsutsumi Y, Tateishi K, Ogata T, Tanaka H, Ueyama T, Takahashi T, Takamatsu T, Fukushima M, Komeda M, Yamagishi M, Yaku H, Tabata Y, Matsubara H, Oh H. Controlled delivery of basic fibroblast growth factor promotes human cardiosphere-derived cell engraftment to enhance cardiac repair for chronic myocardial infarction. *J Am Coll Cardiol* 2008; **52**: 1858-1865 [PMID: 19038683 DOI: 10.1016/j.jacc.2008.06.052]

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

231 **Ishigami S**, Ohtsuki S, Tarui S, Ousaka D, Eitoku T, Kondo M, Okuyama M, Kobayashi J, Baba K, Arai S, Kawabata T, Yoshizumi K, Tateishi A, Kuroko Y, Iwasaki T, Sato S, Kasahara S, Sano S, Oh H. Intracoronary autologous cardiac progenitor cell transfer in patients with hypoplastic left heart syndrome: the TICAP prospective phase 1 controlled trial. *Circ Res* 2015; **116**: 653-664 [PMID: 25403163 DOI: 10.1161/CIRCRESAHA.116.304671]

232 **Ishigami S**, Ohtsuki S, Eitoku T, Ousaka D, Kondo M, Kurita Y, Hirai K, Fukushima Y, Baba K, Goto T, Horio N, Kobayashi J, Kuroko Y, Kotani Y, Arai S, Iwasaki T, Sato S, Kasahara S, Sano S, Oh H. Intracoronary Cardiac Progenitor Cells in Single Ventricle Physiology: The PERSEUS (Cardiac Progenitor Cell Infusion to Treat Univentricular Heart Disease) Randomized Phase 2 Trial. *Circ Res* 2017; **120**: 1162-1173 [PMID: 28052915 DOI: 10.1161/CIRCRESAHA.116.310253]

233 **Tarui S**, Ishigami S, Ousaka D, Kasahara S, Ohtsuki S, Sano S, Oh H. Transcoronary infusion of cardiac progenitor cells in hypoplastic left heart syndrome: Three-year follow-up of the Transcoronary Infusion of Cardiac Progenitor Cells in Patients With Single-Ventricle Physiology (TICAP) trial. *J Thorac Cardiovasc Surg* 2015; **150**: 1198-1207, 1208.e1 [PMID: 26232942 DOI: 10.1016/j.jtcvs.2015.06.076]

234 **Bergmann O**, Zdunek S, Felker A, Salehpour M, Alkass K, Bernard S, Sjostrom SL, Szewczykowska M, Jackowska T, Dos Remedios C, Malm T, Andrä M, Jashari R, Nyengaard JR, Possnert G, Jovinge S, Druid H, Frisén J. Dynamics of Cell Generation and Turnover in the Human Heart. *Cell* 2015; **161**: 1566-1575 [PMID: 26073943 DOI: 10.1016/j.cell.2015.05.026]

235 **Doppler SA**, Deutsch MA, Lange R, Krane M. Cardiac regeneration: current therapies-future concepts. *J Thorac Dis* 2013; **5**: 683-697 [PMID: 24255783 DOI: 10.3978/j.issn.2072-1439.2013.08.71]

236 **Hatzistergos KE**, Quevedo H, Oskouei BN, Hu Q, Feigenbaum GS, Margitich IS, Mazhari R, Boyle AJ, Zambrano JP, Rodriguez JE, Dulce R, Pattany PM, Valdes D, Revilla C, Heldman AW, McNiece I, Hare JM. Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. *Circ Res* 2010; **107**: 913-922 [PMID: 20671238 DOI: 10.1161/CIRCRESAHA.110.222703]

237 **Williams AR**, Hatzistergos KE, Addicott B, McCall F, Carvalho D, Suncion V, Morales AR, Da Silva J, Sussman MA, Heldman AW, Hare JM. Enhanced effect of combining human cardiac stem cells and bone marrow mesenchymal stem cells to reduce infarct size and to restore cardiac function after myocardial infarction. *Circulation* 2013; **127**: 213-223 [PMID: 23224061 DOI: 10.1161/CIRCULATIONAHA.112.131110]

238 **Karantalis V**, Suncion-Loescher VY, Bagno L, Golpanian S, Wolf A, Sanina C, Premer C, Kanelidis AJ, McCall F, Wang B, Balkan W, Rodriguez J, Rosado M, Morales A, Hatzistergos K,

Natsumeda M, Margitich I, Schulman IH, Gomes SA, Mushtaq M, DiFede DL, Fishman JE, Pattany P, Zambrano JP, Heldman AW, Hare JM. Synergistic Effects of Combined Cell Therapy for Chronic Ischemic Cardiomyopathy. *J Am Coll Cardiol* 2015; **66**: 1990-1999 [PMID: 26516002 DOI: 10.1016/j.jacc.2015.08.879]

239 **Natsumeda M**, Florea V, Rieger AC, Tompkins BA, Banerjee MN, Golpanian S, Fritsch J, Landin AM, Kashikar ND, Karantalis V, Loescher VY, Hatzistergos KE, Bagno L, Sanina C, Mushtaq M, Rodriguez J, Rosado M, Wolf A, Collon K, Vincent L, Kanelidis AJ, Schulman IH, Mitrani R, Heldman AW, Balkan W, Hare JM. A Combination of Allogeneic Stem Cells Promotes Cardiac Regeneration. *J Am Coll Cardiol* 2017; **70**: 2504-2515 [PMID: 29145950 DOI: 10.1016/j.jacc.2017.09.036]

240 **Zhang Z**, Yang J, Yan W, Li Y, Shen Z, Asahara T. Pretreatment of Cardiac Stem Cells With Exosomes Derived From Mesenchymal Stem Cells Enhances Myocardial Repair. *J Am Heart Assoc* 2016; **5** [PMID: 26811168 DOI: 10.1161/jaha.115.002856]

241 **Quijada P**, Salunga HT, Hariharan N, Cubillo JD, El-Sayed FG, Moshref M, Bala KM, Emathinger JM, De La Torre A, Ormachea L, Alvarez R Jr, Gude NA, Sussman MA. Cardiac Stem Cell Hybrids Enhance Myocardial Repair. *Circ Res* 2015; **117**: 695-706 [PMID: 26228030 DOI: 10.1161/CIRCRESAHA.115.306838]

242 **Firouzi F**, Sinha Choudhury S, Broughton K, Salazar A, Bailey B, Sussman MA. Human CardioChimeras: Creation of a Novel "Next-Generation" Cardiac Cell. *J Am Heart Assoc* 2020; **9**: e013452 [PMID: 31902324 DOI: 10.1161/JAHA.119.013452]

243 **Monsanto MM**, Wang BJ, Ehrenberg ZR, Echeagaray O, White KS, Alvarez R Jr, Fisher K, Sengphanith S, Muliono A, Gude NA, Sussman MA. Enhancing myocardial repair with CardioClusters. *Nat Commun* 2020; **11**: 3955 [PMID: 32769998 DOI: 10.1038/s41467-020-17742-z]

244 **Yokoyama R**, Ii M, Masuda M, Tabata Y, Hoshiga M, Ishizaka N, Asahi M. Cardiac Regeneration by Statin-Polymer Nanoparticle-Loaded Adipose-Derived Stem Cell Therapy in Myocardial Infarction. *Stem Cells Transl Med* 2019; **8**: 1055-1067 [PMID: 31157513 DOI: 10.1002/sctm.18-0244]

245 **Magouroux T**, Extermann J, Hoffmann P, Mugnier Y, Le Dantec R, Jaconi ME, Kasparian C, Ciepielewski D, Bonacina L, Wolf JP. High-speed tracking of murine cardiac stem cells by harmonic nanodoublers. *Small* 2012; **8**: 2752-2756 [PMID: 22859385 DOI: 10.1002/smll.201200366]

World Journal of Stem Cells

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

World J Stem Cells 2022 January 26; 14(1): 41-53

DOI: 10.4252/wjsc.v14.i1.41

ISSN 1948-0210 (online)

REVIEW

Multiple roles of mothers against decapentaplegic homolog 4 in tumorigenesis, stem cells, drug resistance, and cancer therapy

Chuan-Jing Dai, Yu-Ting Cao, Fang Huang, Yi-Gang Wang

ORCID number: Chuan-Jing Dai 0000-0003-0760-1418; Yu-Ting Cao 0000-0002-4044-1244; Fang Huang 0000-0002-3115-8703; Yi-Gang Wang 0000-0003-4546-8179.

Author contributions: Dai CJ wrote the paper; Cao YT and Huang F wrote the partial manuscript, and contributed equally to this work; Wang YG designed the layout of the review and edited the manuscript.

Conflict-of-interest statement: The authors do not have any possible conflicts of interest to disclose.

Supported by the National Natural Science Foundation of China, No. 8180306; the Natural Science Foundation of Zhejiang Province, No. LY18C070002; and the 521 Talent Project of Zhejiang Sci-Tech University, No. 2021437620 and No. 2019337459.

Country/Territory of origin: China

Specialty type: Cell and tissue engineering

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0

Chuan-Jing Dai, Yu-Ting Cao, Yi-Gang Wang, College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou 310018, Zhejiang Province, China

Fang Huang, Department of Pathology, Zhejiang Provincial People's Hospital of Hangzhou Medical University, Hangzhou 310014, Zhejiang Province, China

Corresponding author: Yi-Gang Wang, PhD, Professor, College of Life Sciences and Medicine, Zhejiang Sci-Tech University, No. 2 Street of Xiasha District, Hangzhou 310018, Zhejiang Province, China. wangyigang43@163.com

Abstract

The transforming growth factor (TGF)-β signaling pathway controls many cellular processes, including proliferation, differentiation, and apoptosis. Abnormalities in the TGF-β signaling pathway and its components are closely related to the occurrence of many human diseases, including cancer. Mothers against decapentaplegic homolog 4 (Smad4), also known as deleted in pancreatic cancer locus 4, is a typical tumor suppressor candidate gene locating at q21.1 of human chromosome 18 and the common mediator of the TGF-β/Smad and bone morphogenetic protein/Smad signaling pathways. It is believed that Smad4 inactivation correlates with the development of tumors and stem cell fate decisions. Smad4 also interacts with cytokines, miRNAs, and other signaling pathways, jointly regulating cell behavior. However, the regulatory function of Smad4 in tumorigenesis, stem cells, and drug resistance is currently controversial. In addition, Smad4 represents an attractive therapeutic target for cancer. Elucidating the specific role of Smad4 is important for understanding the mechanism of tumorigenesis and cancer treatment. Here, we review the identification and characterization of Smad4, the canonical TGF-β/Smad pathway, as well as the multiple roles of Smad4 in tumorigenesis, stem cells, and drug resistance. Furthermore, we provide novel insights into the prospects of Smad4-targeted cancer therapy and the challenges that it will face in the future.

Key Words: Cancer therapy; Drug resistance; Mothers against decapentaplegic homolog 4; Stemness; Transforming growth factor-β; Tumorigenesis

Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 16, 2021
Peer-review started: March 16, 2021
First decision: May 5, 2021
Revised: May 13, 2021
Accepted: December 21, 2021
Article in press: December 21, 2021
Published online: January 26, 2022

P-Reviewer: Zhou P
S-Editor: Wang JJ
L-Editor: Wang TQ
P-Editor: Wang JJ

Core Tip: Mothers against decapentaplegic homolog 4 (Smad4) is regarded as a tumor suppressor. Recent studies have shown that Smad4 plays a tumor-promoting role in specific types of cancer, rather than a tumor-suppressing role. Smad4 also correlates with the stem cells fate and drug resistance of cancer cells. Elucidating the specific role of Smad4 is of positive guiding significance for understanding the mechanism of tumorigenesis and cancer treatment. In this review, we focus on the multiple roles of Smad4 in tumorigenesis, stem cells, and drug resistance, and provide novel insights into the prospect of Smad4 in combination therapy.

Citation: Dai CJ, Cao YT, Huang F, Wang YG. Multiple roles of mothers against decapentaplegic homolog 4 in tumorigenesis, stem cells, drug resistance, and cancer therapy. *World J Stem Cells* 2022; 14(1): 41-53
URL: https://www.wjgnet.com/1948-0210/full/v14/i1/41.htm
DOI: https://dx.doi.org/10.4252/wjsc.v14.i1.41

INTRODUCTION

The transforming growth factor (TGF)-β signaling pathway controls several cell behaviors, including proliferation, inflammation, differentiation, and apoptosis[1,2]. Abnormalities of the TGF-β signaling pathway and its components are related to many human diseases such as fibrosis[3], immune diseases[4], and cancer[5]. Although TGF-β signals are mainly transmitted to the nucleus through the TGF-β/mothers against decapentaplegic homolog (Smad) signaling pathway[6], TGF-β superfamily ligands often interact with other signaling pathways, including JNK/p38, PI3K/AKT, ERK/MAPK, and integrin signaling pathways, regulating various cellular responses in a non-Smad-dependent manner[7-9]. The function of TGF-β in tumorigenesis is frequently described as a double-edged sword, but the precise mechanism for this phenomenon is still unclear[5,10,11]. In addition, TGF-β plays a role in the development of cardiac[12] and kidney[3] fibrosis and interacts with integrins to mediate asthmatic remodeling of the airway[13]. Moreover, TGF-β exerts an immunosuppressive function[14]. In recent years, studies on TGF-β have been focused on elucidating the regulation and effects of its upstream and downstream components on various diseases, as well as the interaction between different factors and TGF-β[10,15,16]. Understanding of these processes may improve the diagnosis and treatment of these diseases in the future.

Cancer has become a major threat to human health worldwide. In 2020 alone, 19.3 million new cases were diagnosed, and nearly 10 million people died of cancer[17]. Unfortunately, our current understanding of the mechanism of tumorigenesis is not enough to completely overcome cancer[18]. Smad4, a key component of the canonical TGF-β signaling pathway, exhibits varying degrees of inactivation among cancers[19], and its expression is significantly correlated with tumor development and prognosis in cancer patients[20,21]. Therefore, Smad4 is potently regarded as a tumor suppressor. Moreover, considering the critical role of Smad4 in tumor progression, Smad4 can be an attractive target for cancer treatment[22]. Additional novel options for cancer therapy were revealed by demonstrating that many RNAs directly/indirectly targeting Smad4, including miRNAs, circular (circ) RNAs, and long noncoding (lnc) RNAs, are dysregulated during cancer progression[23-25]. In the past decade, it has been found that Smad4 seems to play a tumor-promoting role in certain types of cancer, such as hepatocellular carcinoma[26,27]. In addition, an increasing number of studies demonstrated a close association of Smad4 with stem cell fate[28] and drug resistance of cancer cells[29]. Elucidating the specific role of Smad4 is important for understanding the mechanism of tumorigenesis and cancer treatment. Here, we review the identification and characteristics of Smad4, and the canonical TGF-β signaling pathway, and summarize the multiple regulatory functions of Smad4 in tumorigenesis, stem cell fate, and drug resistance. In addition, we provide new insights into the prospects of Smad4-targeting cancer therapy and its challenges in the future.

IDENTIFICATION AND CHARACTERISTICS OF SMAD4

Smad4, also known as deleted in pancreatic cancer locus 4 (*DPC4*), was initially found as a tumor suppressor candidate gene in human pancreatic carcinoma in 1996[30]. The term "Smad" was a combination of the *sma* gene of *Caenorhabditis elegans* and the *mad* gene of *Drosophila melanogaster*[31]. Subsequently, Smad4 mutations were found in additional types of tumors, such as gastrointestinal carcinoid[32], prostate cancer[33], squamous cell carcinoma[34], and lung cancer[35]. The gene coding for Smad4 is located at human chromosome locus 18q21.1 (Figure 1A) and is composed of 12 exons and 10 introns[36]. The 12th exon was named exon 0 because it was discovered after the identification of 11 exons and is located upstream of exon 1[32].

Smad4 protein consists of 552 amino acids and has a molecular weight of 60 kDa [36]. It is composed of the N-terminal mad homology domain 1 (MH1), the middle linker region including nuclear export signal and Smad activation domain (SAD), as well as the C-terminal MH2[37]. The MH1 domain of Smad4 is involved in DNA binding by recognizing the Smad-binding site of DNA. The SAD of the linker region and MH2 domain are responsible for transcriptional activity. Additionally, the MH2 domain interacts with the MH1 domain of other Smads, enabling the transduction of various signaling pathways, including the TGF-β signaling (Figure 1B).

TGF-β/SMAD AND BMP/SMAD SIGNALING PATHWAYS

The canonical TGF-β signaling pathway is an uncomplicated linear cascade, which involves TGF-β superfamily ligands, receptors, and signal transducers (Figure 2)[38]. At present, there are 33 known TGF-β ligands encoded by mammalian genomes, including activin, nodal, TGF-βs, bone morphogenetic proteins (BMPs), and growth differentiation factors (GDFs)[39,40]. According to the difference in structure and function, these polypeptides can be subdivided into two families: TGF-βs (TGF-β, GDF, nodal, and activin) and BMPs (BMP2, BMP4, and BMP7). The TGF-β type I and type II receptors (TβRI and TβRII) are composed of several pairs of serine/threonine protein kinases[41,42].

The eight known Smads are divided into three categories, including a common Smad (Co-Smad, Smad4), two inhibitory Smads (I-Smads, Smad6, and Smad7), and receptor-regulated Smads (R-Smads, Smad2/3 transducing TGF-β signaling, and Smad1/5/8 transducing BMP signaling). Co-Smad and Smad4/DPC4 bind to R-Smads, forming two different complexes, Smad4/Smad2/3 and Smad4/Smad1/5/8. Subsequently, the heteromeric complexes are translocated into the nucleus, where they interact with transcriptional factors and bind to regulatory elements of target genes, affecting their expression[43]. Smad6 and Smad7 can both inhibit the transcriptional activity of target genes, thus blocking TGF-β signal transmission. It must be emphasized that Smad6 acts in a manner that is not always consistent with that of Smad7[44, 45].

MULTIPLE ROLES OF SMAD4 IN TUMORIGENESIS

Due to the multiple interactions of environmental chemicals, genes, and endogenous signals, the process of carcinogenesis is extremely complex[46]. As a tumor suppressor gene, *Smad4* exerts its inhibitory effect on tumor cells primarily *via* the canonical TGF-β signaling pathway[47,48]. The mechanism of this inhibition, which prevents carcinogenesis, involves the role of Smad4 in inhibiting the tumor-promoting activity of proinflammatory cytokines, inducing the cell cycle arrest, and promoting apoptosis through activating the TGF-β/BMP/Smad4 axis. However, once the *Smad4* gene is mutated, TGF-β cannot induce G1 or G2 cell cycle arrest and switch from tumor suppressor to tumor promoter, leading to tumor growth and metastasis[10,49]. In addition, Smad4 is indispensable for the tumor suppressor function of TGF-β[50]. So far, there are many contradictory results and conclusions about the role of Smad4 in tumorigenesis. Here, we focus on pancreatic cancer and hepatocellular carcinoma (HCC) to discuss the role of Smad4 in cancer progression (Figure 3).

It is recognized that Smad4 acts as a tumor suppressor in pancreatic cancer. *Smad4* mutation or deletion is found in > 50% of pancreatic cancer and is associated with the proliferation and metastasis of tumor cells[51,52]. The alterations in the *Smad4* gene mainly include deletion, frameshift mutation, point mutation, amplification, and translocation[19,22]. Furthermore, *Smad4* gene mutation is associated with stages of

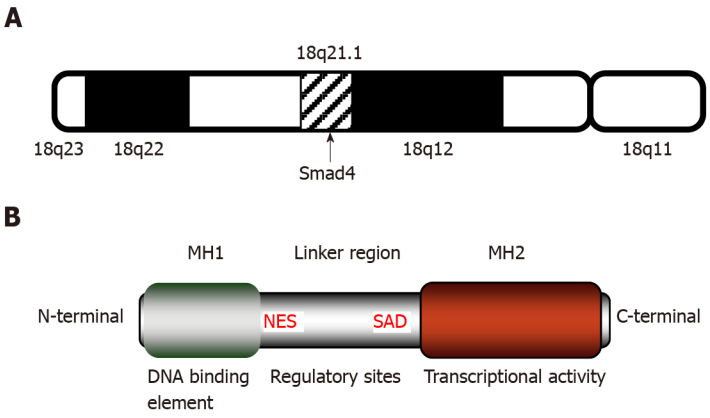

Figure 1 Structure and features of mothers against decapentaplegic homolog 4. A: Location of the mothers against decapentaplegic homolog 4 (*Smad4*) gene. *Smad4* is located at q21.1 of human chromosome 18; B: Structural diagram of Smad4 protein. Smad4 protein consists of three domains: Mad homology domain 1 (MH1) at the N-terminal, MH2 at the C-terminal, and the linker region connecting MH1 and MH2. Functionally, MH1 is responsible for binding to the Smad-binding site, and MH2 is indispensable for transforming growth factor-β transcriptional activity. The middle linker is rich in regulatory sites controlling various signaling pathways and kinases and is essential for the regulation of transcriptional activity. MH1: Mad homology domain 1; MH2: Mad homology domain 2; NES: Nuclear export signal; SAD: Smad activation domain; Smad4: Mothers against decapentaplegic homolog 4.

pancreatic cancer. A study by Notta *et al*[53] showed that the inactivation rate of Smad4 in mid-advanced pancreatic cancer was higher than it in early pancreatic cancer. Therefore, Smad4 dysfunction may be considered an advanced event in pancreatic cancer. Mechanically, the behavior that Smad4 deletion accelerates the progression of pancreatic cancer may be related to the increased expression of HNF4G, PAR-4, and PGK-1[51,54,55]. Additionally, Smad4 mutation in mice does not directly contribute to the formation of pancreatic tumors[56], indicating that a single Smad4 mutation is not sufficient to initiate pancreatic carcinogenesis. Thus, mutations in *Smad4* are likely to cooperate with mutations in other genes in promoting pancreatic cancer progression. For example, Izeradjene *et al*[57] revealed that the formation of mucinous cystic neoplasms is induced by synergistic effects of Kras-G12D and Smad4 mutations. The role of Smad4 in other human cancers, such as colorectal cancer (CRC)[58], gastric cancer[59], ovarian cancer[60] and head and neck squamous cell carcinoma (HNSCC)[61], is similar to that in pancreatic cancer. Downregulation of Smad4 is considered an early event of HNSCC, which is different from pancreatic cancer.

An interesting fact regarding HCC is that the role of Smad4 in HCC differs significantly from that in cancers mentioned above. The nuclear level of Smad4 in liver cancer tissue is markedly higher than that in the adjacent noncancerous tissue[26]. The ability of liver cancer cells to form colonies and migrate was significantly reduced after *Smad4* gene knockout in mouse models. Yuan *et al*[62] showed that ubiquitin-specific proteases promote HCC cell migration and invasion by deubiquitinating and stabilizing Smad4 protein. These findings seem to indicate that Smad4 plays a tumor-promoting rather than tumor-suppressing role in HCC. However, the underlying mechanism of Smad4 in the pathogenesis of HCC remains elusive. We propose that this difference may be explained by the fact that the cellular behaviors of the TGF-β/Smad4 signaling pathway varies with types of cells, their extracellular matrix, TGF-β concentration, and tumor microenvironment. These possibilities warrant further investigation. It is certain that Smad4 plays multiple regulatory roles in carcinogenesis, and whether it has a tumor-promoting or tumor-suppressing function may depend on the microenvironment of tumor cells and surrounding stromal cells.

SMAD4 IN STEM CELLS

Besides tumorigenesis, Smad4 is involved in the self-renewal and pluripotency of human stem cells. It has been reported that several miRNAs targeting Smad4 negatively regulate the differentiation of human mesenchymal stem cells (hMSCs)[63,64], but the underlying mechanisms of stem cell differentiation are poorly understood. The Smad4/TAZ axis might be involved in this process. TAZ protein, a transcriptional coactivator with PDZ-binding motif, can bind to Smad4 protein and translocate to the nucleus, where it enhances the expression of osteogenic genes, promoting the

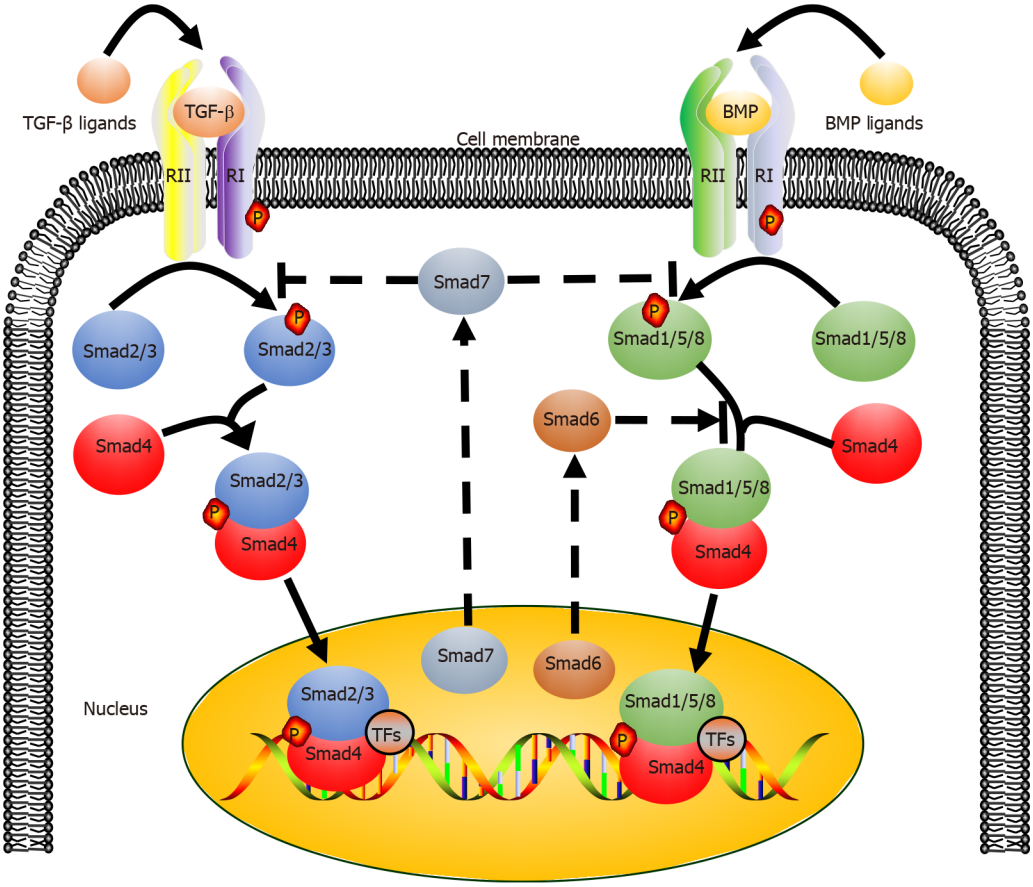

Figure 2 Transforming growth factor-β/mothers against decapentaplegic homolog 4 and bone morphogenetic protein/mothers against decapentaplegic homolog signaling pathways. Activated transforming growth factor-β (TGF-β) ligands or bone morphogenetic protein ligands bind to type II receptor (TβRII) on the cell membrane and recruit type I receptors (TβRI), forming receptor complexes (TβRI and TβRII). Phosphorylated receptor complexes phosphorylate mothers against decapentaplegic homolog (Smad)2/3 and Smad1/5/8 (R-Smads). Smad4 (Co-Smad) binds with R-Smads to form two different complexes (Smad4/Smad2/3 and Smad4/Smad1/5/8). The heteromeric complexes are translocated to the nucleus, where they regulate the expression of specific genes. Smad6 and Smad7 (I-Smads), which are typically located in the nucleus, can enter the cytoplasm upon activation and regulate the transcriptional activity of the TGF-β signaling pathway. Smad6 mainly competes with R-Smads for binding Smad4 to inhibit transcriptional activity, while Smad7 inhibits transcriptional activity mostly by preventing the phosphorylation of R-Smads. BMP: Bone morphogenetic protein; TFs: Transcription factors; Smad1-8: Mothers against decapentaplegic homolog 1-8; TGF-β: Transforming growth factor-β.

osteogenic differentiation of hMSCs[65]. These findings suggest that Smad4 is essential for stem cell differentiation. However, Avery *et al*[28] demonstrated that human embryonic stem cells (hESCs) remain undifferentiated after *Smad4* gene knockdown, indicating that the single event of Smad4 inactivation does not induce stem cell differentiation. Similarly, Smad4 inactivation does not directly induce self-renewal of stem cells. For instance, Smad4 mutation has no effect on hESC self-renewal but is essential for their differentiation into cardiac mesodermal precursors[66].

BMP signaling is also indispensable for regulation of the proliferation of stem cells and maintenance of metabolic homeostasis[67]. BMP binds with leukemia inhibitory factor to maintain the self-renewal of ESCs[68]. Subsequent studies have documented that stem cell fate decisions induced by BMP may be related to Smad4. The BMP/Smad axis regulates the proliferation and differentiation of alveolar stem cells. BMP suppresses proliferation of alveolar type 2 epithelial cells (AT2s), while antagonists promoted the self-renewal of AT2s at the expense of differentiation[67]. BMP restricts the self-renewal of intestinal Lgr5$^+$ stem cells by Smad4-mediated transcriptional repression and thus prevents excessive proliferation of epithelial cells[69]. Therefore, the inactivation of Smad4 may counteract the inhibitory effect of BMP on stem cell proliferation, contributing in this manner to the occurrence of cancer cells.

Cancer stem cells (CSCs) have many characteristics similar to stem cells. However, in contrast to stem cells, they exhibit tumorigenicity and invasiveness[70]. The gradual accumulation of genetic mutations in stem cells during the life of the organism is associated with the development of cancer[71], and results in tumor heterogeneity[72]. Therefore, elucidating the relationship between Smad4 and CSC fate may provide a clinical diagnostic index or potential therapeutic target for cancer. Xia *et al*[73] found

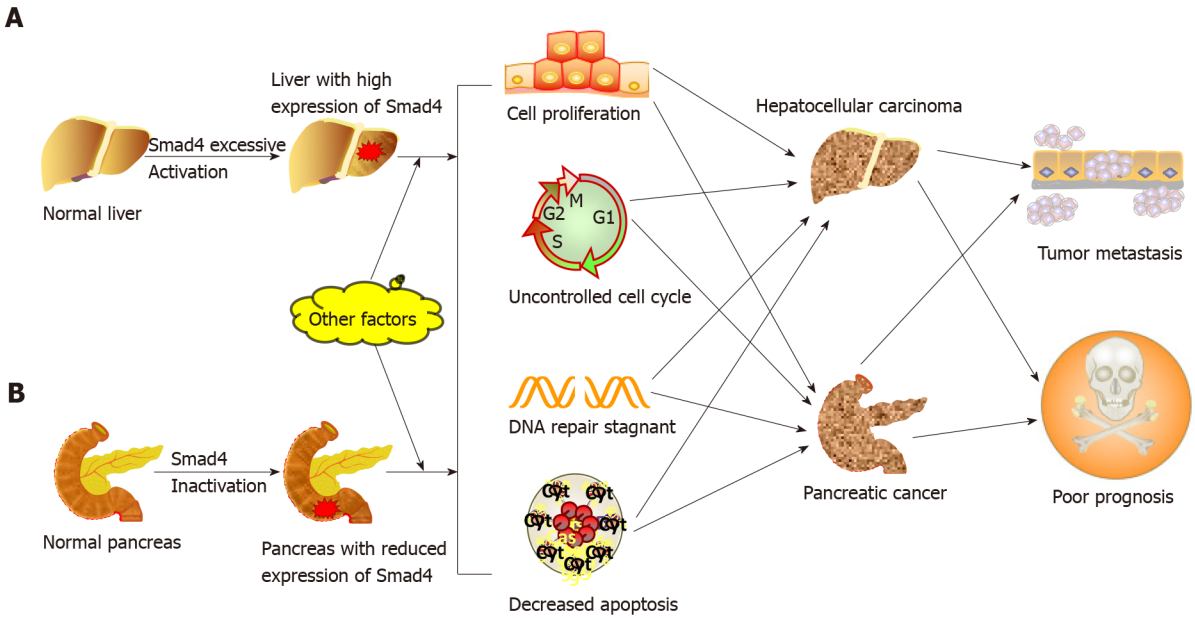

Figure 3 Different roles of mothers against decapentaplegic homolog 4 in the development of hepatocellular carcinoma and pancreatic cancer. A: The role of Smad4 in liver carcinogenesis. During the development of hepatocellular carcinoma, the transcriptional activity of mothers against decapentaplegic homolog 4 (Smad4) is abnormally increased, and in combination with gene mutations, alcohol, pathogen infection, as well as other carcinogenic factors, contributes to the loss of cell cycle control, suppression of DNA damage repair, increased proliferation, and decreased apoptosis. These factors result in a higher metastatic ability of cancer cells and poor patient prognosis; B: The role of Smad4 in pancreatic carcinogenesis. During pancreatic cancer progression, the deletion or mutation of Smad4 leads to loss of Smad4 activity. The absence of Smad4 function, together with other carcinogenic factors such as gene mutations and pancreatic tissue injury, results in the loss of cell cycle control, suppression of DNA damage repair, increased proliferation, and decreased apoptosis. These factors result in a higher metastatic ability of cancer cells and poor patient prognosis. Smad4: Mothers against decapentaplegic homolog 4.

that cyclin D1 interacts with Smad2/3 and Smad4, activating the cyclin D1/Smad pathway and upregulating the expression of stemness genes in liver CSCs. This result implies that the use of Smad inhibitors may be an effective strategy for targeting liver CSCs. Wen *et al*[74] showed that blocking the TGF-β/Smad/EMT pathway inhibited the self-renewal and metastasis of ovarian CSCs. In another study[75], Smad4 mutation was introduced into organoids derived from intestinal epithelium through the CRISPR-Cas9 system. Upon injection of these organoids into the spleen of mice, they formed micrometastases containing invading tumor cells, but they could not colonize the liver, suggesting that Smad4 mutation must be combined with additional factors to promote tumor invasion.

SMAD4 IN DRUG RESISTANCE

Drug resistance is the main cause of chemotherapy failure and tumor recurrence in cancer patients[76]. Drug resistance has been shown to be related to the activation of autophagy[77], a highly conserved catabolic process in which large cellular structures are degraded. Autophagy contributes to cell survival by recycling constituents of cellular structures[78]. Activation of autophagy not only mediates the resistance to chemotherapy but also induces autophagy-mediated cell death, which helps to eliminate tumor cells[52,79]. For instance, autophagy protects breast cancer cells from epirubicin-induced apoptosis and promotes the development of epirubicin resistance [80]. Peptidylarginine deiminase IV, a protein involved in many pathological processes, induces the resistance of HCC to chemotherapy by activating autophagy [81]. Autophagy may promote the development of multidrug resistance. Fortunately, inducing autophagy-mediated cell death can help overcome the resistance to chemotherapy, and there are many clinically available drugs that can regulate autophagy, such as chloroquine or hydroxychloroquine[82]. These autophagy regulators inhibit autophagy mainly by reducing autolysosome fusion[83]. In addition, TGF-β activates autophagy activation by both Smad and non-Smad pathways, promoting the survival of cancer cells[84,85]. Thus, Smad4 plays an indispensable role in TGF-β-induced autophagy and drug resistance (Figure 4).

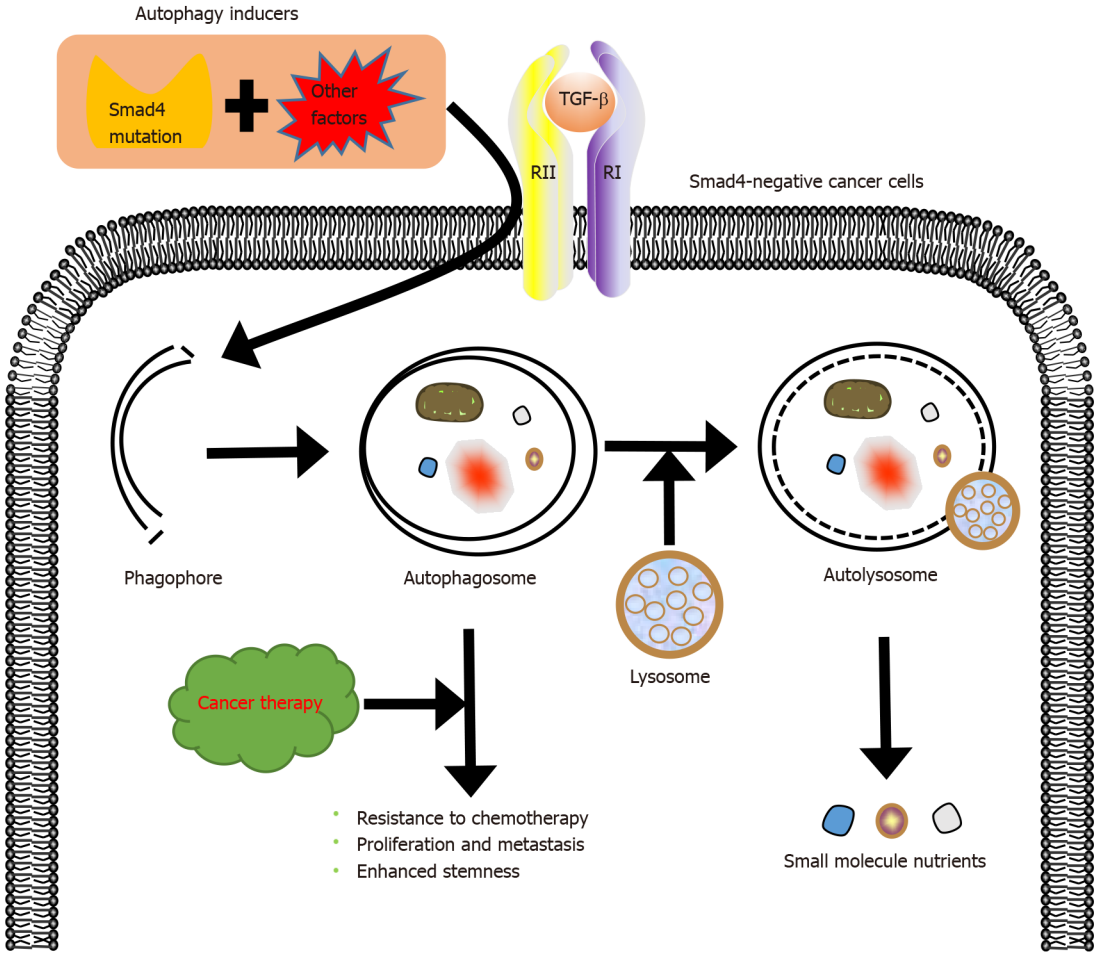

Figure 4 Role of mothers against decapentaplegic homolog 4 in autophagy and drug resistance induced by transforming growth factor-β. In cancer cells with decreased or absent expression of mothers against decapentaplegic homolog 4 (Smad4) (Smad4-negative cancer cells), Smad4 mutation combined with other autophagy inducing factors (autophagy inducers) activates autophagy *via* the transforming growth factor-β signaling pathway, so that cancer cells can obtain nutrition through autophagy, develop resistance to chemotherapy, and show enhanced proliferation, metastasis, and stemness. Smad4: Mothers against decapentaplegic homolog 4; TGF-β: Transforming growth factor-β.

Smad4 inactivation not only involves the development of drug resistance in cancer but also contributes to the crosstalk between TGF-β and other signaling pathways, accelerating this process. Zhang *et al*[86] demonstrated that Smad4 deletion induces the resistance of colon cancer cells to 5-fluorouracil (5-FU)-based therapy by activating the AKT pathway. Moreover, Smad4 deficiency inhibits 5-FU-mediated apoptosis[87]. Besides, the inactivation of Smad4 makes tumors resistant to other chemotherapeutic drugs. Smad4 mutation contributes to platinum resistance in non-small cell lung carcinoma[35] and to cetuximab resistance in HNSCC[29]. Targeting the rapamycin-insensitive companion of mTOR, a component of mammalian target of rapamycin complex 2, increases the sensitivity of Smad4-negative colon cancer to irinotecan[88]. Recent studies have demonstrated that Smad4 deficiency correlates with the development of resistance to chemotherapy and radiotherapy[89]. Therefore, Smad4 modifications may be a critical marker predicting the resistance of tumor cells to chemoradiation therapy[90]. In our view, elucidating the specific role of Smad4 in resistance of tumors to drugs and developing rational therapeutic applications of autophagy will help to improve the outcomes of treatment of drug-resistant tumors.

SMAD4 IN CANCER THERAPY

Given the key role of Smad4 in tumorigenesis, Smad4 is expected to be an attractive therapeutic target for tumors resistant to radiotherapy and chemotherapy. Many miRNAs, such as miR-224[91], miR-34a[92], and miR-205[93], are essential regulators of TGF-β-induced tumor suppression by affecting the TGF-β/Smad signaling pathway. Therefore, Smad4-targeting miRNAs may become novel therapeutic agents for cancer

treatment. Other RNAs, including circRNAs[94] and lncRNAs[95], regulate the level of Smad4 protein by targeting Smad4-targeting miRNAs directly or indirectly. Proteins, such as ALK[96] and tripartite motif 47[97] that promote, respectively, Smad4 phosphorylation and ubiquitination, diminish the tumor suppressor effect of Smad4, indicating that suppressor molecules or enzymes that inhibit Smad4 activity may be a new option for the treatment of Smad4-negative cancers.

Many inhibitors targeting the TGF-β signaling pathway are being developed clinically[98]. Molecules such as TGF-β antibodies, antisense oligonucleotides, and small molecule inhibitors of TGF-β receptor kinase activity show immense clinical potential[99]. Although inhibition of the TGF-β signaling pathway is one of the strategies for cancer treatment, the clinical outcomes of targeting TGF-β signaling and its superfamily members are not satisfactory, suggesting that single target-based therapies are not sufficient to inhibit tumors[10].

Combination therapy is an increasingly important part of anticancer therapies, and has more advantages than single-drug therapy[100-102]. The combination of Smad4 targeting and traditional/nontraditional therapies may become a novel choice for anticancer treatment. Mariathasan *et al*[103] showed that the combined application of TGF-β inhibitors together with anti-PD-L1 antibodies decreased the TGF-β level and promoted the infiltration of T-cells into tumor cells, thereby enhancing antitumor immune response and leading to tumor apoptosis. Kassardjian and Wang[104] found that Smad4-positive tumors had a better response to neoadjuvant therapy, and the lymph node metastasis rate of Smad4-positive tumors was significantly lower, suggesting that Smad4 plays an important role in neoadjuvant therapy. In our previous research[105], we combined oncolytic virus therapy and targeted gene therapy to design a new oncolytic adenovirus CD55-Smad4, which can stably produce Smad4 protein *in vitro* and *in vivo*. CD55-Smad4 significantly inhibited proliferation, metastasis, and stemness of CRC cells. All these show that the combination therapy targeting Smad4 has clinical potential. Therefore, we believe that combination of Smad4-targeted therapy with traditional therapies of surgery, radiotherapy, and chemotherapy, and with emerging treatments such as PD-LI inhibitors, chimeric antigen receptor cell therapy, as well as our targeting gene virotherapy will significantly improve the outcomes of anticancer therapies.

CONCLUSION

Targeting abnormal signal transduction or abnormal metabolic pathway has always been the focus of anticancer research. *Smad4* gene mutations and its abnormal expression have been confirmed to dysregulate the TGF-β signaling pathway, transforming its function from a tumor suppressor to a tumor promoter[106]. This signaling disorder accelerates tumor progression, but the precise mechanism by which Smad4 affects tumor development remains unclear. An increasing number of studies have shown that targeting oncogenic miRNAs, circRNAs, and other RNAs can inhibit TGF-β-induced tumorigenesis by directly or indirectly acting on Smad4, increasing susceptibility of tumors to chemoradiation and improving the survival rate of cancer patients. Although the possibility of regulating tumor progression by these RNAs shows clinical potential, the underlying mechanisms are poorly understood.

Fortunately, the vigorous development of genome editing technologies such as CRISPR-Cas9 systems and AAV gene vectors has enabled the transformation of Smad4-targeted therapies into clinical reality[107,108]. Furthermore, it is asserted that the presence of Smad4 protein promotes the cell-to-cell spread of the vaccinia virus, which enables combining *Smad4* gene therapy with oncolytic virus therapy to effectively eliminate tumors[109]. In summary, although the specific mechanisms by which Smad4 affects carcinogenesis, metastasis, drug resistance, and other malignant features of tumors have not been completely clarified, Smad4-targeted therapies coupled with traditional therapies and emerging anticancer treatments have achieved good antitumor effects in animal models. Smad4 combination therapy shows potential for future clinical applications.

REFERENCES

1 **Blank U**, Karlsson S. TGF-β signaling in the control of hematopoietic stem cells. *Blood* 2015; **125**: 3542-3550 [PMID: 25833962 DOI: 10.1182/blood-2014-12-618090]

2 **Haque S**, Morris JC. Transforming growth factor-β: A therapeutic target for cancer. *Hum Vaccin*

Immunother 2017; **13**: 1741-1750 [PMID: 28575585 DOI: 10.1080/21645515.2017.1327107]
3 **Meng XM**, Nikolic-Paterson DJ, Lan HY. TGF-β: the master regulator of fibrosis. *Nat Rev Nephrol* 2016; **12**: 325-338 [PMID: 27108839 DOI: 10.1038/nrneph.2016.48]
4 **Batlle E**, Massagué J. Transforming Growth Factor-β Signaling in Immunity and Cancer. *Immunity* 2019; **50**: 924-940 [PMID: 30995507 DOI: 10.1016/j.immuni.2019.03.024]
5 **Derynck R**, Akhurst RJ, Balmain A. TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 2001; **29**: 117-129 [PMID: 11586292 DOI: 10.1038/ng1001-117]
6 **Tzavlaki K**, Moustakas A. TGF-β Signaling. *Biomolecules* 2020; **10** [PMID: 32210029 DOI: 10.3390/biom10030487]
7 **Luo K**. Signaling Cross Talk between TGF-β/Smad and Other Signaling Pathways. *Cold Spring Harb Perspect Biol* 2017; **9** [PMID: 27836834 DOI: 10.1101/cshperspect.a022137]
8 **Guo X**, Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell Res* 2009; **19**: 71-88 [PMID: 19002158 DOI: 10.1038/cr.2008.302]
9 **Jahan N**, Hannila SS. Transforming growth factor β-induced expression of chondroitin sulfate proteoglycans is mediated through non-Smad signaling pathways. *Exp Neurol* 2015; **263**: 372-384 [PMID: 25446723 DOI: 10.1016/j.expneurol.2014.10.023]
10 **Colak S**, Ten Dijke P. Targeting TGF-β Signaling in Cancer. *Trends Cancer* 2017; **3**: 56-71 [PMID: 28718426 DOI: 10.1016/j.trecan.2016.11.008]
11 **Wang Y**, Xue Q, Zheng Q, Jin Y, Shen X, Yang M, Zhou X, Li Y. SMAD4 mutation correlates with poor prognosis in non-small cell lung cancer. *Lab Invest* 2021; **101**: 463-476 [PMID: 33303972 DOI: 10.1038/s41374-020-00517-x]
12 **Khalil H**, Kanisicak O, Prasad V, Correll RN, Fu X, Schips T, Vagnozzi RJ, Liu R, Huynh T, Lee SJ, Karch J, Molkentin JD. Fibroblast-specific TGF-β-Smad2/3 signaling underlies cardiac fibrosis. *J Clin Invest* 2017; **127**: 3770-3783 [PMID: 28891814 DOI: 10.1172/JCI94753]
13 **Tatler AL**, John AE, Jolly L, Habgood A, Porte J, Brightling C, Knox AJ, Pang L, Sheppard D, Huang X, Jenkins G. Integrin αvβ5-mediated TGF-β activation by airway smooth muscle cells in asthma. *J Immunol* 2011; **187**: 6094-6107 [PMID: 22025551 DOI: 10.4049/jimmunol.1003507]
14 **Travis MA**, Sheppard D. TGF-β activation and function in immunity. *Annu Rev Immunol* 2014; **32**: 51-82 [PMID: 24313777 DOI: 10.1146/annurev-immunol-032713-120257]
15 **Caja L**, Dituri F, Mancarella S, Caballero-Diaz D, Moustakas A, Giannelli G, Fabregat I. TGF-β and the Tissue Microenvironment: Relevance in Fibrosis and Cancer. *Int J Mol Sci* 2018; **19** [PMID: 29701666 DOI: 10.3390/ijms19051294]
16 **Derynck R**, Budi EH. Specificity, versatility, and control of TGF-β family signaling. *Sci Signal* 2019; **12** [PMID: 30808818 DOI: 10.1126/scisignal.aav5183]
17 **Sung H**, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; **71**: 209-249 [PMID: 33538338 DOI: 10.3322/caac.21660]
18 **Qiu L**, Hu X, Jing Q, Zeng X, Chan KM, Han J. Mechanism of cancer: Oncohistones in action. *J Genet Genomics* 2018; **45**: 227-236 [PMID: 29804713 DOI: 10.1016/j.jgg.2018.04.004]
19 **Cicenas J**, Kvederaviciute K, Meskinyte I, Meskinyte-Kausiliene E, Skeberdyte A, Cicenas J. KRAS, TP53, CDKN2A, SMAD4, BRCA1, and BRCA2 Mutations in Pancreatic Cancer. *Cancers (Basel)* 2017; **9** [PMID: 28452926 DOI: 10.3390/cancers9050042]
20 **Ahmed S**, Bradshaw AD, Gera S, Dewan MZ, Xu R. The TGF-β/Smad4 Signaling Pathway in Pancreatic Carcinogenesis and Its Clinical Significance. *J Clin Med* 2017; **6** [PMID: 28067794 DOI: 10.3390/jcm6010005]
21 **Rosic J**, Dragicevic S, Miladinov M, Despotovic J, Bogdanovic A, Krivokapic Z, Nikolic A. SMAD7 and SMAD4 expression in colorectal cancer progression and therapy response. *Exp Mol Pathol* 2021; **123**: 104714 [PMID: 34717960 DOI: 10.1016/j.yexmp.2021.104714]
22 **Qian Y**, Gong Y, Fan Z, Luo G, Huang Q, Deng S, Cheng H, Jin K, Ni Q, Yu X, Liu C. Molecular alterations and targeted therapy in pancreatic ductal adenocarcinoma. *J Hematol Oncol* 2020; **13**: 130 [PMID: 33008426 DOI: 10.1186/s13045-020-00958-3]
23 **Yan W**, Liu Z, Yang W, Wu G. miRNA expression profiles in Smad4-positive and Smad4-negative SW620 human colon cancer cells detected by next-generation small RNA sequencing. *Cancer Manag Res* 2018; **10**: 5479-5490 [PMID: 30519096 DOI: 10.2147/CMAR.S178261]
24 **Ma C**, Wang X, Yang F, Zang Y, Liu J, Xu X, Li W, Jia J, Liu Z. Circular RNA hsa_circ_0004872 inhibits gastric cancer progression *via* the miR-224/Smad4/ADAR1 successive regulatory circuit. *Mol Cancer* 2020; **19**: 157 [PMID: 33172486 DOI: 10.1186/s12943-020-01268-5]
25 **Zhang C**, Hao Y, Wang Y, Xu J, Teng Y, Yang X. TGF-β/SMAD4-Regulated LncRNA-LINP1 Inhibits Epithelial-Mesenchymal Transition in Lung Cancer. *Int J Biol Sci* 2018; **14**: 1715-1723 [PMID: 30416386 DOI: 10.7150/ijbs.27197]
26 **Hernanda PY**, Chen K, Das AM, Sideras K, Wang W, Li J, Cao W, Bots SJ, Kodach LL, de Man RA, Ijzermans JN, Janssen HL, Stubbs AP, Sprengers D, Bruno MJ, Metselaar HJ, ten Hagen TL, Kwekkeboom J, Peppelenbosch MP, Pan Q. SMAD4 exerts a tumor-promoting role in hepatocellular carcinoma. *Oncogene* 2015; **34**: 5055-5068 [PMID: 25531314 DOI: 10.1038/onc.2014.425]
27 **Hiwatashi K**, Ueno S, Sakoda M, Kubo F, Tateno T, Kurahara H, Mataki Y, Maemura K, Ishigami S, Shinchi H, Natsugoe S. Strong Smad4 expression correlates with poor prognosis after surgery in patients with hepatocellular carcinoma. *Ann Surg Oncol* 2009; **16**: 3176-3182 [PMID: 19626374 DOI: 10.1245/s10434-009-0614-2]

28 **Avery S**, Zafarana G, Gokhale PJ, Andrews PW. The role of SMAD4 in human embryonic stem cell self-renewal and stem cell fate. *Stem Cells* 2010; **28**: 863-873 [PMID: 20235236 DOI: 10.1002/stem.409]

29 **Ozawa H**, Ranaweera RS, Izumchenko E, Makarev E, Zhavoronkov A, Fertig EJ, Howard JD, Markovic A, Bedi A, Ravi R, Perez J, Le QT, Kong CS, Jordan RC, Wang H, Kang H, Quon H, Sidransky D, Chung CH. SMAD4 Loss Is Associated with Cetuximab Resistance and Induction of MAPK/JNK Activation in Head and Neck Cancer Cells. *Clin Cancer Res* 2017; **23**: 5162-5175 [PMID: 28522603 DOI: 10.1158/1078-0432.CCR-16-1686]

30 **Hahn SA**, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996; **271**: 350-353 [PMID: 8553070 DOI: 10.1126/science.271.5247.350]

31 **Derynck R**, Gelbart WM, Harland RM, Heldin CH, Kern SE, Massagué J, Melton DA, Mlodzik M, Padgett RW, Roberts AB, Smith J, Thomsen GH, Vogelstein B, Wang XF. Nomenclature: vertebrate mediators of TGFbeta family signals. *Cell* 1996; **87**: 173 [PMID: 8861901 DOI: 10.1016/s0092-8674(00)81335-5]

32 **Mamot C**, Mild G, Reuter J, Laffer U, Metzger U, Terracciano L, Boulay JL, Herrmann R, Rochlitz C. Infrequent mutation of the tumour-suppressor gene Smad4 in early-stage colorectal cancer. *Br J Cancer* 2003; **88**: 420-423 [PMID: 12569386 DOI: 10.1038/sj.bjc.6600733]

33 **Horvath LG**, Henshall SM, Kench JG, Turner JJ, Golovsky D, Brenner PC, O'Neill GF, Kooner R, Stricker PD, Grygiel JJ, Sutherland RL. Loss of BMP2, Smad8, and Smad4 expression in prostate cancer progression. *Prostate* 2004; **59**: 234-242 [PMID: 15042598 DOI: 10.1002/pros.10361]

34 **Hernandez AL**, Young CD, Wang JH, Wang XJ. Lessons learned from SMAD4 Loss in squamous cell carcinomas. *Mol Carcinog* 2019; **58**: 1648-1655 [PMID: 31140647 DOI: 10.1002/mc.23049]

35 **Tan X**, Tong L, Li L, Xu J, Xie S, Ji L, Fu J, Liu Q, Shen S, Liu Y, Xiao Y, Gao F, Moses RE, Bardeesy N, Wang Y, Zhang J, Tang L, Wong K-K, Song D, Yang X, Liu J, Li X. Loss of Smad4 promotes aggressive lung cancer metastasis by de-repression of PAK3 *via* miRNA regulation. *Nat Commun* 2021; **12** (1): 4853 [PMID: 34381046 DOI: 10.1038/s41467-021-24898-9]

36 **Shi Y**, Hata A, Lo RS, Massagué J, Pavletich NP. A structural basis for mutational inactivation of the tumour suppressor Smad4. *Nature* 1997; **388**: 87-93 [PMID: 9214508 DOI: 10.1038/40431]

37 **Zhao M**, Mishra L, Deng CX. The role of TGF-β/SMAD4 signaling in cancer. *Int J Biol Sci* 2018; **14**: 111-123 [PMID: 29483830 DOI: 10.7150/ijbs.23230]

38 **Budi EH**, Duan D, Derynck R. Transforming Growth Factor-β Receptors and Smads: Regulatory Complexity and Functional Versatility. *Trends Cell Biol* 2017; **27**: 658-672 [PMID: 28552280 DOI: 10.1016/j.tcb.2017.04.005]

39 **Morikawa M**, Derynck R, Miyazono K. TGF-β and the TGF-β Family: Context-Dependent Roles in Cell and Tissue Physiology. *Cold Spring Harb Perspect Biol* 2016; **8** [PMID: 27141051 DOI: 10.1101/cshperspect.a021873]

40 **Weiss A**, Attisano L. The TGFbeta superfamily signaling pathway. *Wiley Interdiscip Rev Dev Biol* 2013; **2**: 47-63 [PMID: 23799630 DOI: 10.1002/wdev.86]

41 **David CJ**, Massagué J. Contextual determinants of TGFβ action in development, immunity and cancer. *Nat Rev Mol Cell Biol* 2018; **19**: 419-435 [PMID: 29643418 DOI: 10.1038/s41580-018-0007-0]

42 **Vander Ark A**, Cao J, Li X. TGF-β receptors: In and beyond TGF-β signaling. *Cell Signal* 2018; **52**: 112-120 [PMID: 30184463 DOI: 10.1016/j.cellsig.2018.09.002]

43 **Massagué J**, Seoane J, Wotton D. Smad transcription factors. *Genes Dev* 2005; **19**: 2783-2810 [PMID: 16322555 DOI: 10.1101/gad.1350705]

44 **Hata A**, Lagna G, Massagué J, Hemmati-Brivanlou A. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev* 1998; **12**: 186-197 [PMID: 9436979 DOI: 10.1101/gad.12.2.186]

45 **Kavsak P**, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* 2000; **6**: 1365-1375 [PMID: 11163210 DOI: 10.1016/s1097-2765(00)00134-9]

46 **Peters JM**, Gonzalez FJ. The Evolution of Carcinogenesis. *Toxicol Sci* 2018; **165**: 272-276 [PMID: 30629266 DOI: 10.1093/toxsci/kfy184]

47 **Levy L**, Hill CS. Smad4 dependency defines two classes of transforming growth factor {beta} (TGF-{beta}) target genes and distinguishes TGF-{beta}-induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. *Mol Cell Biol* 2005; **25**: 8108-8125 [PMID: 16135802 DOI: 10.1128/MCB.25.18.8108-8125.2005]

48 **Ke Y**, Wang XJ. TGFβ Signaling in Photoaging and UV-Induced Skin Cancer. *J Invest Dermatol* 2021; **141**: 1104-1110 [PMID: 33358021 DOI: 10.1016/j.jid.2020.11.007]

49 **Hao Y**, Baker D, Ten Dijke P. TGF-β-Mediated Epithelial-Mesenchymal Transition and Cancer Metastasis. *Int J Mol Sci* 2019; **20** [PMID: 31195692 DOI: 10.3390/ijms20112767]

50 **Li X**, Li J, Cai Y, Peng S, Wang J, Xiao Z, Wang Y, Tao Y, Leng Q, Wu D, Yang S, Ji Z, Han Y, Li L, Gao X, Zeng C, Wen X. Hyperglycaemia-induced miR-301a promotes cell proliferation by repressing p21 and Smad4 in prostate cancer. *Cancer Lett* 2018; **418**: 211-220 [PMID: 29331421 DOI: 10.1016/j.canlet.2018.01.031]

51 **Liang C**, Shi S, Qin Y, Meng Q, Hua J, Hu Q, Ji S, Zhang B, Xu J, Yu XJ. Localisation of PGK1 determines metabolic phenotype to balance metastasis and proliferation in patients with SMAD4-negative pancreatic cancer. *Gut* 2020; **69**: 888-900 [PMID: 31611300 DOI:

10.1136/gutjnl-2018-317163]

52 **Fei N**, Wen S, Ramanathan R, Hogg ME, Zureikat AH, Lotze MT, Bahary N, Singhi AD, Zeh HJ, Boone BA. SMAD4 loss is associated with response to neoadjuvant chemotherapy plus hydroxychloroquine in patients with pancreatic adenocarcinoma. *Clin Transl Sci* 2021; **14**: 1822-1829 [PMID: 34002944 DOI: 10.1111/cts.13029]

53 **Notta F**, Chan-Seng-Yue M, Lemire M, Li Y, Wilson GW, Connor AA, Denroche RE, Liang SB, Brown AM, Kim JC, Wang T, Simpson JT, Beck T, Borgida A, Buchner N, Chadwick D, Hafezi-Bakhtiari S, Dick JE, Heisler L, Hollingsworth MA, Ibrahimov E, Jang GH, Johns J, Jorgensen LG, Law C, Ludkovski O, Lungu I, Ng K, Pasternack D, Petersen GM, Shlush LI, Timms L, Tsao MS, Wilson JM, Yung CK, Zogopoulos G, Bartlett JM, Alexandrov LB, Real FX, Cleary SP, Roehrl MH, McPherson JD, Stein LD, Hudson TJ, Campbell PJ, Gallinger S. A renewed model of pancreatic cancer evolution based on genomic rearrangement patterns. *Nature* 2016; **538**: 378-382 [PMID: 27732578 DOI: 10.1038/nature19823]

54 **Wang C**, Zhang T, Liao Q, Dai M, Guo J, Yang X, Tan W, Lin D, Wu C, Zhao Y. Metformin inhibits pancreatic cancer metastasis caused by SMAD4 deficiency and consequent HNF4G upregulation. *Protein Cell* 2021; **12**: 128-144 [PMID: 32737864 DOI: 10.1007/s13238-020-00760-4]

55 **Mohd Faheem M**, Rasool RU, Ahmad SM, Jamwal VL, Chakraborty S, Katoch A, Gandhi SG, Bhagat M, Goswami A. Par-4 mediated Smad4 induction in PDAC cells restores canonical TGF-β/Smad4 axis driving the cells towards lethal EMT. *Eur J Cell Biol* 2020; **99**: 151076 [PMID: 32439219 DOI: 10.1016/j.ejcb.2020.151076]

56 **Colvin EK**, Scarlett CJ. A historical perspective of pancreatic cancer mouse models. *Semin Cell Dev Biol* 2014; **27**: 96-105 [PMID: 24685616 DOI: 10.1016/j.semcdb.2014.03.025]

57 **Izeradjene K**, Combs C, Best M, Gopinathan A, Wagner A, Grady WM, Deng CX, Hruban RH, Adsay NV, Tuveson DA, Hingorani SR. Kras(G12D) and Smad4/Dpc4 haploinsufficiency cooperate to induce mucinous cystic neoplasms and invasive adenocarcinoma of the pancreas. *Cancer Cell* 2007; **11**: 229-243 [PMID: 17349581 DOI: 10.1016/j.ccr.2007.01.017]

58 **Ogawa R**, Yamamoto T, Hirai H, Hanada K, Kiyasu Y, Nishikawa G, Mizuno R, Inamoto S, Itatani Y, Sakai Y, Kawada K. Loss of SMAD4 Promotes Colorectal Cancer Progression by Recruiting Tumor-Associated Neutrophils *via* the CXCL1/8-CXCR2 Axis. *Clin Cancer Res* 2019; **25**: 2887-2899 [PMID: 30705034 DOI: 10.1158/1078-0432.CCR-18-3684]

59 **Leng A**, Liu T, He Y, Li Q, Zhang G. Smad4/Smad7 balance: a role of tumorigenesis in gastric cancer. *Exp Mol Pathol* 2009; **87**: 48-53 [PMID: 19341727 DOI: 10.1016/j.yexmp.2009.03.003]

60 **Wang J**, Li Y, Zhou JH, Shen FR, Shi X, Chen YG. CircATRNL1 activates Smad4 signaling to inhibit angiogenesis and ovarian cancer metastasis *via* miR-378. *Mol Oncol* 2021; **15**: 1217-1233 [PMID: 33372356 DOI: 10.1002/1878-0261.12893]

61 **Lin LH**, Chang KW, Cheng HW, Liu CJ. *SMAD4* Somatic Mutations in Head and Neck Carcinoma Are Associated With Tumor Progression. *Front Oncol* 2019; **9**: 1379 [PMID: 31867281 DOI: 10.3389/fonc.2019.01379]

62 **Yuan T**, Chen Z, Yan F, Qian M, Luo H, Ye S, Cao J, Ying M, Dai X, Gai R, Yang B, He Q, Zhu H. Deubiquitinating enzyme USP10 promotes hepatocellular carcinoma metastasis through deubiquitinating and stabilizing Smad4 protein. *Mol Oncol* 2020; **14**: 197-210 [PMID: 31721429 DOI: 10.1002/1878-0261.12596]

63 **Ma X**, Fan C, Wang Y, Du Y, Zhu Y, Liu H, Lv L, Liu Y, Zhou Y. MiR-137 knockdown promotes the osteogenic differentiation of human adipose-derived stem cells *via* the LSD1/BMP2/SMAD4 signaling network. *J Cell Physiol* 2020; **235**: 909-919 [PMID: 31241766 DOI: 10.1002/jcp.29006]

64 **Gu Z**, Long J, Li Y, Wang X, Wang H. MiR-125a-3p negatively regulates osteoblastic differentiation of human adipose derived mesenchymal stem cells by targeting Smad4 and Jak1. *Am J Transl Res* 2019; **11**: 2603-2615 [PMID: 31105866]

65 **Park JS**, Kim M, Song NJ, Kim JH, Seo D, Lee JH, Jung SM, Lee JY, Lee J, Lee YS, Park KW, Park SH. A Reciprocal Role of the Smad4-Taz Axis in Osteogenesis and Adipogenesis of Mesenchymal Stem Cells. *Stem Cells* 2019; **37**: 368-381 [PMID: 30444564 DOI: 10.1002/stem.2949]

66 **Xu J**, Gruber PJ, Chien KR. SMAD4 Is Essential for Human Cardiac Mesodermal Precursor Cell Formation. *Stem Cells* 2019; **37**: 216-225 [PMID: 30376214 DOI: 10.1002/stem.2943]

67 **Chung MI**, Bujnis M, Barkauskas CE, Kobayashi Y, Hogan BLM. Niche-mediated BMP/SMAD signaling regulates lung alveolar stem cell proliferation and differentiation. *Development* 2018; **145** [PMID: 29752282 DOI: 10.1242/dev.163014]

68 **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]

69 **Qi Z**, Li Y, Zhao B, Xu C, Liu Y, Li H, Zhang B, Wang X, Yang X, Xie W, Li B, Han JJ, Chen YG. BMP restricts stemness of intestinal $Lgr5^+$ stem cells by directly suppressing their signature genes. *Nat Commun* 2017; **8**: 13824 [PMID: 28059064 DOI: 10.1038/ncomms13824]

70 **Batlle E**, Clevers H. Cancer stem cells revisited. *Nat Med* 2017; **23**: 1124-1134 [PMID: 28985214 DOI: 10.1038/nm.4409]

71 **Vlashi E**, Pajonk F. Cancer stem cells, cancer cell plasticity and radiation therapy. *Semin Cancer Biol* 2015; **31**: 28-35 [PMID: 25025713 DOI: 10.1016/j.semcancer.2014.07.001]

72 **Prasetyanti PR**, Medema JP. Intra-tumor heterogeneity from a cancer stem cell perspective. *Mol*

Cancer 2017; **16**: 41 [PMID: 28209166 DOI: 10.1186/s12943-017-0600-4]
73 **Xia W**, Lo CM, Poon RYC, Cheung TT, Chan ACY, Chen L, Yang S, Tsao GSW, Wang XQ. Smad inhibitor induces CSC differentiation for effective chemosensitization in cyclin D1- and TGF-β /Smad-regulated liver cancer stem cell-like cells. *Oncotarget* 2017; **8**: 38811-38824 [PMID: 28415588 DOI: 10.18632/oncotarget.16402]
74 **Wen H**, Qian M, He J, Li M, Yu Q, Leng Z. Inhibiting of self-renewal, migration and invasion of ovarian cancer stem cells by blocking TGF-β pathway. *PLoS One* 2020; **15**: e0230230 [PMID: 32214328 DOI: 10.1371/journal.pone.0230230]
75 **Matano M**, Date S, Shimokawa M, Takano A, Fujii M, Ohta Y, Watanabe T, Kanai T, Sato T. Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. *Nat Med* 2015; **21**: 256-262 [PMID: 25706875 DOI: 10.1038/nm.3802]
76 **Assaraf YG**, Brozovic A, Gonçalves AC, Jurkovicova D, Linē A, Machuqueiro M, Saponara S, Sarmento-Ribeiro AB, Xavier CPR, Vasconcelos MH. The multi-factorial nature of clinical multidrug resistance in cancer. *Drug Resist Updat* 2019; **46**: 100645 [PMID: 31585396 DOI: 10.1016/j.drup.2019.100645]
77 **Zamame Ramirez JA**, Romagnoli GG, Kaneno R. Inhibiting autophagy to prevent drug resistance and improve anti-tumor therapy. *Life Sci* 2021; **265**: 118745 [PMID: 33186569 DOI: 10.1016/j.lfs.2020.118745]
78 **Smith AG**, Macleod KF. Autophagy, cancer stem cells and drug resistance. *J Pathol* 2019; **247**: 708-718 [PMID: 30570140 DOI: 10.1002/path.5222]
79 **Su Z**, Yang Z, Xu Y, Chen Y, Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. *Mol Cancer* 2015; **14**: 48 [PMID: 25743109 DOI: 10.1186/s12943-015-0321-5]
80 **Sun WL**, Chen J, Wang YP, Zheng H. Autophagy protects breast cancer cells from epirubicin-induced apoptosis and facilitates epirubicin-resistance development. *Autophagy* 2011; **7**: 1035-1044 [PMID: 21646864 DOI: 10.4161/auto.7.9.16521]
81 **Fan T**, Zhang C, Zong M, Zhao Q, Yang X, Hao C, Zhang H, Yu S, Guo J, Gong R, Fan S, Wei L, Fan L. Peptidylarginine deiminase IV promotes the development of chemoresistance through inducing autophagy in hepatocellular carcinoma. *Cell Biosci* 2014; **4**: 49 [PMID: 25922661 DOI: 10.1186/2045-3701-4-49]
82 **Amaravadi RK**, Kimmelman AC, Debnath J. Targeting Autophagy in Cancer: Recent Advances and Future Directions. *Cancer Discov* 2019; **9**: 1167-1181 [PMID: 31434711 DOI: 10.1158/2159-8290.CD-19-0292]
83 **Mauthe M**, Orhon I, Rocchi C, Zhou X, Luhr M, Hijlkema KJ, Coppes RP, Engedal N, Mari M, Reggiori F. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy* 2018; **14**: 1435-1455 [PMID: 29940786 DOI: 10.1080/15548627.2018.1474314]
84 **Kiyono K**, Suzuki HI, Matsuyama H, Morishita Y, Komuro A, Kano MR, Sugimoto K, Miyazono K. Autophagy is activated by TGF-beta and potentiates TGF-beta-mediated growth inhibition in human hepatocellular carcinoma cells. *Cancer Res* 2009; **69**: 8844-8852 [PMID: 19903843 DOI: 10.1158/0008-5472.CAN-08-4401]
85 **Liang C**, Xu J, Meng Q, Zhang B, Liu J, Hua J, Zhang Y, Shi S, Yu X. TGFB1-induced autophagy affects the pattern of pancreatic cancer progression in distinct ways depending on SMAD4 status. *Autophagy* 2020; **16**: 486-500 [PMID: 31177911 DOI: 10.1080/15548627.2019.1628540]
86 **Zhang B**, Zhang B, Chen X, Bae S, Singh K, Washington MK, Datta PK. Loss of Smad4 in colorectal cancer induces resistance to 5-fluorouracil through activating Akt pathway. *Br J Cancer* 2014; **110**: 946-957 [PMID: 24384683 DOI: 10.1038/bjc.2013.789]
87 **Papageorgis P**, Cheng K, Ozturk S, Gong Y, Lambert AW, Abdolmaleky HM, Zhou JR, Thiagalingam S. Smad4 inactivation promotes malignancy and drug resistance of colon cancer. *Cancer Res* 2011; **71**: 998-1008 [PMID: 21245094 DOI: 10.1158/0008-5472.CAN-09-3269]
88 **Wong CK**, Lambert AW, Ozturk S, Papageorgis P, Lopez D, Shen N, Sen Z, Abdolmaleky HM, Győrffy B, Feng H, Thiagalingam S. Targeting RICTOR Sensitizes SMAD4-Negative Colon Cancer to Irinotecan. *Mol Cancer Res* 2020; **18**: 414-423 [PMID: 31932471 DOI: 10.1158/1541-7786.MCR-19-0525]
89 **Jiang D**, Wang X, Wang Y, Philips D, Meng W, Xiong M, Zhao J, Sun L, He D, Li K. Mutation in BRAF and SMAD4 associated with resistance to neoadjuvant chemoradiation therapy in locally advanced rectal cancer. *Virchows Arch* 2019; **475**: 39-47 [PMID: 31056731 DOI: 10.1007/s00428-019-02576-y]
90 **Ezrova Z**, Nahacka Z, Stursa J, Werner L, Vlcak E, Kralova Viziova P, Berridge MV, Sedlacek R, Zobalova R, Rohlena J, Boukalova S, Neuzil J. SMAD4 loss limits the vulnerability of pancreatic cancer cells to complex I inhibition *via* promotion of mitophagy. *Oncogene* 2021; **40**: 2539-2552 [PMID: 33686239 DOI: 10.1038/s41388-021-01726-4]
91 **Zhou J**, Hu M, Wang F, Song M, Huang Q, Ge B. miR-224 Controls Human Colorectal Cancer Cell Line HCT116 Proliferation by Targeting Smad4. *Int J Med Sci* 2017; **14**: 937-942 [PMID: 28924364 DOI: 10.7150/ijms.19565]
92 **Qi Y**, Zhao A, Yang P, Jin L, Hao C. miR-34a-5p Attenuates EMT through targeting SMAD4 in silica-induced pulmonary fibrosis. *J Cell Mol Med* 2020; **24**: 12219-12224 [PMID: 32929850 DOI: 10.1111/jcmm.15853]
93 **Chu P**, Liang A, Jiang A, Zong L. miR-205 regulates the proliferation and invasion of ovarian cancer cells *via* suppressing PTEN/SMAD4 expression. *Oncol Lett* 2018; **15**: 7571-7578 [PMID: 29725462 DOI: 10.3892/ol.2018.8313]

94 **Liu J**, Dai X, Guo X, Cheng A, Mac SM, Wang Z. Circ-OXCT1 Suppresses Gastric Cancer EMT and Metastasis by Attenuating TGF-β Pathway Through the Circ-OXCT1/miR-136/SMAD4 Axis. *Onco Targets Ther* 2020; **13**: 3987-3998 [PMID: 32523351 DOI: 10.2147/OTT.S239789]

95 **Xiao T**, Zou Z, Xue J, Syed BM, Sun J, Dai X, Shi M, Li J, Wei S, Tang H, Zhang A, Liu Q. LncRNA H19-mediated M2 polarization of macrophages promotes myofibroblast differentiation in pulmonary fibrosis induced by arsenic exposure. *Environ Pollut* 2021; **268**: 115810 [PMID: 33162208 DOI: 10.1016/j.envpol.2020.115810]

96 **Zhang Q**, Xiao M, Gu S, Xu Y, Liu T, Li H, Yu Y, Qin L, Zhu Y, Chen F, Wang Y, Ding C, Wu H, Ji H, Chen Z, Zu Y, Malkoski S, Li Y, Liang T, Ji J, Qin J, Xu P, Zhao B, Shen L, Lin X, Feng XH. ALK phosphorylates SMAD4 on tyrosine to disable TGF-β tumour suppressor functions. *Nat Cell Biol* 2019; **21**: 179-189 [PMID: 30664791 DOI: 10.1038/s41556-018-0264-3]

97 **Liang Q**, Tang C, Tang M, Zhang Q, Gao Y, Ge Z. TRIM47 is up-regulated in colorectal cancer, promoting ubiquitination and degradation of SMAD4. *J Exp Clin Cancer Res* 2019; **38**: 159 [PMID: 30979374 DOI: 10.1186/s13046-019-1143-x]

98 **Huynh LK**, Hipolito CJ, Ten Dijke P. A Perspective on the Development of TGF-β Inhibitors for Cancer Treatment. *Biomolecules* 2019; **9** [PMID: 31744193 DOI: 10.3390/biom9110743]

99 **Syed V**. TGF-β Signaling in Cancer. *J Cell Biochem* 2016; **117**: 1279-1287 [PMID: 26774024 DOI: 10.1002/jcb.25496]

100 **Nastiuk KL**, Krolewski JJ. Opportunities and challenges in combination gene cancer therapy. *Adv Drug Deliv Rev* 2016; **98**: 35-40 [PMID: 26724249 DOI: 10.1016/j.addr.2015.12.005]

101 **Colli LM**, Machiela MJ, Zhang H, Myers TA, Jessop L, Delattre O, Yu K, Chanock SJ. Landscape of Combination Immunotherapy and Targeted Therapy to Improve Cancer Management. *Cancer Res* 2017; **77**: 3666-3671 [PMID: 28446466 DOI: 10.1158/0008-5472.CAN-16-3338]

102 **Martin NT**, Bell JC. Oncolytic Virus Combination Therapy: Killing One Bird with Two Stones. *Mol Ther* 2018; **26**: 1414-1422 [PMID: 29703699 DOI: 10.1016/j.ymthe.2018.04.001]

103 **Mariathasan S**, Turley SJ, Nickles D, Castiglioni A, Yuen K, Wang Y, Kadel EE III, Koeppen H, Astarita JL, Cubas R, Jhunjhunwala S, Banchereau R, Yang Y, Guan Y, Chalouni C, Ziai J, Şenbabaoğlu Y, Santoro S, Sheinson D, Hung J, Giltnane JM, Pierce AA, Mesh K, Lianoglou S, Riegler J, Carano RAD, Eriksson P, Höglund M, Somarriba L, Halligan DL, van der Heijden MS, Loriot Y, Rosenberg JE, Fong L, Mellman I, Chen DS, Green M, Derleth C, Fine GD, Hegde PS, Bourgon R, Powles T. TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature* 2018; **554**: 544-548 [PMID: 29443960 DOI: 10.1038/nature25501]

104 **Kassardjian A**, Wang HL. SMAD4-Expressing Pancreatic Ductal Adenocarcinomas Have Better Response to Neoadjuvant Therapy and Significantly Lower Lymph Node Metastasis Rates. *Pancreas* 2020; **49**: 1153-1160 [PMID: 32897998 DOI: 10.1097/MPA.0000000000001636]

105 **Xiao B**, Zhang L, Liu H, Fang H, Wang C, Huang B, Liu X, Zhou X, Wang Y. Oncolytic Adenovirus CD55-Smad4 Suppresses Cell Proliferation, Metastasis, and Tumor Stemness in Colorectal Cancer by Regulating Wnt/β-Catenin Signaling Pathway. *Biomedicines* 2020; **8** [PMID: 33322272 DOI: 10.3390/biomedicines8120593]

106 **Itatani Y**, Kawada K, Sakai Y. Transforming Growth Factor-β Signaling Pathway in Colorectal Cancer and Its Tumor Microenvironment. *Int J Mol Sci* 2019; **20** [PMID: 31756952 DOI: 10.3390/ijms20235822]

107 **Hudry E**, Vandenberghe LH. Therapeutic AAV Gene Transfer to the Nervous System: A Clinical Reality. *Neuron* 2019; **101**: 839-862 [PMID: 30844402 DOI: 10.1016/j.neuron.2019.02.017]

108 **Maeder ML**, Gersbach CA. Genome-editing Technologies for Gene and Cell Therapy. *Mol Ther* 2016; **24**: 430-446 [PMID: 26755333 DOI: 10.1038/mt.2016.10]

109 **Gowripalan A**, Abbott CR, McKenzie C, Chan WS, Karupiah G, Levy L, Newsome TP. Cell-to-cell spread of vaccinia virus is promoted by TGF-β-independent Smad4 signalling. *Cell Microbiol* 2020; **22**: e13206 [PMID: 32237038 DOI: 10.1111/cmi.13206]

World Journal of Stem Cells

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

World J Stem Cells 2022 January 26; 14(1): 54-75

DOI: 10.4252/wjsc.v14.i1.54

ISSN 1948-0210 (online)

REVIEW

Modified mesenchymal stem cells in cancer therapy: A smart weapon requiring upgrades for wider clinical applications

Carla Vicinanza, Elisabetta Lombardi, Francesco Da Ros, Miriam Marangon, Cristina Durante, Mario Mazzucato, Francesco Agostini

ORCID number: Carla Vicinanza 0000-0001-8660-6668; Elisabetta Lombardi 0000-0002-3688-9281; Francesco Da Ros 0000-0002-9869-077X; Miriam Marangon 0000-0002-9577-0774; Cristina Durante 0000-0002-6622-9786; Mario Mazzucato 0000-0001-8319-053X; Francesco Agostini 0000-0002-2808-9348.

Author contributions: Vicinanza C wrote the manuscript; Lombardi E, Da Ros F, Marangon M, Durante C and Mazzucato M edited the manuscript; Agostini F conceived, designed and wrote the article, and provided final approval; all authors have read and approved the final manuscript.

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

Supported by the grant from the Italian Ministry of Health "Ricerca Corrente" funding (J34I19003280007), the organization "Alleanza Contro il Cancro (ACC)" (J34I20000600001), the association "Finchè ci siete voi ci sono anch'io" (J31I17000440007)".

Country/Territory of origin: Italy

Specialty type: Cell and tissue engineering

Provenance and peer review:

Carla Vicinanza, Elisabetta Lombardi, Francesco Da Ros, Miriam Marangon, Cristina Durante, Mario Mazzucato, Francesco Agostini, Stem Cell Unit, Centro di Riferimento Oncologico di Aviano, IRCCS, Aviano 33081, Italy

Corresponding author: Francesco Agostini, PhD, Senior Researcher, Senior Scientist, Stem Cell Unit, Centro di Riferimento Oncologico di Aviano, IRCCS, *Via* F. Gallini, 2, Aviano 33081, Italy. fagostini@cro.it

Abstract

Mesenchymal stem stromal cells (MSC) are characterized by the intriguing capacity to home toward cancer cells after systemic administration. Thus, MSC can be harnessed as targeted delivery vehicles of cytotoxic agents against tumors. In cancer patients, MSC based advanced cellular therapies were shown to be safe but their clinical efficacy was limited. Indeed, the amount of systemically infused MSC actually homing to human cancer masses is insufficient to reduce tumor growth. Moreover, induction of an unequivocal anticancer cytotoxic phenotype in expanded MSC is necessary to achieve significant therapeutic efficacy. *Ex vivo* cell modifications are, thus, required to improve anti-cancer properties of MSC. MSC based cellular therapy products must be handled in compliance with good manufacturing practice (GMP) guidelines. In the present review we include MSC-improving manipulation approaches that, even though actually tested at pre-clinical level, could be compatible with GMP guidelines. In particular, we describe possible approaches to improve MSC homing on cancer, including genetic engineering, membrane modification and cytokine priming. Similarly, we discuss appropriate modalities aimed at inducing a marked cytotoxic phenotype in expanded MSC by direct chemotherapeutic drug loading or by genetic methods. In conclusion, we suggest that, to configure MSC as a powerful weapon against cancer, combinations of clinical grade compatible modification protocols that are currently selected, should be introduced in the final product. Highly standardized cancer clinical trials are required to test the efficacy of ameliorated MSC based cell therapies.

Key Words: Cancer; Mesenchymal stem stromal cells; Good manufacturing practice; Homing; Targeted therapy; *Ex vivo* cell modification

Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 26, 2021
Peer-review started: March 26, 2021
First decision: July 18, 2021
Revised: August 6, 2021
Accepted: December 22, 2021
Article in press: December 22, 2021
Published online: January 26, 2022

P-Reviewer: Farouk S
S-Editor: Wang JJ
L-Editor: Webster JR
P-Editor: Wang JJ

Core Tip: Natural tropism towards a tumor mass and the cytotoxic potential of mesenchymal stem stromal cells (MSC) need to be *ex vivo* ameliorated in order to improve clinical effectiveness of cell therapies against cancer. We review genetic engineering, membrane modification and other approaches to upgrade migration and tumor killing activity of MSC. As cell manipulation must be compliant with good manufacturing practice (GMP) guidelines, *ex vivo* cell modification protocols were selected as potentially compatible with GMP regulations, after appropriate protocol design and validation. Modified cell products must be tested for their clinical relevance in cancer patients within highly standardized clinical trials.

Citation: Vicinanza C, Lombardi E, Da Ros F, Marangon M, Durante C, Mazzucato M, Agostini F. Modified mesenchymal stem cells in cancer therapy: A smart weapon requiring upgrades for wider clinical applications. *World J Stem Cells* 2022; 14(1): 54-75
URL: https://www.wjgnet.com/1948-0210/full/v14/i1/54.htm
DOI: https://dx.doi.org/10.4252/wjsc.v14.i1.54

INTRODUCTION

In multicellular organisms, continuous regeneration and functional maintenance of adult tissues are assured by a stem cell reservoir. The word "stem" is derived, in fact, from the Latin *stamen*, *i.e.*, the warp thread composing a tissue. In the early 70s Friedenstein *et al*[1,2] identified, within the bone marrow, rare multipotent non-hematopoietic fibroblast-like cells characterized by the capacity to differentiate into osteoblasts. As previously reviewed[3], such mesenchymal precursors of stromal cells were shown to play a crucial role in hematopoietic stem cell differentiation and maintenance within the bone marrow niche. In light of their capacity to differentiate into chondrocytes and adipocytes and bone osteocytes[4,5] they were named mesenchymal stem cells[6]. In a position statement of the International Society for Cell Therapies, the definition of such cells was further improved to multipotent mesenchymal stem stromal cells (MSC)[7]. In the same work, the International Society for Cell Therapies proposed three criteria to define MSC. Adherence to a tissue culture plastic substrate is the first mandatory condition for MSC expansion in standard culture medium. A second requirement, flow cytometry analysis must demonstrate that at least 95% of expanded cells express CD105, CD73 and CD90 and that less than 2% express CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leukocyte antigen class II. Finally, MSC must show the above-mentioned tri-lineage differentiation capacity into chon-drocytes, adipocytes and osteocytes.

MSC can be derived from virtually all post-natal human tissues[8] with different abundances. Perinatal tissues such as amniotic fluid[9], umbilical cord blood[10] and Wharton jelly[11] are considered relevant sources of MSC. Precursors are very rare in adult circulating blood[12], while adipose mesenchymal stem cells (ASC) are particularly abundant in fat tissue[13].

In a previously published seminal work, induced pluripotent stem cells (iPSC) were obtained by reprogramming differentiated human somatic cells through artificial introduction of multiple genes and the same work showed that iPSC were characterized by the capacity to induce teratomas *in vivo*[14]. Plating iPSC and sorting cells by expression of selected cell surface markers allowed successful isolation of cells meeting minimal criteria to be defined as MSC[15].

In this review, we focus on MSC related applications as an advanced therapeutic tool against cancer. General MSC biological properties are summarized, but relevant features motivating the choice of MSC as a potential tool against tumor progression are emphasized. Manipulation of cellular therapy products for application in human patients must be performed in compliance with strict regulations warranting safety and efficacy. Thus, we describe published strategies aimed at improving MSC anticancer action, choosing approaches that we consider to be potentially compatible with clinical grade production guidelines and regulatory limitations.

MSC BIOLOGIC PROPERTIES

Regenerative potential

Mainly through paracrine mechanisms, MSC can stimulate tissue regeneration. In particular, soluble factors secreted by MSC were shown to ameliorate cardiac regeneration in a murine model[16]. Similarly, MSC were previously embedded in an agarose scaffold enriched with MSC-released factors, and administration of such products improved regeneration of rat bone calvarial defects[17]. MSC bone repair potential could be further upgraded by tuning administration routes, scaffold types and local angiogenesis[18]. Several studies have previously reported that, exploiting their paracrine action, MSC can sustain regeneration and repair of cartilage in osteoarthritis models[19,20].

Immune system modulation

In addition to their regenerative potential, MSC as well as iPSC derived MSC, can efficiently modulate reactivity of the recipient immune system mainly acting as suppressing agents[21,22]. MSC mediated immune regulation was shown to be dependent on microenvironmental cues[21,23]: In particular, MSC exposure to a low grade inflammatory milieu was shown, in murine models, to enhance inflammatory processes such as monocyte mobilization[24]. Such an MSC mediated effect was shown to be determined by secretion of specific chemokines, in turn recruiting lymphocytes[25]. As characterized in the literature[26], MSC exposure to elevated concentrations of proinflammatory mediators (licensing) can trigger their anti-inflammatory properties[25]. Coculturing MSC with monocytes, after application of sufficient pro-inflammatory stimuli, was shown to promote polarization of macrophages to the anti-inflammatory M2 phenotype[27]. Similarly, expanded MSC were shown to induce in culture a regulatory T cell phenotype in CD4+ cells[28]. Moreover, previous works reported that appropriate MSC licensing by stimulatory cytokines, such as interferon gamma (IFN-γ) together with tumor necrosis factor alpha (TNF-α) can properly stimulate and enhance their capacity to downregulate inflammation[26].

Homing to inflamed or cancer tissues

As previously reviewed[29], MSC are characterized by the peculiar capacity to spontaneously reach damaged or inflamed tissues as well as primary or metastatic cancer masses (Figure 1). Although not fully elucidated, mechanisms regulating such processes are analogous to the leukocyte model of adhesion and invasion[30]. When in contact with endothelial cells within an inflamed microenvironment, circulating MSC can set transient and repeated physical interactions, resulting in cell tethering and rolling: This represents a crucial and rate limiting step in the cell adhesion process[29,30]. Selectin expression on the endothelial surface is known to mediate leukocyte tethering and rolling on the internal vessel lumen[31]. Accordingly, MSC can bind *in vitro* and *in vivo* selectins expressed on inflamed endothelial cells[32]. The capacity of MSC to interact with the endothelium through selectins, was questioned: MSC were, in fact, shown not to normally express the P-selectin glycoprotein ligand-1 containing the active interaction domain Sialyl Lewis^{x} (SLeX)[33-35]. Further investigations are required to fully clarify mechanisms explaining MSC early interaction with inflamed endothelial cells.

Following loose contacts with endothelial cells, MSC activation can trigger firm cell adhesion. This process is mainly mediated by the interaction between stromal derived factor-1 (SDF-1), a ligand expressed on endothelial cells, and the C-X-C chemokine receptor type 4 (CXCR4) exposed on MSC[36]. In a clinical trial testing the efficacy of modified MSC against glioblastoma, the authors showed that migration capacities and expression levels of selected adhesion molecules (*e.g.*, CXCR1 and CXCR4) were higher in MSC derived from responding patients *vs* non-responders[37]. MSC activation by chemokine interaction with the receptor can fully stabilize cell adhesion, increasing integrin affinity for extracellular matrix proteins or for other adhesion molecules[38-40]. In particular, SDF-1 interaction with CXCR4 can activate integrins such as very late antigen-4 (VLA-4), in turn promoting MSC adhesion through vascular cell adhesion molecule 1 (VCAM-1)[41]. Interestingly, preclinical studies demonstrated that binding between VLA-4, expressed on MSC, and VCAM-1, on endothelial cells, can actively contribute to MSC interaction with the vessel lumen[32,41]. Recently, MSC expressing higher levels of the integrin lymphocyte function-associated antigen 1 were shown to adhere on endothelial cells through Intercellular adhesion molecule 1[42].

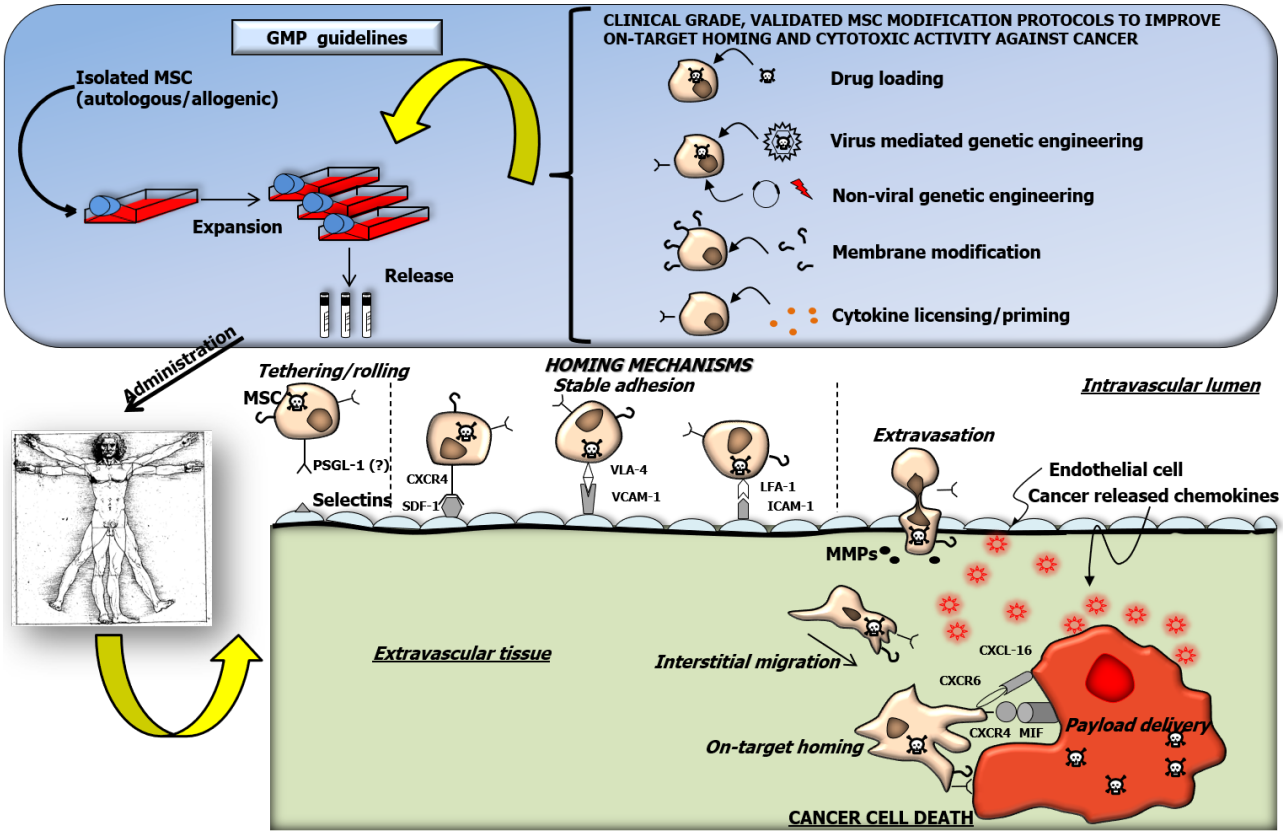

Figure 1 Graphic summary. A graphic simplified summary of mesenchymal stem stromal cells (MSC) *ex vivo* handling and of possible cell modification strategies under good manufacturing practice regulations is reported in Figure 1. In particular, the possibility of improving MSC homing capacity through viral/non-viral genetic engineering, membrane modification and cytokine licensing/priming is reported. In parallel, genetic engineering and direct drug loading are illustrated as a mean of inducing a cytotoxic phenotype in MSC. In the lower section of the figure, relevant molecular mechanisms controlling distinct phases (tethering/rolling, firm adhesion, extravasation, interstitial migration) of the homing process to the cancer mass, potentially occurring after systemic administration of modified MSC to human patients are illustrated. PSGL-1: P-selectin glycoprotein ligand-1; CXCR4: C-X-C chemokine receptor type 4; SDF-1: Stromal derived factor-1; VLA-4: Very late antigen-4; VCAM-1: Vascular cell adhesion molecule 1; LFA-1: Lymphocyte function-associated antigen 1; ICAM-1: Intercellular adhesion molecule 1; MMPs: Metalloproteases; CXCR6: C-X-C chemokine receptor type 6; CXCL16: C-X-C motif ligand 16; MIF: Macrophage migration inhibitory factor; MSC: Mesenchymal stem stromal cells; GMP: Good manufacturing practice.

Firmly adhering MSC can extravasate crossing the inflamed endothelium mainly through paracellular and transcellular diapedesis[41]. Inflammation elicited activation and secretion of metalloproteases (MMP) plays a crucial role in this step, paving the way to final interstitial migration toward the target site[41,43]. Together with CXCR4, MMP-2 is involved in MSC tropism to subcutaneous and lung metastatic prostate tumors *in vitro*[44].

Final MSC migration toward the target site occurs in response to various and poorly defined chemotactic stimuli released by inflamed tissues. Interaction between CXCR4 and SDF-1 was proven to be important for MSC final nesting within bone marrow[45]. Interestingly, CXCR4 binding to macrophage migration inhibitory factor released by cancer cells, was considered as one of the dominant signals regulating MSC homing into the tumor microenvironment: In fact, downregulation of either macrophage migration inhibitory factor or CXCR4 abrogated MSC *in vivo* migration to pulmonary tumor metastasis[46]. Additional receptors expressed by MSC were shown to be involved in their cancer homing capacity: Through paired CXCR4 and CXCR7 interaction with SDF-1, MSC can get trapped in the lung and, in turn, they can migrate toward pulmonary cancer nodules[47]. Such evidence was confirmed by subsequent work showing that CXCR7 promotes MSC adhesion and migration toward osteosarcoma cells *in vitro*[48]. Pathways, *e.g.*, C-X-C motif ligand 16 binding with the CXCR6 receptor expressed by MSC, can mediate cell docking into tumor masses[49]. Further knowledge of molecular mechanisms mediating specific migration to the cancer mass could contribute to improving the effectiveness of MSC therapeutic potential.

CLINICAL APPLICATIONS

Due to their biological properties, MSC can be used for therapeutic applications in humans. In 2020, more than 1100 clinical trials were registered at the clinicaltrial.gov database, with a steep increase from 2005[50]. The majority (around 50%) of such studies was focused on traumatology, pneumology and neurology fields. The results were disclosed and published only in a relatively small fraction of registered clinical trials. Improved cardiac function was demonstrated after MSC administration in clinical settings of dilated cardiomyopathy[51,52] and heart failure[53]. Encouraging results were also reported in cartilage lesions and osteoarthritis studies, in which pain reduction and joint function amelioration were demonstrated following application of MSC[54]. Strikingly, MSC were also proposed as a potential therapy against coronavirus disease 2019[55] and preliminary encouraging reports were published[56]. MSC were also administered in cancer clinical trials to reduce steroid-resistant graft-*versus*-host disease and successful results were reported both in adult and pediatric patients[57-59]. In a recently published work, iPSC derived MSC, obtained by non-integrating episomal reprogramming, were successfully applied in a clinical trial against graft-*versus*-host disease[60]. MSC co-transplantation with hematopoietic stem cells was also shown to be a potentially effective and safe treatment to improve engraftment in children and adolescents with severe aplastic anemia[61]. MSC were used in a few clinical trials as a therapeutic product directly aimed at counteracting solid cancer progression (Table 1). Results were published only from a minor number of such studies. In the TREAT-ME study, genetically modified autologous MSC induced disease stabilization in 5 out of 10 patients suffering from end-stage gastrointestinal tumors even though immunological or cancer markers were not clearly affected. Similarly, modified MSC were administered in adults and children affected by neuroblastoma and stabilization of neoplastic progression was demonstrated in a subgroup of patients. The above mentioned results derived from preclinical experiments or from studies performed in human patients suggest that mesenchymal stem cells can be a clinically relevant therapeutic option in different disease conditions, but significant efforts are required to obtain satisfactory results, especially in human cancer patients.

MSC EXPANSION FOR CLINICAL USE

As previously mentioned, MSC precursors can be obtained from different human source tissues such as bone marrow, adipose tissue, cord blood or Wharton jelly. Upon isolation, the absolute number of cells is not sufficient for clinical applications in humans. To obtain a sufficient amount of cells to be administered as an autologous or allogenic Advanced Cell Therapy Product, *ex vivo* cell expansion is mandatory. When intended for therapeutic applications, MSC must be isolated and cultured in accordance with good manufacturing practice (GMP) rules for medicinal products (European Cgmp-Annex 1: Manufacture of sterile medicinal products). For this reason, procedures must be performed in appropriate facilities allowing strict control of environmental air quality. Contamination levels of environments are classified from the cleanest "A" to "D". Authorized personnel can progressively access from external not-classified areas to class "B" operational rooms wearing disposable sterile coats. Class "A" air contamination level is obtained by taking advantage of a sterile laminar flow biological cabinet that must be located within the class B environment. Maintenance of air quality within defined standards is obtained by setting positive pressure differences between the highest and lowest classified areas. Rigorous environmental microbiological tests must be routinely performed to demonstrate compliance with required standards. The cell product must be manipulated with validated procedures and standardized quality control tests must be carried out in order to warrant product safety, identity and compliance with intended use[62,63]. Only GMP certified devices, disposables and growth media can be used within the production pipeline. Reagents and additives must be non-toxic, highly standardized, and they must not contain animal derived components (European cGMP-Annex 1: Manufacture of sterile medicinal products). The final product can be "released" for human applications, only after approval by an authorized Qualified Person, carefully checking full compliance with defined requirements and standards.

In a recently published work, we focused on the identification of a substitute for fetal bovine serum, as a source of growth factors to promote cell expansion[64]: The adoption of such an animal derived additive is, in fact, not recommended for GMP

Table 1 Human mesenchymal stem cells based clinical trials for solid tumors

Clinical trial ID	Source of MSC	Diagnosis	Trial phase	Route of administration	Cell product name (modifying factor)	Status
NCT03298763	Umbilical cord MSC (not specified)	Adenocarcinoma of lung	I/II	Intravenous	MSCTRAIL (TRAIL)	Recruiting
NCT02530047	Bone marrow MSC (not specified)	Ovarian cancer	I	Intraperitoneal	MSC-INFβ (INF-β)	Completed
NCT02068794	ASC (not specified)	Ovarian, primary peritoneal or fallopian tube cancer	I/II	Intraperitoneal	(MV-NIS)	Recruiting
NCT02079324	Not specified (not specified)	Head and neck cancer	I	Intratumoral	GX-051 (IL-12)	Unknown
NCT04657315	Not specified (not specified)	Recurrent glioblastoma	I/II	Intratumoral	MSC11FCD (CD)	Not yet recruiting
NCT01983709	Bone marrow MSC (allogenic)	Prostate cancer	I	Intravenous	Not modified	Terminated
NCT02008539	Bone marrow MSC (autologous)	Advanced gastrointestinal cancer	I/II	Infusion	MSC_apceth_101 (HSV-TK)	Terminated
2015-000520-29	Bone marrow MSC (allogenic)	Advanced gastrointestinal adenocarcinoma	I/II	Intravenous	MSC_apceth_111	Prematurely ended
NCT01844661	Bone marrow MSC (autologous)	Metastatic and refractory solid tumors	I/II	Intravenous	CELYVIR (ICOVIR5)	Completed
2019-001154-26	Bone marrow MSC (allogenic)	Relapsed or refractory extracranial solid tumors	I	Intravenous	AloCELYVIR (ICOVIR-5)	Recruiting
NCT04758533	Bone marrow MSC (allogenic)	Diffuse intrinsic pontine glioma or medulloblastoma	I/II	Infusion	AloCELYVIR (ICOVIR-5)	Not yet recruiting

Table 1 reports data regarding selected clinical trials investigating the impact of modified mesenchymal stem cells (MSC) against solid tumors. Beside the clinical trial identification code (Clinical trial ID), were reported (from left to right): Source of MSC and the related use in brackets (Source of MSC), diagnosis of enrolled patients (Diagnosis), clinical trial phase (Trial phase), route of cell administration to patients (Route of administration), cell product name and related modification approach [Cell product name (modifying factor)] and the actual trial status (Status). MSC: Mesenchymal stem stromal cells; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; INFβ: Interferon beta; MV-NIS: Oncolytic measles virus encoding thyroidal sodium iodide symporter; IL-12: Interleukin-12; CD: Cytosine deaminase; HSV-TK: Herpes simplex virus-thymidine kinase; ICOVIR5: Modified oncolytic adenovirus.

compliant cell therapy production protocols. We took advantage of a supernatant rich in growth factors (SRGF) derived from a platelet apheresis product[65] in which the coagulation cascade was triggered by the addition of a standardized concentration of $CaCl_2$. We previously demonstrated that SRGF is characterized by elevated concentrations of crucial growth factors involved in cell cycle progression such as platelet derived growth factor isoforms AA, AB, and BB, as well as epidermal growth factor and fibroblast growth factor[64]. SRGF was shown to increase, when compared to fetal bovine serum, the proliferation rate of ASC also at extended passages, without affecting cell phenotype, differentiation and clonogenic potential, as well as karyotype stability[64]. Of note, by exposing ASC to a medium containing 5% SRGF we obtained in less than two weeks the same cell yield reached when expanding cells for two months in the presence of 10% fetal bovine serum. Growth factor concentrates derived from platelets can also be obtained by other means *e.g.*, repeated freeze and thaw cycles to disrupt platelet cell membranes, and such a platelet lysate was previously shown to efficiently surrogate fetal bovine serum in GMP compliant culture[66]. We also demonstrated that, when compared to a platelet lysate, SRGF induced a higher bone marrow MSC proliferation rate: This effect was reasonably shown to be mediated by increased platelet derived growth factor concentrations in SRGF[67]. As previously mentioned, standardization of ancillary medium additives is fundamental for GMP guidelines in order to warrant a safe and consistent product expansion. Pooling together single donor derived platelet products can efficiently minimize biological variability between medium additive batches[68], but the definition of the optimal pool size is not trivial, especially for academic GMP facilities. We demonstrated that to

obtain stable SRGF batches, that equally stimulate MSC proliferation rate, at least 16 different SRGF products derived from single donors must be mixed together[69]: To achieve this aim, we adopted a predictive mathematical approach, followed by "wet biology" validation. In order to identify, in compliance with GMP requirements, a reliable and comprehensive quality control assay for SRGF, we manufactured from platelet concentrates several medium additive types differently promoting ASC growth rate[70]. Interestingly, while integrative analysis of growth factor concentration changes was shown to be insufficiently sensitive, ^{1}H-NMR and MALDI-TOF MS could clearly identify differences between product isoforms. Thus, we concluded that a single analysis using such metabolomic approaches could rapidly predict and classify the potential biological activity of our GMP compatible ancillary product.

MSC HOMING IMPROVEMENT STRATEGIES

MSC can be administered *in situ* (intramuscular or direct injection) or by systemic infusion (intravenous, intraarterial)[71]. Systemic administration can be easily performed as it allows for rapid product availability for the entire organism: These are clear advantages, especially in cancer patients. Nevertheless, intrinsic homing properties of MSC are limited, especially after *in vitro* expansion[72]: Only a very small percentage of the infused cells can home to targets[73] and, in a clinical trial, expanded MSC failed to be detected within prostate cancer masses after systemic administration in patients[74]. Topical applications can circumvent limitations linked to restricted naïve MSC homing properties: Even though injection modality and flow rate were previously investigated[75], a standardized and appropriate local cell delivery approach was not yet defined. Local MSC injection in cancer patients is limited to surgically accessible neoplastic lesions; thus, cell modification approaches improving systemically administered MSC homing capacities are required. When planning the best technical procedures aimed to potentially improve MSC features, restrictions related to GMP requirements for clinical grade cell production must be strictly adhered to. In the next sections, we will report selected evidence derived from preclinical studies that involved potential homing improving methods in compliance with future GMP applications.

Genetic modifications

Genetic modification is one of the most frequently used approaches to tailor MSC properties: MSC are prone to infection with high efficiency by replication-deficient recombinant viruses leading to increased expression of a selected protein[76,77]. Adenoviruses, retroviruses and lentiviruses are used to induce stable expression of the exogenous protein through integration in the host genome, while insertion fails to occur when using baculoviruses[78]. While high transduction efficiency can encourage the use of viral gene editing systems, the possible insertional mutagenesis secondary to integration in the patient's genome could increase the risk of cell transformation [79]. In addition, virus mediated application in gene editing could lead to undesired immune responses in patients[80]. Elevated costs of virus production and management as well as regulatory requirements, may represent a constraint to the obtainment of a genetically modified cell therapy product. Interestingly, MSC can also be modified by non-viral approaches[81]. Such approaches can circumvent virus related drawbacks, but transfection efficiency is known to be poor: Technical protocols improving such limitations and maintaining compliance to GMP rules, are required [81].

Both viral and non-viral methods are accepted for application in GMP compliant clinical settings: Examples of preclinical investigations regarding both approaches are reported below. As mentioned above, SDF-1 interaction with the chemokine receptor CXCR4 is known to guide MSC migration to the target site in bone defects[82]. Overexpression of CXCR4 gene by lentivirus, enhanced MSC *in vitro* migration to osteosarcoma and this effect was demonstrated to occur through the Phosphoinositide 3-kinase/Protein kinase B/Nuclear Factor kB signaling pathway[83]. Non-viral overexpression of CXCR4 increased in a dose-dependent manner the migration capacity of MSC toward glioblastoma cells both *in vitro* and in a human malignant glioma xenograft model[84]. Interestingly, reduced MSC interaction with osteosarcoma and hepatocellular carcinoma cells secondary to selective inhibition of CXCR4 strengthens the role of such receptors in the regulation of MSC migratory capacity[85]. Nevertheless, a previously published work showed that, even though CXCR4 inhibition impaired MSC migration, its viral overexpression failed to promote *in vitro*

transmigration toward glioma cancer conditioned medium, as chemoattractant[86]. Thus, the exact involvement of CXCR4 in MSC migration toward cancer cells was not univocally clarified. Identification of selected homing controlling factors, whose non-viral overexpression could improve MSC targeting to cancer masses, could simplify the GMP compliant obtainment of a cell product with ameliorated therapeutic effectiveness.

Cell membrane modification

Specific targeting or adhesion moieties can be added, by different means, to the cell membrane of expanded MSC. In principle, using certified reagents and performing an appropriate product validation, membrane modification can be performed in compliance with GMP guidelines. As mentioned above, selectin mediated rolling is a crucial and rate limiting step in the cell adhesion process[31,33]. In order to increase the fraction of rolling cells in dynamic conditions, in a seminal work by Sackstein *et al* [34], the normally expressed CD44 antigen on MSC was converted, by alpha-1,3-fucosyltransferase, to E-selectin/L-selectin ligand (HCELL), which is expressed in bone marrow hematopoietic stem cells. In addition, HCELL over expression increased MSC trans-endothelial migration[87]. Furthermore, covalent modifications or lipidic particles addition were adopted[35] to load biotin on the MSC cell surface, as a docking site for specific streptavidin-bound ligands: Using such strategies, MSC were decorated with the active integrin binding factor SLeX to improve cell-substrate interaction in *in vitro* dynamic flow conditions. Furthermore, palmitated protein A/G as well as bi-specific antibodies were used to enrich MSC membranes with specific antigens or receptors improving the migratory properties of MSC[88]. Palmitic acid conjugated peptides can be easily coated on MSC membranes to tailor their homing potential[89,90]. To our knowledge, even though deserving investigation, the efficacy of such cell membrane modification protocols has not yet been tested as a strategy to improve the fraction of MSC selectively homing to cancer.

In vitro priming

As mentioned above, MSC behavior can be modulated by the so called "licensing" approach, *i.e.*, cell exposure to selected cytokine(s) in culture. This simple approach was included in the present review as, running appropriate validation and quality controls, it could be easily translated to production processes under GMP guidelines. MSC priming was previously investigated to direct cells toward a sharp anti-inflammatory phenotype[26] and can be applied to tailor and ameliorate general migration and homing properties of MSC. Incubating MSC in the presence of appropriate TNF-α concentrations can, in fact, trigger the enhanced expression of CXCR4[91], in turn potentially ameliorating the homing efficiency of such cells. MSC pre-exposure to TNF-α was also shown to improve MSC adhesion to endothelial cells *in vitro* and in rat ischemic hind limbs, through upregulation of VCAM-1[92]. Similarly, TNF-α preconditioned MSC could better migrate *in vitro* toward selected chemokines such as the above-mentioned SDF-1, but this effect could not be correlated to CXCR4 expression levels[93]. In parallel, migration of MSC was also shown to be enhanced by exposure to transforming growth factor beta (TGF-β)[94], even though, in other studies[95], the same cytokine was also shown to downregulate migration of MSCs in response to SDF-1 stimuli. Interestingly, pre-exposure of MSC to TGF-β resulted in enhanced CXCR4 mediated migration toward glioblastoma cells[96]. The migration rate of interleukin (IL) 1β primed MSC was enhanced through upregulation of CXCR4 expression[97,98] and through increased expression of MMP-1 and MMP-9[99]; by contrast previous work reported that IL-1β did not improve MSC trans-migration potential[93]. Interestingly, supplementation of growth medium with IFN-γ[100] and insulin-like growth factor-1[101] increased MSC migration capacity toward chemokines released within inflamed tissues. Similarly, a blend of different factors such as fms-related tyrosine kinase 3 ligand, stem cell factor, IL-3 and IL-6 as well as hepatocyte growth factor[102] increased MSC migration toward SDF-1 as a chemoattractant. In an interesting published work[103], the authors demonstrated that transient exposure of MSC to conditioned medium from glioma cells increased MSC migration potential toward glioblastoma itself, both *in vitro* (static and microfluidic conditions) and *in vivo* (mouse model). In the same work, the authors showed that the conditioned medium contained higher levels of IFN-γ, IL-6, IL-8 and TNF-α.

In addition, preventive exposure of MSC to valproic acid[104], as well as to erythropoietin and granulocyte colony-stimulating factor[105] was shown to ameliorate their homing properties toward inflamed tissues. Finally, culturing MSC in hypoxic conditions increased the number of migrating MSC as a consequence of hypoxia inducible factor-1α and SDF-1 overexpression[106]. The aforementioned

evidence suggests that appropriately priming MSC in culture can improve their capacity to reach inflamed tissues after systemic administration. Considering cancer as a never-healing wound that secretes inflammatory cytokines and chemotactic factors (*e.g.*, monocyte chemotactic protein-1, SDF-1, TGF-β, TNF-α, ILs), MSC licensing can be considered a potentially GMP compatible and simple option to improve MSC homing toward tumor masses[107,108].

We recently demonstrated that modification of culture conditions can improve ASC homing properties *in vitro*: We showed that, when compared to fetal bovine serum expanded MSC, SRGF cultured cells could better adhere in microfluidic conditions on a layer of fibrosarcoma (HT1080) or glioblastoma (T98G) cells[109]. Cell interaction with selected cancer tissues was shown to be specific because MSC expanded using SRGF additive displayed lower affinity for hepatocarcinoma cells and for unspecific interaction sites, *i.e.*, mixed extracellular matrix proteins[109]. We also showed that cell activation, evidenced by intracellular calcium concentration changes, occurred upon the adhesion of SRGF expanded ASC on cancer cells and extracellular matrix proteins [109].

ARMING MSC TOWARD CANCER

As previously reviewed[110], unmodified expanded or naïve endogenous MSC can play a dual role towards cancer cells. MSC were previously shown to support tumor expansion directly, by playing an antiapoptotic role[111] or indirectly, by suppressing, patient immune responses against tumor cells, upon release of soluble mediators[112]. Moreover, MSC were shown to promote angiogenesis[113] and epithelial-to-mesenchymal transition[114] in turn favoring invasion and metastasis[115,116]. MSC are involved in the architecture of the tumor stroma where they can become intra-tumor associated fibroblasts[44] promoting drug resistance[117] or leading to higher nodule formation in mice[118]. Interestingly, in a previous paper, iPSC derived MSCs, when compared to adult bone marrow MSC, were characterized by a weaker capacity to promote cancer cell growth and invasion *in vitro*[119]. On the other hand, unmodified MSC were also shown to actively counteract cancer expansion. In particular, MSC were demonstrated to induce cell cycle arrest in hepatoma cells *in vitro* and *in vivo*, promoting p21 expression[120] and such cells were shown to trigger apoptosis through caspase-3 and caspase-9 induction in cancer cells[121]. When expanded at high density, MSC overexpressed IFN-β, which directly induced *in vitro* breast cancer cell death[122]. In an attempt to apply MSC as a therapeutic tool against cancer, such a potentially bivalent role toward tumor cells must be clearly overcome, by introducing appropriate cell modifications that confer an unequivocal on-target cytotoxic behavior. In the following sections, we will discuss selected approaches to modify MSC that, in principle, could rapidly be translated to clinical applications, following GMP rules.

MSC as tools for chemotherapeutic drug delivery

After transient exposure in culture vessels, MSC can uptake chemotherapeutic drugs such as doxorubicin, paclitaxel, or gemcitabine[123]. Following drug removal, MSC can locally release their payload by passive diffusion, and exosome secretion[124] in turn inducing cancer cell death. Thus, after migration and homing toward cancer cells, MSC can release active substances in the tumor stroma, inducing localized cancer cytolysis.

Doxorubicin loaded MSC were effective against breast and thyroid cancer *in vitro* and *in vivo* in mice[125] as well as in counteracting oral squamous cell carcinoma[126]. MSC exposure to nanoparticles with adsorbed doxorubicin was adopted as a strategy to control drug release: Such an approach was effective in reducing the proliferation of breast cancer, lung melanoma metastasis and glioblastoma in mice[127,128]. Purified exosomes obtained from doxorubicin loaded MSC were shown to be a potentially effective cell-free targeted therapy against osteosarcoma cells[129]. Furthermore, linking doxorubicin-loaded liposomes on MSC outer membranes, a specific cytotoxic effect against colon adenocarcinoma was observed *in vitro* and in mice, with a limited impact on MSC as carrier cells[130].

Paclitaxel loaded MSC were shown to be effective against pancreatic[131] and brain cancer[132], as well as squamous cell carcinoma[126], mesothelioma[133], metastatic lung cancer[47] and leukemia[134]. In a recent work[135], drug pharmacokinetics and pharmacodynamics after administration of MSC containing paclitaxel loaded nanoparticles were analyzed, and the authors demonstrated that mouse orthotopic human lung tumors were completely eradicated after administration of 2×10^6 MSC

(equivalent to 50 μg or 2.5 mg/kg of paclitaxel). In analogy, MSC containing paclitaxel loaded nanoparticles were shown *in vitro* and *in vivo* to be a promising treatment for glioma and lung carcinoma targeted therapy[136,137]. Moreover, functionalization of MSC cell membranes with a transcription activating peptide, improved intracellular accumulation of nanoparticles in MSC as well as paclitaxel mediated cytotoxic activity against target lung cancer cells[138].

Furthermore, gemcitabine-releasing MSC were able to inhibit the growth of human pancreatic cancer[139] and of squamous cell carcinoma[126] without altering MSC multi-lineage differentiation potential and surface marker expression pattern[140].

Induction of MSC cytotoxic phenotype by lentiviral transduction

Taking advantage of recombinant lentiviruses, MSC can be modified to over express cytotoxic proteins to kill cancer cells after MSC specific homing. As previously mentioned, this approach could be compliant with GMP rules but its potential therapeutic efficacy was previously tested mainly *in vitro* and in animal models. Administration of MSC over expressing TRAIL by lentivirus transduction were shown to reduce the growth of pancreatic cancer and sarcomas[141,142] as well as colorectal carcinoma[143]. Similarly, MSC modified to actively secrete IFN-γ, induced apoptosis in lung tumor cells through caspase-3 activation[144]. Moreover, administration of MSC in which the IFN-β was transduced could lower brain tumor expansion[77] and similarly modified cells could specifically target lung cancer lesions[145] in mice. Interestingly, IL-18 and IFN-β lentiviral overexpression synergically inhibited tumor growth in a rat intracranial glioma model[146].

MSC were previously transduced by lentiviral or retroviral vectors to induce the expression of herpes simplex virus-thymidine kinase (HSV-TK), an enzyme converting the prodrug ganciclovir to is triphosphate toxic metabolite: After systemic administration of transduced MSC together with ganciclovir, efficient suppression of tumor growth was observed in implanted glioma cells[147-149]. Retroviral approaches were also used in MSC to induce the expression of cytosine deaminase::uracil phosphoribosyltransferase (CD::UPRT), the enzyme that converts 5-fluorocytosin (5-FC) to an active drug[150]: Such modified MSC actively inhibited prostate cancer growth after intravenous administration in mice. Retroviral MSC modification with HSV-TK, combined with CD::UPRT, synergically counteracted the growth of breast cancer cells and related lung metastases in mice[151]. MSC were also engineered by a lentivirus to play a localized anti-angiogenic role within cancer masses through the secretion of fms-like tyrosine kinase-1; this modification inhibited tumor growth and prolonged survival in a mouse hepatocarcinoma model[152]. After intravenous administration, lentivirus treated MSC co-expressing the angiogenesis inhibitor kringle 5 of human plasminogen and the human sodium-iodide symporter (involved in radioisotope uptake), decreased tumor growth and improved the survival rate of glioblastoma bearing mice[153]. MSC, transduced with the hepatocyte growth factor inhibitor NK4, suppressed the growth of gastric cancer xenografts[154] after systemic administration and this effect was also mediated by impaired intra-tumoral vascularization.

Locally released exosomes from MSC, modified by lentivirus infection to upregulate microRNA (miR) miR-199a or miR-124a, improved hepatocellular carcinoma sensitivity to doxorubicin and eradicated brain cancer in preclinical animal models, respectively[155,156].

MSC modification by adenovirus and baculovirus transduction

In addition to lentiviruses, MSC engineering can be performed in GMP compatible conditions by also taking advantage of recombinant adenovirus infection potential. MSC overexpressing the proinflammatory IL-21 were shown in mice to efficiently counteract disseminated B-cell lymphoma through induction of systemic immunity [157].

Adenoviral transduced TRAIL expression in MSC have shown antitumor effects on esophageal cancer xenografts in mice[158] and, similarly, NK4 modified MSC inhibited liver cancer growth and migration in animal models[159]. MSC transduced to express HSV-TK and TRAIL, induced long-term remission of murine metastatic renal cell carcinoma after three injections (100% survival of tumor-bearing mice)[160]. In comparison, systemic administration of IL-2, IL-12 or IL-18 overexpressing MSC by adenoviral transduction, reduced cancer masses and improved survival after administration in a glioma murine model[161,162].

Similarly, injection of MSC in which the expression of HSV-TK was induced by baculovirus-based transduction, inhibited tumor growth and prolonged survival in glioblastoma-bearing mice[163]. Interestingly, in a recent paper, a hybrid baculovirus vector containing key transfection enhancing elements of adeno-associated viruses

was defined as a promising targeted-delivery vehicle to counteract hypopharyngeal carcinoma[164].

MSC as oncolytic adenovirus carriers

MSC were shown to be efficient delivery vehicles for oncolytic adenoviruses directed against gliomas[165]. In particular, MSC loaded with the oncolytic adenovirus Delta-24-RGD could eradicate murine glioblastomas[166] and the same approach was applied in healthy dogs to demonstrate its technical feasibility in a more complex model[167]. Oncolytic adenoviruses delivered by MSC efficiently challenged hepatocellular carcinomas with reduced toxicity in healthy liver tissues[168]. Appropriately modified MSC to support viral replication were loaded with an oncolytic adenovirus expressing p14 and p53: Such engineered cells efficiently suppressed prostate cancer progression in mice[169]. Similarly, MSC loaded with a cytolytic adenovirus, additionally expressing TRAIL, efficiently counteracted pancreatic cancer cells *in vitro* and in xenografted live chick embryos[170]. Administration of MSC carrying an adenoviral oncolytic virus with the addition of a replication defective vector encoding inducible caspase-9, enabled efficient antitumor activity in a non-small-cell lung cancer murine model and improved overall survival[171]. In a clinical trial, involving advanced metastatic neuroblastoma pediatric patients, autologous MSC carrying an oncolytic adenovirus were safely administered and disease stabilization occurred in nearly half of patients[37].

Arming MSC by non-viral genetic modification approaches

MSC can be successfully engineered through non-viral vectors achieving transient but sustained gene overexpression. Infusion of MSC overexpressing TRAIL through non-viral vectors were shown to efficiently induce pancreatic or liver cancer cell death[172, 173]. In a murine melanoma model, significant cancer mass reduction was obtained by MSC stably overexpressing IFN-γ through a non-viral method involving PhiC31 recombinase and piggyBac transposase[174].

In mice, intravenously applied MSC transfected to express HSV-TK, reduced primary pancreatic tumor growth and the incidence of metastases[175] and, after tissue specific expression, inhibited expansion of hepatocellular carcinoma cells[176]. In a mouse model, pulmonary cancer nodules were efficiently targeted by MSC induced to express CMV-TK by non-viral methods[177]. Polyethylenimine based polymers were used to transiently engineer MSC with HSV-TK, together with TRAIL: These modified cells were effective *in vitro* and *in vivo* against glioma through increased apoptosis and reduced angiogenesis[178]. MSC expressing CDy::UPRT by the same transfection method significantly inhibited *in vivo* temozolomide resistant glioma tumors[179] as well as 5-fluorouracil resistant colorectal adenocarcinoma cells [180].

In addition, bone morphogenetic protein 4 overexpression achieved by a non-viral method was demonstrated to induce a reduction of brain tumor cell growth in rats, after intranasal administration and homing within the tumor mass[181]. Interestingly, the same study showed that bone morphogenetic protein 4 engineered MSC treatment significantly improved survival of tumor bearing rats.

Transfected MSC can deliver growth inhibiting miR to tumors: In particular, by direct intercellular communication or locally releasing microvesicles, MSC were demonstrated to transport anti-miR-9 to glioblastoma cells, in turn reversing drug resistance in these cells[182]. Similarly, recent studies have shown that exosomes released from MSC containing elevated amounts of miR-381-3p, miR-34a, miR-193a and miR146a were effective against triple negative breast cancer, non-small cell lung carcinoma and ovarian cancer[183-186].

CONCLUDING REMARKS

In this review, we briefly reported the biological features of MSC, focusing on cell properties and on mechanisms that could play a crucial role in MSC applications for cancer therapy. The importance of MSC modification to improve their naïve homing properties and to induce a clear cytotoxic behavior was also discussed. Such features are not the only parameters potentially affecting the final clinical outcome related to MSC administration in patients: A thorough discussion regarding this issue is beyond the scope of this review. Briefly, the impact on MSC therapeutic performance mediated by cell origin, expansion protocol, and dosage has not previously been defined[71, 187]. ASC and MSC derived from bone marrow share several biological features and

they are both frequently applied in clinical trials. In particular, ASC as well as the stromal vascular fraction derived from adipose tissue, are often used for regenerative medicine purposes[188-190], while bone marrow MSC are principally adopted to counteract, among others, graft-*versus*-host disease[57] or acute renal failure (NCT01275612) in cancer clinical trials. However, the clinical efficacy of bone marrow MSC and of ASC was never compared in the same experimental study. Even if iPSC are to be characterized by great expansion potential[60], such MSC applications in humans are still at very early development stages. Expansion conditions, *e.g.*, cell seeding density[191] or culture medium additives[109], are known to affect MSC properties but the optimal production approach was not defined in relation to the desired clinical applications. Introduction of automated cell expansion protocols should be strongly encouraged, as it can improve reproducibility of cell growth in GMP environments[192]. Expanded MSC were previously administered in patients in a wide dosage range (from 1 to 4 million cells/kg) by single or multiple administrations[193]. A potentially appropriate minimal effective dose of MSC was previously proposed by analyzing published clinical trial results[187]: The authors suggested that clinical benefits were evident when 100-150 million cells/patient were systemically administered. Significant clinical effects were not registered when less than 70 million cells/patient or, interestingly, over 200 million cells/patient were administered[187].

In addition to the parameters requiring standardization, as stated above, core MSC properties requiring amelioration to improve their clinical effectiveness in tumor patients are homing potential and the capacity to actively counteract cancer growth. In this review, we reported the efficacy of published preclinical modification protocols aimed at improving such MSC features. Approaches were selected as they were considered potentially suitable for future translation to cell therapy production, in compliance with GMP guidelines. We can hypothesize that both modifications improving homing and cancer killing activity of MSC should be introduced in the same cell therapy product. The definition of comprehensive GMP compliant protocols could allow safe translation to clinical trials in humans.

CONCLUSION

In conclusion, it is agreed that MSC represent a powerful weapon against cancer but significant efforts are needed to introduce in human clinical trials combinations of relevant MSC modification protocols that were shown to be effective in preclinical studies. The study design of such experimental campaigns in human patients should be highly standardized in order to allow comparison and critical discussion of obtained positive or negative results.

ACKNOWLEDGEMENTS

We are grateful to Dr. Gonzalo Almanza (The Laboratory of Immunology, Department of Medicine and Moores Cancer Center, University of California, San Diego, La Jolla, California) for careful manuscript editing as a native English speaker.

REFERENCES

1 **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]

2 **Friedenstein AJ**, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation* 1974; **17**: 331-340 [PMID: 4150881 DOI: 10.1097/00007890-197404000-00001]

3 **Charbord P**. Bone marrow mesenchymal stem cells: historical overview and concepts. *Hum Gene Ther* 2010; **21**: 1045-1056 [PMID: 20565251 DOI: 10.1089/hum.2010.115]

4 **Jiang Y**, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002; **418**: 41-49 [PMID: 12077603 DOI: 10.1038/nature00870]

5 **Pittenger MF**, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science*

1999; **284**: 143-147 [PMID: 10102814 DOI: 10.1126/science.284.5411.143]
6 **Caplan AI**. Mesenchymal stem cells. *J Orthop Res* 1991; **9**: 641-650 [PMID: 1870029 DOI: 10.1002/jor.1100090504]
7 **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]
8 **da Silva Meirelles L**, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006; **119**: 2204-2213 [PMID: 16684817 DOI: 10.1242/jcs.02932]
9 **Fei X**, Jiang S, Zhang S, Li Y, Ge J, He B, Goldstein S, Ruiz G. Isolation, culture, and identification of amniotic fluid-derived mesenchymal stem cells. *Cell Biochem Biophys* 2013; **67**: 689-694 [PMID: 23508888 DOI: 10.1007/s12013-013-9558-z]
10 **Harris DT**. Umbilical cord tissue mesenchymal stem cells: characterization and clinical applications. *Curr Stem Cell Res Ther* 2013; **8**: 394-399 [PMID: 23895058 DOI: 10.2174/1574888x11308050006]
11 **Mennan C**, Wright K, Bhattacharjee A, Balain B, Richardson J, Roberts S. Isolation and characterisation of mesenchymal stem cells from different regions of the human umbilical cord. *Biomed Res Int* 2013; **2013**: 916136 [PMID: 23984420 DOI: 10.1155/2013/916136]
12 **Roufosse CA**, Direkze NC, Otto WR, Wright NA. Circulating mesenchymal stem cells. *Int J Biochem Cell Biol* 2004; **36**: 585-597 [PMID: 15010325 DOI: 10.1016/j.biocel.2003.10.007]
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 **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]
15 **Lian Q**, Zhang Y, Zhang J, Zhang HK, Wu X, Lam FF, Kang S, Xia JC, Lai WH, Au KW, Chow YY, Siu CW, Lee CN, Tse HF. Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. *Circulation* 2010; **121**: 1113-1123 [PMID: 20176987 DOI: 10.1161/CIRCULATIONAHA.109.898312]
16 **Park SJ**, Kim RY, Park BW, Lee S, Choi SW, Park JH, Choi JJ, Kim SW, Jang J, Cho DW, Chung HM, Moon SH, Ban K, Park HJ. Dual stem cell therapy synergistically improves cardiac function and vascular regeneration following myocardial infarction. *Nat Commun* 2019; **10**: 3123 [PMID: 31311935 DOI: 10.1038/s41467-019-11091-2]
17 **Osugi M**, Katagiri W, Yoshimi R, Inukai T, Hibi H, Ueda M. Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects. *Tissue Eng Part A* 2012; **18**: 1479-1489 [PMID: 22443121 DOI: 10.1089/ten.TEA.2011.0325]
18 **Ciapetti G**, Granchi D, Baldini N. The combined use of mesenchymal stromal cells and scaffolds for bone repair. *Curr Pharm Des* 2012; **18**: 1796-1820 [PMID: 22352754 DOI: 10.2174/138161212799859648]
19 **Savkovic V**, Li H, Seon JK, Hacker M, Franz S, Simon JC. Mesenchymal stem cells in cartilage regeneration. *Curr Stem Cell Res Ther* 2014; **9**: 469-488 [PMID: 25005451 DOI: 10.2174/1574888x09666140709111444]
20 **Vonk LA**, van Dooremalen SFJ, Liv N, Klumperman J, Coffer PJ, Saris DBF, Lorenowicz MJ. Mesenchymal Stromal/stem Cell-derived Extracellular Vesicles Promote Human Cartilage Regeneration *In Vitro*. *Theranostics* 2018; **8**: 906-920 [PMID: 29463990 DOI: 10.7150/thno.20746]
21 **Jiang W**, Xu J. Immune modulation by mesenchymal stem cells. *Cell Prolif* 2020; **53**: e12712 [PMID: 31730279 DOI: 10.1111/cpr.12712]
22 **Ng J**, Hynes K, White G, Sivanathan KN, Vandyke K, Bartold PM, Gronthos S. Immunomodulatory Properties of Induced Pluripotent Stem Cell-Derived Mesenchymal Cells. *J Cell Biochem* 2016; **117**: 2844-2853 [PMID: 27167148 DOI: 10.1002/jcb.25596]
23 **Bernardo ME**, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* 2013; **13**: 392-402 [PMID: 24094322 DOI: 10.1016/j.stem.2013.09.006]
24 **Shi C**, Jia T, Mendez-Ferrer S, Hohl TM, Serbina NV, Lipuma L, Leiner I, Li MO, Frenette PS, Pamer EG. Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. *Immunity* 2011; **34**: 590-601 [PMID: 21458307 DOI: 10.1016/j.immuni.2011.02.016]
25 **Li W**, Ren G, Huang Y, Su J, Han Y, Li J, Chen X, Cao K, Chen Q, Shou P, Zhang L, Yuan ZR, Roberts AI, Shi S, Le AD, Shi Y. Mesenchymal stem cells: a double-edged sword in regulating immune responses. *Cell Death Differ* 2012; **19**: 1505-1513 [PMID: 22421969 DOI: 10.1038/cdd.2012.26]
26 **Krampera M**. Mesenchymal stromal cell 'licensing': a multistep process. *Leukemia* 2011; **25**: 1408-1414 [PMID: 21617697 DOI: 10.1038/leu.2011.108]
27 **Eggenhofer E**, Hoogduijn MJ. Mesenchymal stem cell-educated macrophages. *Transplant Res* 2012; **1**: 12 [PMID: 23369493 DOI: 10.1186/2047-1440-1-12]
28 **Maccario R**, Podestà M, Moretta A, Cometa A, Comoli P, Montagna D, Daudt L, Ibatici A, Piaggio G, Pozzi S, Frassoni F, Locatelli F. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing

a regulatory/suppressive phenotype. *Haematologica* 2005; **90**: 516-525 [PMID: 15820948]
29 **Ullah M**, Liu DD, Thakor AS. Mesenchymal Stromal Cell Homing: Mechanisms and Strategies for Improvement. *iScience* 2019; **15**: 421-438 [PMID: 31121468 DOI: 10.1016/j.isci.2019.05.004]
30 **De Becker A**, Riet IV. Homing and migration of mesenchymal stromal cells: How to improve the efficacy of cell therapy? *World J Stem Cells* 2016; **8**: 73-87 [PMID: 27022438 DOI: 10.4252/wjsc.v8.i3.73]
31 **Lawrence MB**, Bainton DF, Springer TA. Neutrophil tethering to and rolling on E-selectin are separable by requirement for L-selectin. *Immunity* 1994; **1**: 137-145 [PMID: 7534197 DOI: 10.1016/1074-7613(94)90107-4]
32 **Rüster B**, Göttig S, Ludwig RJ, Bistrian R, Müller S, Seifried E, Gille J, Henschler R. Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 2006; **108**: 3938-3944 [PMID: 16896152 DOI: 10.1182/blood-2006-05-025098]
33 **Lo CY**, Antonopoulos A, Dell A, Haslam SM, Lee T, Neelamegham S. The use of surface immobilization of P-selectin glycoprotein ligand-1 on mesenchymal stem cells to facilitate selectin mediated cell tethering and rolling. *Biomaterials* 2013; **34**: 8213-8222 [PMID: 23891082 DOI: 10.1016/j.biomaterials.2013.07.033]
34 **Sackstein R**, Merzaban JS, Cain DW, Dagia NM, Spencer JA, Lin CP, Wohlgemuth R. Ex vivo glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. *Nat Med* 2008; **14**: 181-187 [PMID: 18193058 DOI: 10.1038/nm1703]
35 **Sarkar D**, Spencer JA, Phillips JA, Zhao W, Schafer S, Spelke DP, Mortensen LJ, Ruiz JP, Vemula PK, Sridharan R, Kumar S, Karnik R, Lin CP, Karp JM. Engineered cell homing. *Blood* 2011; **118**: e184-e191 [PMID: 22034631 DOI: 10.1182/blood-2010-10-311464]
36 **Lau TT**, Wang DA. Stromal cell-derived factor-1 (SDF-1): homing factor for engineered regenerative medicine. *Expert Opin Biol Ther* 2011; **11**: 189-197 [PMID: 21219236 DOI: 10.1517/14712598.2011.546338]
37 **Melen GJ**, Franco-Luzón L, Ruano D, González-Murillo Á, Alfranca A, Casco F, Lassaletta Á, Alonso M, Madero L, Alemany R, García-Castro J, Ramírez M. Influence of carrier cells on the clinical outcome of children with neuroblastoma treated with high dose of oncolytic adenovirus delivered in mesenchymal stem cells. *Cancer Lett* 2016; **371**: 161-170 [PMID: 26655276 DOI: 10.1016/j.canlet.2015.11.036]
38 **Constantin G**, Majeed M, Giagulli C, Piccio L, Kim JY, Butcher EC, Laudanna C. Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* 2000; **13**: 759-769 [PMID: 11163192 DOI: 10.1016/s1074-7613(00)00074-1]
39 **Li P**, Liu F, Sun L, Zhao Z, Ding X, Shang D, Xu Z, Sun C. Chemokine receptor 7 promotes cell migration and adhesion in metastatic squamous cell carcinoma of the head and neck by activating integrin αvβ3. *Int J Mol Med* 2011; **27**: 679-687 [PMID: 21347514 DOI: 10.3892/ijmm.2011.628]
40 **Lin TH**, Liu HH, Tsai TH, Chen CC, Hsieh TF, Lee SS, Lee YJ, Chen WC, Tang CH. CCL2 increases αvβ3 integrin expression and subsequently promotes prostate cancer migration. *Biochim Biophys Acta* 2013; **1830**: 4917-4927 [PMID: 23845726 DOI: 10.1016/j.bbagen.2013.06.033]
41 **Teo GS**, Ankrum JA, Martinelli R, Boetto SE, Simms K, Sciuto TE, Dvorak AM, Karp JM, Carman CV. Mesenchymal stem cells transmigrate between and directly through tumor necrosis factor-α-activated endothelial cells *via* both leukocyte-like and novel mechanisms. *Stem Cells* 2012; **30**: 2472-2486 [PMID: 22887987 DOI: 10.1002/stem.1198]
42 **Wu TY**, Liang YH, Wu JC, Wang HS. Interleukin-1*β* Enhances Umbilical Cord Mesenchymal Stem Cell Adhesion Ability on Human Umbilical Vein Endothelial Cells *via* LFA-1/ICAM-1 Interaction. *Stem Cells Int* 2019; **2019**: 7267142 [PMID: 31949440 DOI: 10.1155/2019/7267142]
43 **De Becker A**, Van Hummelen P, Bakkus M, Vande Broek I, De Wever J, De Waele M, Van Riet I. Migration of culture-expanded human mesenchymal stem cells through bone marrow endothelium is regulated by matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-3. *Haematologica* 2007; **92**: 440-449 [PMID: 17488654 DOI: 10.3324/haematol.10475]
44 **Hass R**. Role of MSC in the Tumor Microenvironment. *Cancers (Basel)* 2020; **12** [PMID: 32751163 DOI: 10.3390/cancers12082107]
45 **Wynn RF**, Hart CA, Corradi-Perini C, O'Neill L, Evans CA, Wraith JE, Fairbairn LJ, Bellantuono I. A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood* 2004; **104**: 2643-2645 [PMID: 15251986 DOI: 10.1182/blood-2004-02-0526]
46 **Lourenco S**, Teixeira VH, Kalber T, Jose RJ, Floto RA, Janes SM. Macrophage migration inhibitory factor-CXCR4 is the dominant chemotactic axis in human mesenchymal stem cell recruitment to tumors. *J Immunol* 2015; **194**: 3463-3474 [PMID: 25712213 DOI: 10.4049/jimmunol.1402097]
47 **Pessina A**, Leonetti C, Artuso S, Benetti A, Dessy E, Pascucci L, Passeri D, Orlandi A, Berenzi A, Bonomi A, Coccè V, Ceserani V, Ferri A, Dossena M, Mazzuca P, Ciusani E, Ceccarelli P, Caruso A, Portolani N, Sisto F, Parati E, Alessandri G. Drug-releasing mesenchymal cells strongly suppress B16 lung metastasis in a syngeneic murine model. *J Exp Clin Cancer Res* 2015; **34**: 82 [PMID: 26264809 DOI: 10.1186/s13046-015-0200-3]
48 **Han Y**, Wu C, Wang J, Liu N. CXCR7 maintains osteosarcoma invasion after CXCR4 suppression in bone marrow microenvironment. *Tumour Biol* 2017; **39**: 1010428317701631 [PMID: 28468584 DOI: 10.1177/1010428317701631]
49 **Jung Y**, Kim JK, Shiozawa Y, Wang J, Mishra A, Joseph J, Berry JE, McGee S, Lee E, Sun H, Jin

T, Zhang H, Dai J, Krebsbach PH, Keller ET, Pienta KJ, Taichman RS. Recruitment of mesenchymal stem cells into prostate tumours promotes metastasis. *Nat Commun* 2013; **4**: 1795 [PMID: 23653207 DOI: 10.1038/ncomms2766]

50 **Rodríguez-Fuentes DE**, Fernández-Garza LE, Samia-Meza JA, Barrera-Barrera SA, Caplan AI, Barrera-Saldaña HA. Mesenchymal Stem Cells Current Clinical Applications: A Systematic Review. *Arch Med Res* 2021; **52**: 93-101 [PMID: 32977984 DOI: 10.1016/j.arcmed.2020.08.006]

51 **Mushtaq M**, DiFede DL, Golpanian S, Khan A, Gomes SA, Mendizabal A, Heldman AW, Hare JM. Rationale and design of the Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis in Dilated Cardiomyopathy (the POSEIDON-DCM study): a phase I/II, randomized pilot study of the comparative safety and efficacy of transendocardial injection of autologous mesenchymal stem cell vs. allogeneic mesenchymal stem cells in patients with non-ischemic dilated cardiomyopathy. *J Cardiovasc Transl Res* 2014; **7**: 769-780 [PMID: 25354998 DOI: 10.1007/s12265-014-9594-0]

52 **Suncion VY**, Ghersin E, Fishman JE, Zambrano JP, Karantalis V, Mandel N, Nelson KH, Gerstenblith G, DiFede Velazquez DL, Breton E, Sitammagari K, Schulman IH, Taldone SN, Williams AR, Sanina C, Johnston PV, Brinker J, Altman P, Mushtaq M, Trachtenberg B, Mendizabal AM, Tracy M, Da Silva J, McNiece IK, Lardo AC, George RT, Hare JM, Heldman AW. Does transendocardial injection of mesenchymal stem cells improve myocardial function locally or globally? *Circ Res* 2014; **114**: 1292-1301 [PMID: 24449819 DOI: 10.1161/CIRCRESAHA.114.302854]

53 **Butler J**, Epstein SE, Greene SJ, Quyyumi AA, Sikora S, Kim RJ, Anderson AS, Wilcox JE, Tankovich NI, Lipinski MJ, Ko YA, Margulies KB, Cole RT, Skopicki HA, Gheorghiade M. Intravenous Allogeneic Mesenchymal Stem Cells for Nonischemic Cardiomyopathy: Safety and Efficacy Results of a Phase II-A Randomized Trial. *Circ Res* 2017; **120**: 332-340 [PMID: 27856497 DOI: 10.1161/CIRCRESAHA.116.309717]

54 **Soler R**, Orozco L, Munar A, Huguet M, López R, Vives J, Coll R, Codinach M, Garcia-Lopez J. Final results of a phase I-II trial using ex vivo expanded autologous Mesenchymal Stromal Cells for the treatment of osteoarthritis of the knee confirming safety and suggesting cartilage regeneration. *Knee* 2016; **23**: 647-654 [PMID: 26783191 DOI: 10.1016/j.knee.2015.08.013]

55 **Lin F**, Ichim TE, Pingle S, Jones LD, Kesari S, Ashili S. Mesenchymal stem cells as living anti-inflammatory therapy for COVID-19 related acute respiratory distress syndrome. *World J Stem Cells* 2020; **12**: 1067-1079 [PMID: 33178392 DOI: 10.4252/wjsc.v12.i10.1067]

56 **Feng Y**, Huang J, Wu J, Xu Y, Chen B, Jiang L, Xiang H, Peng Z, Wang X. Safety and feasibility of umbilical cord mesenchymal stem cells in patients with COVID-19 pneumonia: A pilot study. *Cell Prolif* 2020; **53**: e12947 [PMID: 33205469 DOI: 10.1111/cpr.12947]

57 **Introna M**, Rambaldi A. Mesenchymal stromal cells for prevention and treatment of graft-versus-host disease: successes and hurdles. *Curr Opin Organ Transplant* 2015; **20**: 72-78 [PMID: 25563994 DOI: 10.1097/MOT.0000000000000158]

58 **Introna M**, Lucchini G, Dander E, Galimberti S, Rovelli A, Balduzzi A, Longoni D, Pavan F, Masciocchi F, Algarotti A, Micò C, Grassi A, Deola S, Cavattoni I, Gaipa G, Belotti D, Perseghin P, Parma M, Pogliani E, Golay J, Pedrini O, Capelli C, Cortelazzo S, D'Amico G, Biondi A, Rambaldi A, Biagi E. Treatment of graft versus host disease with mesenchymal stromal cells: a phase I study on 40 adult and pediatric patients. *Biol Blood Marrow Transplant* 2014; **20**: 375-381 [PMID: 24321746 DOI: 10.1016/j.bbmt.2013.11.033]

59 **Le Blanc K**, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringdén O; Developmental Committee of the European Group for Blood and Marrow Transplantation. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008; **371**: 1579-1586 [PMID: 18468541 DOI: 10.1016/S0140-6736(08)60690-X]

60 **Bloor AJC**, Patel A, Griffin JE, Gilleece MH, Radia R, Yeung DT, Drier D, Larson LS, Uenishi GI, Hei D, Kelly K, Slukvin I, Rasko JEJ. Production, safety and efficacy of iPSC-derived mesenchymal stromal cells in acute steroid-resistant graft versus host disease: a phase I, multicenter, open-label, dose-escalation study. *Nat Med* 2020; **26**: 1720-1725 [PMID: 32929265 DOI: 10.1038/s41591-020-1050-x]

61 **Ding L**, Han DM, Zheng XL, Yan HM, Xue M, Liu J, Zhu L, Li S, Mao N, Guo ZK, Ning HM, Wang HX, Zhu H. A study of human leukocyte antigen-haploidentical hematopoietic stem cells transplantation combined with allogenic mesenchymal stem cell infusion for treatment of severe aplastic anemia in pediatric and adolescent patients. *Stem Cells Transl Med* 2021; **10**: 291-302 [PMID: 32978903 DOI: 10.1002/sctm.20-0345]

62 **Bieback K**, Schallmoser K, Klüter H, Strunk D. Clinical Protocols for the Isolation and Expansion of Mesenchymal Stromal Cells. *Transfus Med Hemother* 2008; **35**: 286-294 [PMID: 21512644 DOI: 10.1159/000141567]

63 **Lechanteur C**, Briquet A, Giet O, Delloye O, Baudoux E, Beguin Y. Clinical-scale expansion of mesenchymal stromal cells: a large banking experience. *J Transl Med* 2016; **14**: 145 [PMID: 27207011 DOI: 10.1186/s12967-016-0892-y]

64 **Agostini F**, Rossi FM, Aldinucci D, Battiston M, Lombardi E, Zanolin S, Massarut S, Parodi PC, Da Ponte A, Tessitori G, Pivetta B, Durante C, Mazzucato M. Improved GMP compliant approach to manipulate lipoaspirates, to cryopreserve stromal vascular fraction, and to expand adipose stem cells in xeno-free media. *Stem Cell Res Ther* 2018; **9**: 130 [PMID: 29751821 DOI: 10.1186/s13287-018-0886-1]

65 **Durante C**, Agostini F, Abbruzzese L, Toffola RT, Zanolin S, Suine C, Mazzucato M. Growth factor release from platelet concentrates: analytic quantification and characterization for clinical applications. *Vox Sang* 2013; **105**: 129-136 [PMID: 23639058 DOI: 10.1111/vox.12039]

66 **Schallmoser K**, Bartmann C, Rohde E, Reinisch A, Kashofer K, Stadelmeyer E, Drexler C, Lanzer G, Linkesch W, Strunk D. Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. *Transfusion* 2007; **47**: 1436-1446 [PMID: 17655588 DOI: 10.1111/j.1537-2995.2007.01220.x]

67 **Bernardi M**, Agostini F, Chieregato K, Amati E, Durante C, Rassu M, Ruggeri M, Sella S, Lombardi E, Mazzucato M, Astori G. The production method affects the efficacy of platelet derivatives to expand mesenchymal stromal cells in vitro. *J Transl Med* 2017; **15**: 90 [PMID: 28460641 DOI: 10.1186/s12967-017-1185-9]

68 **Schallmoser K**, Strunk D. Generation of a pool of human platelet lysate and efficient use in cell culture. *Methods Mol Biol* 2013; **946**: 349-362 [PMID: 23179843 DOI: 10.1007/978-1-62703-128-8_22]

69 **Agostini F**, Polesel J, Battiston M, Lombardi E, Zanolin S, Da Ponte A, Astori G, Durante C, Mazzucato M. Standardization of platelet releasate products for clinical applications in cell therapy: a mathematical approach. *J Transl Med* 2017; **15**: 107 [PMID: 28526045 DOI: 10.1186/s12967-017-1210-z]

70 **Agostini F**, Ruzza M, Corpillo D, Biondi L, Acquadro E, Canepa B, Viale A, Battiston M, Serra F, Aime S, Mazzucato M. 1H-NMR and MALDI-TOF MS as metabolomic quality control tests to classify platelet derived medium additives for GMP compliant cell expansion procedures. *PLoS One* 2018; **13**: e0203048 [PMID: 30188924 DOI: 10.1371/journal.pone.0203048]

71 **Caplan H**, Olson SD, Kumar A, George M, Prabhakara KS, Wenzel P, Bedi S, Toledano-Furman NE, Triolo F, Kamhieh-Milz J, Moll G, Cox CS Jr. Mesenchymal Stromal Cell Therapeutic Delivery: Translational Challenges to Clinical Application. *Front Immunol* 2019; **10**: 1645 [PMID: 31417542 DOI: 10.3389/fimmu.2019.01645]

72 **Rombouts WJ**, Ploemacher RE. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. *Leukemia* 2003; **17**: 160-170 [PMID: 12529674 DOI: 10.1038/sj.leu.2402763]

73 **Barbash IM**, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, Miller L, Guetta E, Zipori D, Kedes LH, Kloner RA, Leor J. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* 2003; **108**: 863-868 [PMID: 12900340 DOI: 10.1161/01.CIR.0000084828.50310.6A]

74 **von Einem JC**, Guenther C, Volk HD, Grütz G, Hirsch D, Salat C, Stoetzer O, Nelson PJ, Michl M, Modest DP, Holch JW, Angele M, Bruns C, Niess H, Heinemann V. Treatment of advanced gastrointestinal cancer with genetically modified autologous mesenchymal stem cells: Results from the phase 1/2 TREAT-ME-1 trial. *Int J Cancer* 2019; **145**: 1538-1546 [PMID: 30801698 DOI: 10.1002/ijc.32230]

75 **Amer MH**, Rose FRAJ, Shakesheff KM, Modo M, White LJ. Translational considerations in injectable cell-based therapeutics for neurological applications: concepts, progress and challenges. *NPJ Regen Med* 2017; **2**: 23 [PMID: 29302358 DOI: 10.1038/s41536-017-0028-x]

76 **Chen W**, Li M, Cheng H, Yan Z, Cao J, Pan B, Sang W, Wu Q, Zeng L, Li Z, Xu K. Overexpression of the mesenchymal stem cell Cxcr4 gene in irradiated mice increases the homing capacity of these cells. *Cell Biochem Biophys* 2013; **67**: 1181-1191 [PMID: 23712865 DOI: 10.1007/s12013-013-9632-6]

77 **Mao J**, Cao M, Zhang F, Zhang J, Duan X, Lu L, Yang Z, Zhang X, Zhu W, Zhang Q, Wang Z, Shen J. Peritumoral administration of IFNβ upregulated mesenchymal stem cells inhibits tumor growth in an orthotopic, immunocompetent rat glioma model. *J Immunother Cancer* 2020; **8** [PMID: 32169868 DOI: 10.1136/jitc-2019-000164]

78 **Lee CS**, Bishop ES, Zhang R, Yu X, Farina EM, Yan S, Zhao C, Zheng Z, Shu Y, Wu X, Lei J, Li Y, Zhang W, Yang C, Wu K, Wu Y, Ho S, Athiviraham A, Lee MJ, Wolf JM, Reid RR, He TC. Adenovirus-Mediated Gene Delivery: Potential Applications for Gene and Cell-Based Therapies in the New Era of Personalized Medicine. *Genes Dis* 2017; **4**: 43-63 [PMID: 28944281 DOI: 10.1016/j.gendis.2017.04.001]

79 **Rothe M**, Modlich U, Schambach A. Biosafety challenges for use of lentiviral vectors in gene therapy. *Curr Gene Ther* 2013; **13**: 453-468 [PMID: 24195603 DOI: 10.2174/15665232113136660006]

80 **Nayak S**, Herzog RW. Progress and prospects: immune responses to viral vectors. *Gene Ther* 2010; **17**: 295-304 [PMID: 19907498 DOI: 10.1038/gt.2009.148]

81 **Abdul Halim NS**, Fakiruddin KS, Ali SA, Yahaya BH. A comparative study of non-viral gene delivery techniques to human adipose-derived mesenchymal stem cell. *Int J Mol Sci* 2014; **15**: 15044-15060 [PMID: 25162825 DOI: 10.3390/ijms150915044]

82 **Zhang H**, Li X, Li J, Zhong L, Chen X, Chen S. SDF-1 mediates mesenchymal stem cell recruitment and migration *via* the SDF-1/CXCR4 axis in bone defect. *J Bone Miner Metab* 2021; **39**: 126-138 [PMID: 33079278 DOI: 10.1007/s00774-020-01122-0]

83 **Zhang H**, Jiang C, Li M, Wang X, Tian F, Fang X, Zhu L, Bian Z. CXCR4 enhances invasion and proliferation of bone marrow stem cells *via* PI3K/AKT/NF-κB signaling pathway. *Int J Clin Exp Pathol* 2017; **10**: 9829-9836 [PMID: 31966870]

84 **Park SA**, Ryu CH, Kim SM, Lim JY, Park SI, Jeong CH, Jun JA, Oh JH, Park SH, Oh W, Jeun SS.

CXCR4-transfected human umbilical cord blood-derived mesenchymal stem cells exhibit enhanced migratory capacity toward gliomas. *Int J Oncol* 2011; **38**: 97-103 [PMID: 21109930]

85 **Fontanella R**, Pelagalli A, Nardelli A, D'Alterio C, Ieranò C, Cerchia L, Lucarelli E, Scala S, Zannetti A. A novel antagonist of CXCR4 prevents bone marrow-derived mesenchymal stem cell-mediated osteosarcoma and hepatocellular carcinoma cell migration and invasion. *Cancer Lett* 2016; **370**: 100-107 [PMID: 26517945 DOI: 10.1016/j.canlet.2015.10.018]

86 **Ho IA**, Yulyana Y, Sia KC, Newman JP, Guo CM, Hui KM, Lam PY. Matrix metalloproteinase-1-mediated mesenchymal stem cell tumor tropism is dependent on crosstalk with stromal derived growth factor 1/C-X-C chemokine receptor 4 axis. *FASEB J* 2014; **28**: 4359-4368 [PMID: 25271298 DOI: 10.1096/fj.14-252551]

87 **Thankamony SP**, Sackstein R. Enforced hematopoietic cell E- and L-selectin ligand (HCELL) expression primes transendothelial migration of human mesenchymal stem cells. *Proc Natl Acad Sci U S A* 2011; **108**: 2258-2263 [PMID: 21257905 DOI: 10.1073/pnas.1018064108]

88 **Ko IK**, Kim BG, Awadallah A, Mikulan J, Lin P, Letterio JJ, Dennis JE. Targeting improves MSC treatment of inflammatory bowel disease. *Mol Ther* 2010; **18**: 1365-1372 [PMID: 20389289 DOI: 10.1038/mt.2010.54]

89 **Huang B**, Jiang XC, Zhang TY, Hu YL, Tabata Y, Chen Z, Pluchino S, Gao JQ. Peptide modified mesenchymal stem cells as targeting delivery system transfected with miR-133b for the treatment of cerebral ischemia. *Int J Pharm* 2017; **531**: 90-100 [PMID: 28827201 DOI: 10.1016/j.ijpharm.2017.08.073]

90 **Kean TJ**, Duesler L, Young RG, Dadabayev A, Olenyik A, Penn M, Wagner J, Fink DJ, Caplan AI, Dennis JE. Development of a peptide-targeted, myocardial ischemia-homing, mesenchymal stem cell. *J Drug Target* 2012; **20**: 23-32 [PMID: 22047107 DOI: 10.3109/1061186X.2011.622398]

91 **Ziaei R**, Ayatollahi M, Yaghobi R, Sahraeian Z, Zarghami N. Involvement of TNF-α in differential gene expression pattern of CXCR4 on human marrow-derived mesenchymal stem cells. *Mol Biol Rep* 2014; **41**: 1059-1066 [PMID: 24395293 DOI: 10.1007/s11033-013-2951-2]

92 **Xiao Q**, Wang SK, Tian H, Xin L, Zou ZG, Hu YL, Chang CM, Wang XY, Yin QS, Zhang XH, Wang LY. TNF-α increases bone marrow mesenchymal stem cell migration to ischemic tissues. *Cell Biochem Biophys* 2012; **62**: 409-414 [PMID: 22081437 DOI: 10.1007/s12013-011-9317-y]

93 **Ponte AL**, Marais E, Gallay N, Langonné A, Delorme B, Hérault O, Charbord P, Domenech J. The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. *Stem Cells* 2007; **25**: 1737-1745 [PMID: 17395768 DOI: 10.1634/stemcells.2007-0054]

94 **Dubon MJ**, Yu J, Choi S, Park KS. Transforming growth factor β induces bone marrow mesenchymal stem cell migration *via* noncanonical signals and N-cadherin. *J Cell Physiol* 2018; **233**: 201-213 [PMID: 28213973 DOI: 10.1002/jcp.25863]

95 **Nam D**, Park A, Dubon MJ, Yu J, Kim W, Son Y, Park KS. Coordinated Regulation of Mesenchymal Stem Cell Migration by Various Chemotactic Stimuli. *Int J Mol Sci* 2020; **21** [PMID: 33202862 DOI: 10.3390/ijms21228561]

96 **Li M**, Zeng L, Liu S, Dangelmajer S, Kahlert UD, Huang H, Han Y, Chi X, Zhu M, Lei T. Transforming Growth Factor-β Promotes Homing and Therapeutic Efficacy of Human Mesenchymal Stem Cells to Glioblastoma. *J Neuropathol Exp Neurol* 2019; **78**: 315-325 [PMID: 30863846 DOI: 10.1093/jnen/nlz016]

97 **Fan H**, Zhao G, Liu L, Liu F, Gong W, Liu X, Yang L, Wang J, Hou Y. Pre-treatment with IL-1β enhances the efficacy of MSC transplantation in DSS-induced colitis. *Cell Mol Immunol* 2012; **9**: 473-481 [PMID: 23085948 DOI: 10.1038/cmi.2012.40]

98 **Nie H**, An F, Mei J, Yang C, Zhan Q, Zhang Q. IL-1*β* Pretreatment Improves the Efficacy of Mesenchymal Stem Cells on Acute Liver Failure by Enhancing CXCR4 Expression. *Stem Cells Int* 2020; **2020**: 1498315 [PMID: 32724311 DOI: 10.1155/2020/1498315]

99 **Magne B**, Dedier M, Nivet M, Coulomb B, Banzet S, Lataillade JJ, Trouillas M. IL-1β-Primed Mesenchymal Stromal Cells Improve Epidermal Substitute Engraftment and Wound Healing *via* Matrix Metalloproteinases and Transforming Growth Factor-β1. *J Invest Dermatol* 2020; **140**: 688-698.e21 [PMID: 31513805 DOI: 10.1016/j.jid.2019.07.721]

100 **Duijvestein M**, Wildenberg ME, Welling MM, Hennink S, Molendijk I, van Zuylen VL, Bosse T, Vos AC, de Jonge-Muller ES, Roelofs H, van der Weerd L, Verspaget HW, Fibbe WE, te Velde AA, van den Brink GR, Hommes DW. Pretreatment with interferon-γ enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. *Stem Cells* 2011; **29**: 1549-1558 [PMID: 21898680 DOI: 10.1002/stem.698]

101 **Li Y**, Yu X, Lin S, Li X, Zhang S, Song YH. Insulin-like growth factor 1 enhances the migratory capacity of mesenchymal stem cells. *Biochem Biophys Res Commun* 2007; **356**: 780-784 [PMID: 17382293 DOI: 10.1016/j.bbrc.2007.03.049]

102 **Shi M**, Li J, Liao L, Chen B, Li B, Chen L, Jia H, Zhao RC. Regulation of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in homing efficiency in NOD/SCID mice. *Haematologica* 2007; **92**: 897-904 [PMID: 17606439 DOI: 10.3324/haematol.10669]

103 **Smith CL**, Chaichana KL, Lee YM, Lin B, Stanko KM, O'Donnell T, Gupta S, Shah SR, Wang J, Wijesekera O, Delannoy M, Levchenko A, Quiñones-Hinojosa A. Pre-exposure of human adipose mesenchymal stem cells to soluble factors enhances their homing to brain cancer. *Stem Cells Transl Med* 2015; **4**: 239-251 [PMID: 25646527 DOI: 10.5966/sctm.2014-0149]

104 **Tsai LK**, Wang Z, Munasinghe J, Leng Y, Leeds P, Chuang DM. Mesenchymal stem cells primed

with valproate and lithium robustly migrate to infarcted regions and facilitate recovery in a stroke model. *Stroke* 2011; **42**: 2932-2939 [PMID: 21836090 DOI: 10.1161/STROKEAHA.110.612788]

105 **Yu Q**, Chen L, You Y, Zou C, Zhang Y, Liu Q, Cheng F. Erythropoietin combined with granulocyte colonystimulating factor enhances MMP-2 expression in mesenchymal stem cells and promotes cell migration. *Mol Med Rep* 2011; **4**: 31-36 [PMID: 21461559 DOI: 10.3892/mmr.2010.387]

106 **Xu W**, Xu R, Li Z, Wang Y, Hu R. Hypoxia changes chemotaxis behaviour of mesenchymal stem cells *via* HIF-1α signalling. *J Cell Mol Med* 2019; **23**: 1899-1907 [PMID: 30628201 DOI: 10.1111/jcmm.14091]

107 **Annaratone L**, Cascardi E, Vissio E, Sarotto I, Chmielik E, Sapino A, Berrino E, Marchiò C. The Multifaceted Nature of Tumor Microenvironment in Breast Carcinomas. *Pathobiology* 2020; **87**: 125-142 [PMID: 32325459 DOI: 10.1159/000507055]

108 **Siveen KS**, Kuttan G. Role of macrophages in tumour progression. *Immunol Lett* 2009; **123**: 97-102 [PMID: 19428556 DOI: 10.1016/j.imlet.2009.02.011]

109 **Agostini F**, Vicinanza C, Di Cintio F, Battiston M, Lombardi E, Golinelli G, Durante C, Toffoli G, Dominici M, Mazzucato M. Adipose mesenchymal stromal/stem cells expanded by a GMP compatible protocol displayed improved adhesion on cancer cells in flow conditions. *Ann Transl Med* 2020; **8**: 533 [PMID: 32411756 DOI: 10.21037/atm.2020.04.25]

110 **Liang W**, Chen X, Zhang S, Fang J, Chen M, Xu Y. Mesenchymal stem cells as a double-edged sword in tumor growth: focusing on MSC-derived cytokines. *Cell Mol Biol Lett* 2021; **26**: 3 [PMID: 33472580 DOI: 10.1186/s11658-020-00246-5]

111 **Karnoub AE**, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, Richardson AL, Polyak K, Tubo R, Weinberg RA. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007; **449**: 557-563 [PMID: 17914389 DOI: 10.1038/nature06188]

112 **Rivera-Cruz CM**, Shearer JJ, Figueiredo Neto M, Figueiredo ML. The Immunomodulatory Effects of Mesenchymal Stem Cell Polarization within the Tumor Microenvironment Niche. *Stem Cells Int* 2017; **2017**: 4015039 [PMID: 29181035 DOI: 10.1155/2017/4015039]

113 **Du WJ**, Chi Y, Yang ZX, Li ZJ, Cui JJ, Song BQ, Li X, Yang SG, Han ZB, Han ZC. Heterogeneity of proangiogenic features in mesenchymal stem cells derived from bone marrow, adipose tissue, umbilical cord, and placenta. *Stem Cell Res Ther* 2016; **7**: 163 [PMID: 27832825 DOI: 10.1186/s13287-016-0418-9]

114 **Xue Z**, Wu X, Chen X, Liu Y, Wang X, Wu K, Nie Y, Fan D. Mesenchymal stem cells promote epithelial to mesenchymal transition and metastasis in gastric cancer though paracrine cues and close physical contact. *J Cell Biochem* 2015; **116**: 618-627 [PMID: 25399738 DOI: 10.1002/jcb.25013]

115 **Fregni G**, Quinodoz M, Möller E, Vuille J, Galland S, Fusco C, Martin P, Letovanec I, Provero P, Rivolta C, Riggi N, Stamenkovic I. Reciprocal modulation of mesenchymal stem cells and tumor cells promotes lung cancer metastasis. *EBioMedicine* 2018; **29**: 128-145 [PMID: 29503225 DOI: 10.1016/j.ebiom.2018.02.017]

116 **Luo J**, Ok Lee S, Liang L, Huang CK, Li L, Wen S, Chang C. Infiltrating bone marrow mesenchymal stem cells increase prostate cancer stem cell population and metastatic ability *via* secreting cytokines to suppress androgen receptor signaling. *Oncogene* 2014; **33**: 2768-2778 [PMID: 23792449 DOI: 10.1038/onc.2013.233]

117 **Houthuijzen JM**, Daenen LG, Roodhart JM, Voest EE. The role of mesenchymal stem cells in anti-cancer drug resistance and tumour progression. *Br J Cancer* 2012; **106**: 1901-1906 [PMID: 22596239 DOI: 10.1038/bjc.2012.201]

118 **Zhang P**, Dong L, Yan K, Long H, Yang TT, Dong MQ, Zhou Y, Fan QY, Ma BA. CXCR4-mediated osteosarcoma growth and pulmonary metastasis is promoted by mesenchymal stem cells through VEGF. *Oncol Rep* 2013; **30**: 1753-1761 [PMID: 23863999 DOI: 10.3892/or.2013.2619]

119 **Zhao Q**, Gregory CA, Lee RH, Reger RL, Qin L, Hai B, Park MS, Yoon N, Clough B, McNeill E, Prockop DJ, Liu F. MSCs derived from iPSCs with a modified protocol are tumor-tropic but have much less potential to promote tumors than bone marrow MSCs. *Proc Natl Acad Sci U S A* 2015; **112**: 530-535 [PMID: 25548183 DOI: 10.1073/pnas.1423008112]

120 **Lu YR**, Yuan Y, Wang XJ, Wei LL, Chen YN, Cong C, Li SF, Long D, Tan WD, Mao YQ, Zhang J, Li YP, Cheng JQ. The growth inhibitory effect of mesenchymal stem cells on tumor cells in vitro and in vivo. *Cancer Biol Ther* 2008; **7**: 245-251 [PMID: 18059192 DOI: 10.4161/cbt.7.2.5296]

121 **Dasari VR**, Velpula KK, Kaur K, Fassett D, Klopfenstein JD, Dinh DH, Gujrati M, Rao JS. Cord blood stem cell-mediated induction of apoptosis in glioma downregulates X-linked inhibitor of apoptosis protein (XIAP). *PLoS One* 2010; **5**: e11813 [PMID: 20676365 DOI: 10.1371/journal.pone.0011813]

122 **Ryu H**, Oh JE, Rhee KJ, Baik SK, Kim J, Kang SJ, Sohn JH, Choi E, Shin HC, Kim YM, Kim HS, Bae KS, Eom YW. Adipose tissue-derived mesenchymal stem cells cultured at high density express IFN-β and suppress the growth of MCF-7 human breast cancer cells. *Cancer Lett* 2014; **352**: 220-227 [PMID: 25016057 DOI: 10.1016/j.canlet.2014.06.018]

123 **Petrella F**, Rimoldi I, Rizzo S, Spaggiari L. Mesenchymal Stromal Cells for Antineoplastic Drug Loading and Delivery. *Medicines (Basel)* 2017; **4** [PMID: 29168760 DOI: 10.3390/medicines4040087]

124 **Pascucci L**, Coccè V, Bonomi A, Ami D, Ceccarelli P, Ciusani E, Viganò L, Locatelli A, Sisto F, Doglia SM, Parati E, Bernardo ME, Muraca M, Alessandri G, Bondiolotti G, Pessina A. Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit in vitro tumor growth: a new approach for drug delivery. *J Control Release* 2014; **192**: 262-270 [PMID: 25084218

DOI: 10.1016/j.jconrel.2014.07.042]

125 **Kalimuthu S**, Zhu L, Oh JM, Gangadaran P, Lee HW, Baek SH, Rajendran RL, Gopal A, Jeong SY, Lee SW, Lee J, Ahn BC. Migration of mesenchymal stem cells to tumor xenograft models and *in vitro* drug delivery by doxorubicin. *Int J Med Sci* 2018; **15**: 1051-1061 [PMID: 30013447 DOI: 10.7150/ijms.25760]

126 **Coccè V**, Farronato D, Brini AT, Masia C, Giannì AB, Piovani G, Sisto F, Alessandri G, Angiero F, Pessina A. Drug Loaded Gingival Mesenchymal Stromal Cells (GinPa-MSCs) Inhibit In Vitro Proliferation of Oral Squamous Cell Carcinoma. *Sci Rep* 2017; **7**: 9376 [PMID: 28839168 DOI: 10.1038/s41598-017-09175-4]

127 **Cao S**, Guo J, He Y, Alahdal M, Tang S, Zhao Y, Yang Z, Gao H, Hu W, Jiang H, Qin L, Jin L. Nano-loaded human umbilical cord mesenchymal stem cells as targeted carriers of doxorubicin for breast cancer therapy. *Artif Cells Nanomed Biotechnol* 2018; **46**: 642-652 [PMID: 29457930 DOI: 10.1080/21691401.2018.1434185]

128 **Zhang X**, Yao S, Liu C, Jiang Y. Tumor tropic delivery of doxorubicin-polymer conjugates using mesenchymal stem cells for glioma therapy. *Biomaterials* 2015; **39**: 269-281 [PMID: 25468377 DOI: 10.1016/j.biomaterials.2014.11.003]

129 **Wei H**, Chen J, Wang S, Fu F, Zhu X, Wu C, Liu Z, Zhong G, Lin J. A Nanodrug Consisting Of Doxorubicin And Exosome Derived From Mesenchymal Stem Cells For Osteosarcoma Treatment In Vitro. *Int J Nanomedicine* 2019; **14**: 8603-8610 [PMID: 31802872 DOI: 10.2147/IJN.S218988]

130 **Takayama Y**, Kusamori K, Tsukimori C, Shimizu Y, Hayashi M, Kiyama I, Katsumi H, Sakane T, Yamamoto A, Nishikawa M. Anticancer drug-loaded mesenchymal stem cells for targeted cancer therapy. *J Control Release* 2021; **329**: 1090-1101 [PMID: 33098911 DOI: 10.1016/j.jconrel.2020.10.037]

131 **Brini AT**, Coccè V, Ferreira LM, Giannasi C, Cossellu G, Giannì AB, Angiero F, Bonomi A, Pascucci L, Falchetti ML, Ciusani E, Bondiolotti G, Sisto F, Alessandri G, Pessina A, Farronato G. Cell-mediated drug delivery by gingival interdental papilla mesenchymal stromal cells (GinPa-MSCs) loaded with paclitaxel. *Expert Opin Drug Deliv* 2016; **13**: 789-798 [PMID: 26986001 DOI: 10.1517/17425247.2016.1167037]

132 **Bonomi A**, Ghezzi E, Pascucci L, Aralla M, Ceserani V, Pettinari L, Coccè V, Guercio A, Alessandri G, Parati E, Brini AT, Zeira O, Pessina A. Effect of canine mesenchymal stromal cells loaded with paclitaxel on growth of canine glioma and human glioblastoma cell lines. *Vet J* 2017; **223**: 41-47 [PMID: 28671070 DOI: 10.1016/j.tvjl.2017.05.005]

133 **Petrella F**, Coccè V, Masia C, Milani M, Salè EO, Alessandri G, Parati E, Sisto F, Pentimalli F, Brini AT, Pessina A, Spaggiari L. Paclitaxel-releasing mesenchymal stromal cells inhibit in vitro proliferation of human mesothelioma cells. *Biomed Pharmacother* 2017; **87**: 755-758 [PMID: 28153512 DOI: 10.1016/j.biopha.2017.01.118]

134 **Pessina A**, Coccè V, Pascucci L, Bonomi A, Cavicchini L, Sisto F, Ferrari M, Ciusani E, Crovace A, Falchetti ML, Zicari S, Caruso A, Navone S, Marfia G, Benetti A, Ceccarelli P, Parati E, Alessandri G. Mesenchymal stromal cells primed with Paclitaxel attract and kill leukaemia cells, inhibit angiogenesis and improve survival of leukaemia-bearing mice. *Br J Haematol* 2013; **160**: 766-778 [PMID: 23293837 DOI: 10.1111/bjh.12196]

135 **Cheng S**, Nethi SK, Al-Kofahi M, Prabha S. Pharmacokinetic-Pharmacodynamic Modeling of Tumor Targeted Drug Delivery Using Nano-Engineered Mesenchymal Stem Cells. *Pharmaceutics* 2021; **13** [PMID: 33445681 DOI: 10.3390/pharmaceutics13010092]

136 **Layek B**, Sadhukha T, Panyam J, Prabha S. Nano-Engineered Mesenchymal Stem Cells Increase Therapeutic Efficacy of Anticancer Drug Through True Active Tumor Targeting. *Mol Cancer Ther* 2018; **17**: 1196-1206 [PMID: 29592881 DOI: 10.1158/1535-7163.MCT-17-0682]

137 **Wang X**, Gao J, Ouyang X, Wang J, Sun X, Lv Y. Mesenchymal stem cells loaded with paclitaxel-poly(lactic-*co*-glycolic acid) nanoparticles for glioma-targeting therapy. *Int J Nanomedicine* 2018; **13**: 5231-5248 [PMID: 30237710 DOI: 10.2147/IJN.S167142]

138 **Moku G**, Layek B, Trautman L, Putnam S, Panyam J, Prabha S. Improving Payload Capacity and Anti-Tumor Efficacy of Mesenchymal Stem Cells Using TAT Peptide Functionalized Polymeric Nanoparticles. *Cancers (Basel)* 2019; **11** [PMID: 30959908 DOI: 10.3390/cancers11040491]

139 **Bonomi A**, Sordi V, Dugnani E, Ceserani V, Dossena M, Coccè V, Cavicchini L, Ciusani E, Bondiolotti G, Piovani G, Pascucci L, Sisto F, Alessandri G, Piemonti L, Parati E, Pessina A. Gemcitabine-releasing mesenchymal stromal cells inhibit in vitro proliferation of human pancreatic carcinoma cells. *Cytotherapy* 2015; **17**: 1687-1695 [PMID: 26481416 DOI: 10.1016/j.jcyt.2015.09.005]

140 **Lopez Perez R**, Münz F, Vidoni D, Rühle A, Trinh T, Sisombath S, Zou B, Wuchter P, Debus J, Grosu AL, Saffrich R, Huber PE, Nicolay NH. Mesenchymal stem cells preserve their stem cell traits after exposure to antimetabolite chemotherapy. *Stem Cell Res* 2019; **40**: 101536 [PMID: 31437767 DOI: 10.1016/j.scr.2019.101536]

141 **Rossignoli F**, Spano C, Grisendi G, Foppiani EM, Golinelli G, Mastrolia I, Bestagno M, Candini O, Petrachi T, Recchia A, Miselli F, Rovesti G, Orsi G, Veronesi E, Medici G, Petocchi B, Pinelli M, Horwitz EM, Conte P, Dominici M. MSC-Delivered Soluble TRAIL and Paclitaxel as Novel Combinatory Treatment for Pancreatic Adenocarcinoma. *Theranostics* 2019; **9**: 436-448 [PMID: 30809285 DOI: 10.7150/thno.27576]

142 **Spano C**, Grisendi G, Golinelli G, Rossignoli F, Prapa M, Bestagno M, Candini O, Petrachi T, Recchia A, Miselli F, Rovesti G, Orsi G, Maiorana A, Manni P, Veronesi E, Piccinno MS, Murgia

A, Pinelli M, Horwitz EM, Cascinu S, Conte P, Dominici M. Soluble TRAIL Armed Human MSC As Gene Therapy For Pancreatic Cancer. *Sci Rep* 2019; **9**: 1788 [PMID: 30742129 DOI: 10.1038/s41598-018-37433-6]

143 **Mueller LP**, Luetzkendorf J, Widder M, Nerger K, Caysa H, Mueller T. TRAIL-transduced multipotent mesenchymal stromal cells (TRAIL-MSC) overcome TRAIL resistance in selected CRC cell lines in vitro and in vivo. *Cancer Gene Ther* 2011; **18**: 229-239 [PMID: 21037557 DOI: 10.1038/cgt.2010.68]

144 **Yang X**, Du J, Xu X, Xu C, Song W. IFN-γ-secreting-mesenchymal stem cells exert an antitumor effect in vivo *via* the TRAIL pathway. *J Immunol Res* 2014; **2014**: 318098 [PMID: 24971369 DOI: 10.1155/2014/318098]

145 **Chen X**, Wang K, Chen S, Chen Y. Effects of mesenchymal stem cells harboring the Interferon-β gene on A549 lung cancer in nude mice. *Pathol Res Pract* 2019; **215**: 586-593 [PMID: 30683475 DOI: 10.1016/j.prp.2019.01.013]

146 **Xu G**, Guo Y, Seng Z, Cui G, Qu J. Bone marrow-derived mesenchymal stem cells co-expressing interleukin-18 and interferon-β exhibit potent antitumor effect against intracranial glioma in rats. *Oncol Rep* 2015; **34**: 1915-1922 [PMID: 26252165 DOI: 10.3892/or.2015.4174]

147 **de Melo SM**, Bittencourt S, Ferrazoli EG, da Silva CS, da Cunha FF, da Silva FH, Stilhano RS, Denapoli PM, Zanetti BF, Martin PK, Silva LM, dos Santos AA, Baptista LS, Longo BM, Han SW. The Anti-Tumor Effects of Adipose Tissue Mesenchymal Stem Cell Transduced with HSV-Tk Gene on U-87-Driven Brain Tumor. *PLoS One* 2015; **10**: e0128922 [PMID: 26067671 DOI: 10.1371/journal.pone.0128922]

148 **Uchibori R**, Okada T, Ito T, Urabe M, Mizukami H, Kume A, Ozawa K. Retroviral vector-producing mesenchymal stem cells for targeted suicide cancer gene therapy. *J Gene Med* 2009; **11**: 373-381 [PMID: 19274675 DOI: 10.1002/jgm.1313]

149 **Uchibori R**, Tsukahara T, Ohmine K, Ozawa K. Cancer gene therapy using mesenchymal stem cells. *Int J Hematol* 2014; **99**: 377-382 [PMID: 24578184 DOI: 10.1007/s12185-014-1537-7]

150 **Cavarretta IT**, Altanerova V, Matuskova M, Kucerova L, Culig Z, Altaner C. Adipose tissue-derived mesenchymal stem cells expressing prodrug-converting enzyme inhibit human prostate tumor growth. *Mol Ther* 2010; **18**: 223-231 [PMID: 19844197 DOI: 10.1038/mt.2009.237]

151 **Matuskova M**, Kozovska Z, Toro L, Durinikova E, Tyciakova S, Cierna Z, Bohovic R, Kucerova L. Combined enzyme/prodrug treatment by genetically engineered AT-MSC exerts synergy and inhibits growth of MDA-MB-231 induced lung metastases. *J Exp Clin Cancer Res* 2015; **34**: 33 [PMID: 25884597 DOI: 10.1186/s13046-015-0149-2]

152 **Li G**, Miao F, Zhu J, Chen Y. Antiangiogenesis gene therapy for hepatocellular carcinoma *via* systemic injection of mesenchymal stem cells engineered to secrete soluble Flt1. *Mol Med Rep* 2017; **16**: 5799-5806 [PMID: 28849176 DOI: 10.3892/mmr.2017.7310]

153 **Shi S**, Zhang M, Guo R, Miao Y, Li B. Bone Marrow-Derived Mesenchymal Stem Cell-Mediated Dual-Gene Therapy for Glioblastoma. *Hum Gene Ther* 2019; **30**: 106-117 [PMID: 29993289 DOI: 10.1089/hum.2018.092]

154 **Zhu Y**, Cheng M, Yang Z, Zeng CY, Chen J, Xie Y, Luo SW, Zhang KH, Zhou SF, Lu NH. Mesenchymal stem cell-based NK4 gene therapy in nude mice bearing gastric cancer xenografts. *Drug Des Devel Ther* 2014; **8**: 2449-2462 [PMID: 25525335 DOI: 10.2147/DDDT.S71466]

155 **Lang FM**, Hossain A, Gumin J, Momin EN, Shimizu Y, Ledbetter D, Shahar T, Yamashita S, Parker Kerrigan B, Fueyo J, Sawaya R, Lang FF. Mesenchymal stem cells as natural biofactories for exosomes carrying miR-124a in the treatment of gliomas. *Neuro Oncol* 2018; **20**: 380-390 [PMID: 29016843 DOI: 10.1093/neuonc/nox152]

156 **Lou G**, Chen L, Xia C, Wang W, Qi J, Li A, Zhao L, Chen Z, Zheng M, Liu Y. MiR-199a-modified exosomes from adipose tissue-derived mesenchymal stem cells improve hepatocellular carcinoma chemosensitivity through mTOR pathway. *J Exp Clin Cancer Res* 2020; **39**: 4 [PMID: 31898515 DOI: 10.1186/s13046-019-1512-5]

157 **Kim N**, Nam YS, Im KI, Lim JY, Lee ES, Jeon YW, Cho SG. IL-21-Expressing Mesenchymal Stem Cells Prevent Lethal B-Cell Lymphoma Through Efficient Delivery of IL-21, Which Redirects the Immune System to Target the Tumor. *Stem Cells Dev* 2015; **24**: 2808-2821 [PMID: 26415081 DOI: 10.1089/scd.2015.0103]

158 **Li L**, Li F, Tian H, Yue W, Li S, Chen G. Human mesenchymal stem cells with adenovirus-mediated TRAIL gene transduction have antitumor effects on esophageal cancer cell line Eca-109. *Acta Biochim Biophys Sin (Shanghai)* 2014; **46**: 471-476 [PMID: 24739635 DOI: 10.1093/abbs/gmu024]

159 **Cai C**, Hou L, Zhang J, Zhao D, Wang Z, Hu H, He J, Guan W, Ma Y. The Inhibitory Effect of Mesenchymal Stem Cells with rAd-NK4 on Liver Cancer. *Appl Biochem Biotechnol* 2017; **183**: 444-459 [PMID: 28353041 DOI: 10.1007/s12010-017-2456-x]

160 **Kim SW**, Kim SJ, Park SH, Yang HG, Kang MC, Choi YW, Kim SM, Jeun SS, Sung YC. Complete regression of metastatic renal cell carcinoma by multiple injections of engineered mesenchymal stem cells expressing dodecameric TRAIL and HSV-TK. *Clin Cancer Res* 2013; **19**: 415-427 [PMID: 23204131 DOI: 10.1158/1078-0432.CCR-12-1568]

161 **Nakamura K**, Ito Y, Kawano Y, Kurozumi K, Kobune M, Tsuda H, Bizen A, Honmou O, Niitsu Y, Hamada H. Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. *Gene Ther* 2004; **11**: 1155-1164 [PMID: 15141157 DOI: 10.1038/sj.gt.3302276]

162 **Xu G**, Jiang XD, Xu Y, Zhang J, Huang FH, Chen ZZ, Zhou DX, Shang JH, Zou YX, Cai YQ, Kou

SB, Chen YZ, Xu RX, Zeng YJ. Adenoviral-mediated interleukin-18 expression in mesenchymal stem cells effectively suppresses the growth of glioma in rats. *Cell Biol Int* 2009; **33**: 466-474 [PMID: 18725309 DOI: 10.1016/j.cellbi.2008.07.023]

163 **Bak XY**, Lam DH, Yang J, Ye K, Wei EL, Lim SK, Wang S. Human embryonic stem cell-derived mesenchymal stem cells as cellular delivery vehicles for prodrug gene therapy of glioblastoma. *Hum Gene Ther* 2011; **22**: 1365-1377 [PMID: 21425958 DOI: 10.1089/hum.2010.212]

164 **Wang J**, Zhu L, Chen X, Huang R, Wang S, Dong P. Human Bone Marrow Mesenchymal Stem Cells Functionalized by Hybrid Baculovirus-Adeno-Associated Viral Vectors for Targeting Hypopharyngeal Carcinoma. *Stem Cells Dev* 2019; **28**: 543-553 [PMID: 30747033 DOI: 10.1089/scd.2018.0252]

165 **Parker Kerrigan BC**, Shimizu Y, Andreeff M, Lang FF. Mesenchymal stromal cells for the delivery of oncolytic viruses in gliomas. *Cytotherapy* 2017; **19**: 445-457 [PMID: 28233640 DOI: 10.1016/j.jcyt.2017.02.002]

166 **Yong RL**, Shinojima N, Fueyo J, Gumin J, Vecil GG, Marini FC, Bogler O, Andreeff M, Lang FF. Human bone marrow-derived mesenchymal stem cells for intravascular delivery of oncolytic adenovirus Delta24-RGD to human gliomas. *Cancer Res* 2009; **69**: 8932-8940 [PMID: 19920199 DOI: 10.1158/0008-5472.CAN-08-3873]

167 **Srinivasan VM**, Gumin J, Camstra KM, Collins DE, Chen MM, Shpall EJ, Parker Kerrigan BC, Johnson JN, Chen SR, Fueyo J, Gomez-Manzano C, Lang FF, Kan P. Endovascular Selective Intra-Arterial Infusion of Mesenchymal Stem Cells Loaded With Delta-24 in a Canine Model. *Neurosurgery* 2020; **88**: E102-E113 [PMID: 33231254 DOI: 10.1093/neuros/nyaa470]

168 **Yoon AR**, Hong J, Li Y, Shin HC, Lee H, Kim HS, Yun CO. Mesenchymal Stem Cell-Mediated Delivery of an Oncolytic Adenovirus Enhances Antitumor Efficacy in Hepatocellular Carcinoma. *Cancer Res* 2019; **79**: 4503-4514 [PMID: 31289131 DOI: 10.1158/0008-5472.CAN-18-3900]

169 **Muhammad T**, Sakhawat A, Khan AA, Ma L, Gjerset RA, Huang Y. Mesenchymal stem cell-mediated delivery of therapeutic adenoviral vectors to prostate cancer. *Stem Cell Res Ther* 2019; **10**: 190 [PMID: 31238944 DOI: 10.1186/s13287-019-1268-z]

170 **Kaczorowski A**, Hammer K, Liu L, Villhauer S, Nwaeburu C, Fan P, Zhao Z, Gladkich J, Groß W, Nettelbeck DM, Herr I. Delivery of improved oncolytic adenoviruses by mesenchymal stromal cells for elimination of tumorigenic pancreatic cancer cells. *Oncotarget* 2016; **7**: 9046-9059 [PMID: 26824985 DOI: 10.18632/oncotarget.7031]

171 **Hoyos V**, Del Bufalo F, Yagyu S, Ando M, Dotti G, Suzuki M, Bouchier-Hayes L, Alemany R, Brenner MK. Mesenchymal Stromal Cells for Linked Delivery of Oncolytic and Apoptotic Adenoviruses to Non-small-cell Lung Cancers. *Mol Ther* 2015; **23**: 1497-1506 [PMID: 26084970 DOI: 10.1038/mt.2015.110]

172 **Moniri MR**, Sun XY, Rayat J, Dai D, Ao Z, He Z, Verchere CB, Dai LJ, Warnock GL. TRAIL-engineered pancreas-derived mesenchymal stem cells: characterization and cytotoxic effects on pancreatic cancer cells. *Cancer Gene Ther* 2012; **19**: 652-658 [PMID: 22767216 DOI: 10.1038/cgt.2012.46]

173 **Sun XY**, Nong J, Qin K, Lu H, Moniri MR, Dai LJ, Warnock GL. MSC(TRAIL)-mediated HepG2 cell death in direct and indirect co-cultures. *Anticancer Res* 2011; **31**: 3705-3712 [PMID: 22110190]

174 **Bahrambeigi V**, Ahmadi N, Moisyadi S, Urschitz J, Salehi R, Haghjooy Javanmard S. PhiC31/PiggyBac modified stromal stem cells: effect of interferon γ and/or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) on murine melanoma. *Mol Cancer* 2014; **13**: 255 [PMID: 25428727 DOI: 10.1186/1476-4598-13-255]

175 **Zischek C**, Niess H, Ischenko I, Conrad C, Huss R, Jauch KW, Nelson PJ, Bruns C. Targeting tumor stroma using engineered mesenchymal stem cells reduces the growth of pancreatic carcinoma. *Ann Surg* 2009; **250**: 747-753 [PMID: 19826249 DOI: 10.1097/SLA.0b013e3181bd62d0]

176 **Niess H**, Bao Q, Conrad C, Zischek C, Notohamiprodjo M, Schwab F, Schwarz B, Huss R, Jauch KW, Nelson PJ, Bruns CJ. Selective targeting of genetically engineered mesenchymal stem cells to tumor stroma microenvironments using tissue-specific suicide gene expression suppresses growth of hepatocellular carcinoma. *Ann Surg* 2011; **254**: 767-74; discussion 774 [PMID: 22042469 DOI: 10.1097/SLA.0b013e3182368c4f]

177 **Zhang TY**, Huang B, Yuan ZY, Hu YL, Tabata Y, Gao JQ. Gene recombinant bone marrow mesenchymal stem cells as a tumor-targeted suicide gene delivery vehicle in pulmonary metastasis therapy using non-viral transfection. *Nanomedicine* 2014; **10**: 257-267 [PMID: 23770065 DOI: 10.1016/j.nano.2013.06.003]

178 **Malik YS**, Sheikh MA, Xing Z, Guo Z, Zhu X, Tian H, Chen X. Polylysine-modified polyethylenimine polymer can generate genetically engineered mesenchymal stem cells for combinational suicidal gene therapy in glioblastoma. *Acta Biomater* 2018; **80**: 144-153 [PMID: 30223091 DOI: 10.1016/j.actbio.2018.09.015]

179 **Ho YK**, Woo JY, Tu GXE, Deng LW, Too HP. A highly efficient non-viral process for programming mesenchymal stem cells for gene directed enzyme prodrug cancer therapy. *Sci Rep* 2020; **10**: 14257 [PMID: 32868813 DOI: 10.1038/s41598-020-71224-2]

180 **Durinikova E**, Plava J, Tyciakova S, Skvara P, Vojs Stanova A, Kozovska Z, Kucerova L, Matuskova M. Cytotoxic response of 5-fluorouracil-resistant cells to gene- and cell-directed enzyme/prodrug treatment. *Cancer Gene Ther* 2018; **25**: 285-299 [PMID: 29925898 DOI: 10.1038/s41417-018-0030-5]

181 **Mangraviti A**, Tzeng SY, Gullotti D, Kozielski KL, Kim JE, Seng M, Abbadi S, Schiapparelli P,

Sarabia-Estrada R, Vescovi A, Brem H, Olivi A, Tyler B, Green JJ, Quinones-Hinojosa A. Non-virally engineered human adipose mesenchymal stem cells produce BMP4, target brain tumors, and extend survival. *Biomaterials* 2016; **100**: 53-66 [PMID: 27240162 DOI: 10.1016/j.biomaterials.2016.05.025]

182 **Munoz JL**, Bliss SA, Greco SJ, Ramkissoon SH, Ligon KL, Rameshwar P. Delivery of Functional Anti-miR-9 by Mesenchymal Stem Cell-derived Exosomes to Glioblastoma Multiforme Cells Conferred Chemosensitivity. *Mol Ther Nucleic Acids* 2013; **2**: e126 [PMID: 24084846 DOI: 10.1038/mtna.2013.60]

183 **Qiu L**, Wang J, Chen M, Chen F, Tu W. Exosomal microRNA146a derived from mesenchymal stem cells increases the sensitivity of ovarian cancer cells to docetaxel and taxane *via* a LAMC2mediated PI3K/Akt axis. *Int J Mol Med* 2020; **46**: 609-620 [PMID: 32626953 DOI: 10.3892/ijmm.2020.4634]

184 **Shojaei S**, Hashemi SM, Ghanbarian H, Sharifi K, Salehi M, Mohammadi-Yeganeh S. Delivery of miR-381-3p Mimic by Mesenchymal Stem Cell-Derived Exosomes Inhibits Triple Negative Breast Cancer Aggressiveness; an In Vitro Study. *Stem Cell Rev Rep* 2021; **17**: 1027-1038 [PMID: 33410095 DOI: 10.1007/s12015-020-10089-4]

185 **Vakhshiteh F**, Rahmani S, Ostad SN, Madjd Z, Dinarvand R, Atyabi F. Exosomes derived from miR-34a-overexpressing mesenchymal stem cells inhibit in vitro tumor growth: A new approach for drug delivery. *Life Sci* 2021; **266**: 118871 [PMID: 33309716 DOI: 10.1016/j.lfs.2020.118871]

186 **Wu H**, Mu X, Liu L, Wu H, Hu X, Chen L, Liu J, Mu Y, Yuan F, Liu W, Zhao Y. Bone marrow mesenchymal stem cells-derived exosomal microRNA-193a reduces cisplatin resistance of non-small cell lung cancer cells *via* targeting LRRC1. *Cell Death Dis* 2020; **11**: 801 [PMID: 32978367 DOI: 10.1038/s41419-020-02962-4]

187 **Kabat M**, Bobkov I, Kumar S, Grumet M. Trends in mesenchymal stem cell clinical trials 2004-2018: Is efficacy optimal in a narrow dose range? *Stem Cells Transl Med* 2020; **9**: 17-27 [PMID: 31804767 DOI: 10.1002/sctm.19-0202]

188 **Guo J**, Nguyen A, Banyard DA, Fadavi D, Toranto JD, Wirth GA, Paydar KZ, Evans GR, Widgerow AD. Stromal vascular fraction: A regenerative reality? *J Plast Reconstr Aesthet Surg* 2016; **69**: 180-188 [PMID: 26546112 DOI: 10.1016/j.bjps.2015.10.014]

189 **Nguyen A**, Guo J, Banyard DA, Fadavi D, Toranto JD, Wirth GA, Paydar KZ, Evans GR, Widgerow AD. Stromal vascular fraction: A regenerative reality? *J Plast Reconstr Aesthet Surg* 2016; **69**: 170-179 [PMID: 26565755 DOI: 10.1016/j.bjps.2015.10.015]

190 **Yoshimura K**, Asano Y, Aoi N, Kurita M, Oshima Y, Sato K, Inoue K, Suga H, Eto H, Kato H, Harii K. Progenitor-enriched adipose tissue transplantation as rescue for breast implant complications. *Breast J* 2010; **16**: 169-175 [PMID: 19912236 DOI: 10.1111/j.1524-4741.2009.00873.x]

191 **Neuhuber B**, Swanger SA, Howard L, Mackay A, Fischer I. Effects of plating density and culture time on bone marrow stromal cell characteristics. *Exp Hematol* 2008; **36**: 1176-1185 [PMID: 18495329 DOI: 10.1016/j.exphem.2008.03.019]

192 **Barckhausen C**, Rice B, Baila S, Sensebé L, Schrezenmeier H, Nold P, Hackstein H, Rojewski MT. GMP-Compliant Expansion of Clinical-Grade Human Mesenchymal Stromal/Stem Cells Using a Closed Hollow Fiber Bioreactor. *Methods Mol Biol* 2016; **1416**: 389-412 [PMID: 27236685 DOI: 10.1007/978-1-4939-3584-0_23]

193 **Strioga M**, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? *Stem Cells Dev* 2012; **21**: 2724-2752 [PMID: 22468918 DOI: 10.1089/scd.2011.0722]

World Journal of Stem Cells

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

World J Stem Cells 2022 January 26; 14(1): 76-91

DOI: 10.4252/wjsc.v14.i1.76

ISSN 1948-0210 (online)

REVIEW

Molecular mechanism of therapeutic approaches for human gastric cancer stem cells

Hsi-Lung Hsieh, Ming-Chin Yu, Li-Ching Cheng, Ta-Sen Yeh, Ming-Ming Tsai

ORCID number: Hsi-Lung Hsieh 0000-0001-8302-2472; Li- Ching Cheng 0000-0003-3214-7798; Ming-Chin Yu 0000-0002-6980-7123; Ta-Sen Yeh 0000-0002-1830-9466; Ming-Ming Tsai 0000-0002-4495-6616.

Author contributions: All authors contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version; Hsieh HL, Yu MC and Cheng LC have contributed equally to this work.

Conflict-of-interest statement: The author has no conflict of interest to declare.

Supported by the Ministry of Science and Technology, Taiwan, No. MOST 108-2320-B-255-002-MY3; Chang Gung Medical Research Foundation, Taoyuan, Taiwan No. CMRPF1I0031, No. CMRPF1I0041, No. CMRPF1I0041-2, and No. CMRPF1L0021; and Chang Gung University of Science and Technology, Taoyuan, Taiwan, No. ZRRPF3J0081, No. ZRRPF3K0111, and No. ZRRPF3L0091.

Country/Territory of origin: Taiwan

Specialty type: Oncology

Provenance and peer review:

Hsi-Lung Hsieh, Li-Ching Cheng, Ming-Ming Tsai, Department of Nursing, Division of Basic Medical Sciences, Chang-Gung University of Science and Technology, Taoyuan 333, Taiwan

Hsi-Lung Hsieh, Ming-Ming Tsai, Research Center for Chinese Herbal Medicine, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan 333, Taiwan

Hsi-Lung Hsieh, Department of Neurology, Chang Gung Memorial Hospital, Taoyuan 333, Taiwan

Ming-Chin Yu, Ta-Sen Yeh, Department of General Surgery, Chang Gung Memorial Hospital at Linkou, Taoyuan 333, Taiwan

Ming-Chin Yu, Ta-Sen Yeh, College of Medicine, Chang Gung University, Taoyuan 333, Taiwan

Ming-Chin Yu, Department of General Surgery, New Taipei Municipal TuCheng Hospital, New Taipei 236, Taiwan

Ming-Ming Tsai, Department of General Surgery, Chang Gung Memorial Hospital, Chiayi 613, Taiwan

Corresponding author: Ming-Ming Tsai, PhD, Associate Professor, Department of Nursing, Division of Basic Medical Sciences, Chang-Gung University of Science and Technology, No. 261 Wen-hwa 1 Road, Taoyuan 333, Taiwan. mmtsai@mail.cgust.edu.tw

Abstract

Gastric cancer (GC) is a primary cause of cancer-related mortality worldwide, and even after therapeutic gastrectomy, survival rates remain poor. The presence of gastric cancer stem cells (GCSCs) is thought to be the major reason for resistance to anticancer treatment (chemotherapy or radiotherapy), and for the development of tumor recurrence, epithelial–mesenchymal transition, and metastases. Additionally, GCSCs have the capacity for self-renewal, differentiation, and tumor initiation. They also synthesize antiapoptotic factors, demonstrate higher performance of drug efflux pumps, and display cell plasticity abilities. Moreover, the tumor microenvironment (TME; tumor niche) that surrounds GCSCs contains secreted growth factors and supports angiogenesis and is thus responsible for the maintenance of the growing tumor. However, the genesis of GCSCs is unclear and exploration of the source of GCSCs is essential. In this review, we provide up-to-date information about GCSC-surface/intracellular markers and GCSC-mediated pathways and their role in tumor development. This information will support

Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: April 18, 2021
Peer-review started: April 18, 2021
First decision: May 12, 2021
Revised: May 15, 2021
Accepted: December 21, 2021
Article in press: December 21, 2021
Published online: January 26, 2022

P-Reviewer: Rojas A
S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM

improved diagnosis, novel therapeutic approaches, and better prognosis using GCSC-targeting agents as a potentially effective treatment choice following surgical resection or in combination with chemotherapy and radiotherapy. To date, most anti-GCSC blockers when used alone have been reported as unsatisfactory anticancer agents. However, when used in combination with adjuvant therapy, treatment can improve. By providing insights into the molecular mechanisms of GCSCs associated with tumors in GC, the aim is to optimize anti-GCSCs molecular approaches for GC therapy in combination with chemotherapy, radiotherapy, or other adjuvant treatment.

Key Words: Gastric cancer stem cells; Stem cell-surface markers; Tumor niche; Tumor microenvironment

Core Tip: In recent years, cancer stem cells (CSCs) have become an extremely important subject in cancer biology. CSCs are considered to be a very small component of tumor tissues, which nevertheless have the capacity for self-renewal, differentiation, and epithelial–mesenchymal transition. CSCs can also explain the clinical phenomenon of resistance to chemotherapy or radiotherapy. Many studies have also found that CSCs are important for cancer initiation and metastasis and play a key role in cancer recurrence. Here, we review GCSC (gastric cancer stem cell)-targeting therapies in GC, including information on GCSC-surface/intracellular markers and GCSC-mediated pathways. This review also provides a brief description of the GCSC niche. Understanding the molecular mechanism of GCSC-targeting agents can help optimize the accuracy of diagnosis and prognosis and the selection of the most appropriate therapy for GC patients.

Citation: Hsieh HL, Yu MC, Cheng LC, Yeh TS, Tsai MM. Molecular mechanism of therapeutic approaches for human gastric cancer stem cells. *World J Stem Cells* 2022; 14(1): 76-91
URL: https://www.wjgnet.com/1948-0210/full/v14/i1/76.htm
DOI: https://dx.doi.org/10.4252/wjsc.v14.i1.76

INTRODUCTION

Despite progress in the diagnosis, prognosis, and therapeutic approaches to gastric cancer (GC), it persists as one of the most commonly identified malignant tumors in the world, and with a challenging mortality rate[1-3].

The discovery of cancer stem cells (CSCs) has explained much cancer behavior. Although CSCs account for only a small percentage of the entire tumor tissues, these cells are the key components that form the entire tumor. CSCs are viewed as a tumor-initiating subpopulation of cells within tumors capable of self-renewal, which can divide and differentiate into various tumor cell types (intratumoral heterogeneity). They are highly tumorigenic, involved in metastasis, relatively resistant to chemotherapy and radiotherapy, secrete antiapoptotic factors, undergo epithelial-mesenchymal transition (EMT), and display a higher performance of drug efflux pumps[4-7]. Opinions regarding the existence, role, and behavior of CSCs differ, suggesting that basic research on CSCs is crucial. Understanding CSCs also gives new ideas for the treatment of cancer. On the premise that targeted killing of CSCs is the fundamental way to completely eliminate tumor tissues, methods specifically focused on CSCs have become a very important topic, the ultimate goal being to design drugs for treatment of GC[8-11].

In recent years, due to the rapid development of CSC research, methods used to identify CSCs include evaluation of tumor formation in immune-deficient mice, tumorigenicity, GCSC generation *in vivo*, spheroid colony formation, metastasis, EMT, chemotherapy resistance, radiotherapy resistance, and expression of specific cell-surface/intracellular markers *in vitro*. The GCSC topics to be discussed in this review focus on GCSC-surface/intracellular markers, intracellular markers, the regulation of

GCSCs in the tumor microenvironment (TME), and the potential for GCSC-targeted treatments in the future (Figure 1).

MAIN CELL-SURFACE/INTRACELLULAR MARKERS OF GCSCS

Since pathologists found that CSCs and SCs have many similarities in histological morphology, the concept that "malignant tumors arise from SCs" is an existing hypothesis[12,13]. Support for this hypothesis was first published in 1994 by Dick *et al* [14], who discovered CSCs in acute myeloid leukemia (AML).

In 2003, Singh *et al*[15] showed that CSCs can also be successfully isolated from several solid tumors. CSCs are a common phenomenon in cancer, not all of a specific cancer[5,15,16]. In solid tumors, the earliest successful isolation of CSCs was in breast cancer, when researchers used cell-surface markers and successfully isolated CSCs [16]. Moreover, high tumor-forming ability is an extremely important biological characteristic of CSCs[15]. To date, various CSCs have been shown to display different stem cell-surface markers. However, CD44 and CD133 are currently the most commonly used cell-surface markers for the identification of CSCs[5]. As noted, because the proportion of each type of CSCs present in solid tumors also differs, it is likely that more cell-surface/intracellular markers will be found in the future that can more accurately determine the presence of CSCs[5].

GCSC-surface markers have also been found using flow cytometry in GC cell lines and primary GC tissues, confirming the presence of CSCs in GC[17-20], as shown in Table 1[21-49], and other potential GCSC-intracellular markers are summarized in Table 2[23,48,50-57].

CD44+, CD44H, CD44 splice variants (CD44v), CD44+/CD24+, CD44+/CD54+, EpCAM+/CD44+

CD44 is a transmembrane glycoprotein that functions as a receptor for hyaluronic acid that is involved in the Wnt/β-catenin pathway that regulates cell migration and homing[21]. Takaishi *et al*[21] showed that CD44+ was first identified as a common GCSC-surface marker using GC cell lines, which demonstrated spheroid formation ability in *vitro* and established tumorigenicity in *vivo* when injected into immune-deficient mice. Chen *et al*[22] reported that CD44+ cells have a sphere-forming ability that can be a cell-surface marker of GCSCs. They also provided a reasonable explanation for the chemoresistance that is frequently observed in GC patients. Takafumi *et al*[23] showed that CD44+ cells have higher potential for peritoneal metastasis associated with GCSC-surface markers.

CD44 has two isoforms: CD44H, which reveals a higher affinity for hyaluronic acid, and a CD44 splice variant, CD44v, which displays prometastatic ability. Ishimoto *et al* [25] reported that CD44v reduced glutathione (GSH) synthesis and provided protection against reactive oxygen species (ROS). Both CD44H and CD44v may act as GCSC-surface markers.

In addition, CD44+/CD24+ have been examined in GC cell lines and primary GC tissues using flow cytometric analysis[29]. It was found that the presence of CD44+/CD24+ was associated with a higher tumorigenicity compared with the control cells (CD44–/CD24–) in an immune-deficient mice model. Hence, these cells have self-renewal and differentiation abilities and the combined expression of CD44+/CD24+ acts as a potential GCSC-surface marker[29].

Combined CD44+/CD54+ cells were isolated from the peripheral blood of GC patients and tumor growth similar to the original human tumor was generated when the cells were injected into immune-deficient mice. The same cells can be differentiated into gastric epithelial cells and self-renewed in vitro. These results suggest that the combined CD44+/CD54+ phenotype could be used as a potential cell-surface marker for GCSCs[30]. Another group reported that GCSCs also isolated from human tumor tissues and peripheral blood carried CD44+/CD54+ GCSC-surface markers[22].

Epithelial cell adhesion molecule (EpCAM)+/CD44+ has also been identified as CSC-surface marker in various types of tumors[58-61]. EpCAM+/CD44+ cells from human GC tissues grew into tumors in an immune-deficient mice model, they maintained differentiation potency, and reproduced the intratumoral heterogeneity of the original gastric tumors. EpCAM+/CD44+ cells owned anticancer agents than other subtypes of cells[31]. Han *et al*[31] demonstrated that EpCAM+/CD44+ cells from GC tissues are viable GCSC-surface markers by using an immune-deficient mice model. However, Rocco *et al*[62] reported that CD44+/CD133+ cells displayed neither SC effects nor revealed tumor-initiating effects.

Table 1 Potential gastric cancer stem cells cell-surface markers by using flow cytometric analysis

Cell-surface markers	Function	Physiological significance					Involved signaling pathways	Therapeutic targets or inhibitor	Prognostic marker of GC	Ref.
		Tumorigenicity/GCSC generation	Spheroid formation	Chemo-resistance	Radio-resistance	EMT/metastasis				
CD44	Hyaluronic acid receptor	•	•	•	ND	•	Wnt/β-catenin pathway	CD44	•	[21-24]
CD44H	Hyaluronic acid receptor	•	•	•	ND	ND	Wnt/β-catenin pathway	CD44	•	[25]
CD44v	Hyaluronic acid receptor	•	•	ND	ND	•	Glutathione metabolism	Glutathione metabolismsulfasalazine (SSZ)	•	[25-28]
CD44+CD24	Cell adhesion molecule	•	•	•	ND	ND	Notch pathway	CD44+CD24	•	[24,29]
CD44+CD54	Cell adhesion molecule	•	•	•	ND	ND	Wnt/β-catenin pathway	CD44+CD54	ND	[22,24, 30]
CD44+EpCAM	Cell adhesion molecule/epithelial cell adhesion molecule	•	•	•	ND	ND	Wnt/β-catenin pathway	ND	ND	[24,31]
CD71-	Transferrin receptor	•	ND	ND	ND	•	uptake of transferrin/iron	CD71	•	[32]
CD90	Immunoglobulin superfamily/Thy-1 cell-surface antigen	•	•	ND	ND	•	Notch pathway	CD90trastuzumab	ND	[24,33]
CD133	Pentaspan transmembrane glycoprotein	•	•	ND	ND	ND	MAPK/ERK pathway	CD133	•	[24]
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5	•	ND	•	ND	•	Notch-mTOR pathway	Notch-mTOR pathwayMiR-132Anti-Lgr5	•	[34-38]
ALDH1	Aldehyde dehydrogenase 1	•	•	•	•	•	Wnt/β-catenin pathway	TGF-	•	[39]
CD44+CD166+ALDH	Activated leukocyte cell adhesion molecule (ALCAM)	•	•	•	ND	•	Wnt/β-catenin pathway	CD44CD166TGF-	•	[24,40, 41]
CXCR4	C-X-C chemokine receptor type 4	•	•	•	ND	•	CXCR4/SDF-1pathway	CXCR4	•	[42]
ABCB1/MDR1ABCG2	ABC transporters	•	•	•	ND	•	JAK/STAT pathwayHH/PTCH1 pathway	ABCB1/MDR1ABCG2	•	[23,33, 43-45]

SP	Side population	•	•	•	ND	•	Wnt/β-catenin pathway	ND	•	[21,23,44,46-48]
Lrig1	Regulatory factor of cell cycle	•	•	•	ND	•	ErbB pathway	Lrig1	•	[49]
2-, 5-, 3- and 5- integrins	Cell adhesion molecule	•	•	ND	ND	•	p38/ERK pathway	Integrins	ND	[23]

•: Determined; ND: Not determined. CD44: Cluster of differentiation 44; CD44v: CD44 splice variant; Lgr5: Leucine-rich repeat-containing G-protein coupled receptor 5; ALDH1: Aldehyde dehydrogenase 1; CXCR4: C-X-C chemokine receptor type 4; GCSCs: Gastric cancer stem cells; GC: Gastric cancer; EpCAM: Epithelial cell adhesion molecule; Lgr5: Leucine-rich repeat-containing G-protein coupled receptor 5; ABCB1: ATP-binding cassette subfamily B member 1; ABCG2: ATP-binding cassette subfamily G member 2; MDR1: Multidrug resistance protein 1; SP: side population; Lrig1: Leucine rich repeats and immunoglobulin like domains protein 1; SDF-1: Stromal cell-derived factor-1; SSZ: Sulfasalazine; TGF-β: Transforming growth factor-β; HH: Hedgehog; PTCH1: Patched homolog 1; MAPK: Mitogen-activated protein kinase; ERK: Extracellular-signal-regulated kinase; JAK :Janus kinase; STAT3: Signal transducer and activator of transcription 3; mTOR: Mechanistic target of rapamycin; EMT: Epithelial–mesenchymal transition; EGFR: Epidermal growth factor receptor; miRNA: MicroRNA.

CD71-

CD71 belongs to the transferrin receptor that mediates the uptake of transferrin/iron complexes on the surface of red cells. Ohkuma *et al*[32] reported that the CD71-cell surface marker was increased in the G1/G0 phase causing cell-cycle arrest, and CD71-cell the invasive heads of cancer motivations, showing CD71- cells have high tumorigenicity, multipotency, and invasiveness abilities. Thus, they suggested that CD71- is a suitable GCSC-surface marker for discovering GC in patients.

CD90

CD90 belongs to the immunoglobulin superfamily and the Thy-1 cell-surface antigen (adhesion molecule) family, which is involved in several signal pathways (such as the Notch pathway). Jiang *et al*[33] tested CD90+, a well-known CSC-surface marker in gastric primary tumors. They found that CD90+ GC cells can format spheroid populations, undergo self-renewal, and form a tumor hierarchy from human gastric primary tumors *in vitro* and tumorigenicity of gastric primary tumor models *in vivo*. Thus, they suggested that CD90+ is a potential GCSC-surface marker.

CD133

CD133 belongs to the pentaspan transmembrane glycoprotein, which is considered to be a hematopoietic SC-surface marker. Zhao *et al*[63] verified that CD133 is significantly related to CSC-surface markers in various tumors; however, whether CD133 is also a significant GCSC-surface marker remains unclear[62,63].

Leucine-rich repeat-containing G protein-coupled receptor 5

Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) was identified as a novel normal SC-surface marker of the gastrointestinal tract[34,35,64]. CD44+, ALDH1+, and CD133+ cell-surface marker cells co-combined with Lgr5+ cells in the SCs of adjacent normal gastric mucosa in GC[34]. One group has shown that Lgr5+

Table 2 Potential gastric cancer stem cells intracellular markers

Intracellular markers	Function	Physiological significance					Involved signaling pathways	Therapeutic targets	Prognostic marker of GC	Ref.
		Tumorigenicity/GCSC generation	Spheroid formation	Chemo-resistance	Radio-resistance	EMT/metastasis				
DOCK6	Dock-C subfamily guanine nucleotide exchanger	•	•	•	•	•	Wnt/β-catenin pathway	DOCK6	•	[50]
Mist1	Transcriptional factor	•	•	•	ND	•	Wnt/β-catenin pathway	Mist1	•	[51]
MSI-1	RNA-binding protein	•	•	•	ND	•	EGFR pathway	MSI-1	•	[48]
NANOG	Transcription factor	•	•	•	ND	•	Wnt/β-catenin pathwayJAK-STAT pathway	NANOG	•	[23,52]
Oct3/4	Octamer-binding transcription factor 3/4	•	•	•	ND	•	Wnt/β-catenin pathwayJAK-STAT pathway	Oct3/4	•	[23,53]
SOX2	Sex determining region Y-box 2	•	•	•	ND	•	Wnt/β-catenin pathwayJAK-STAT pathway	SOX2MiR-134all-trans retinoic acid	•	[52-57]

•: Determined; ND: Not determined; DOCK6: Dedicator of cytokinesis 6; Mist1: Muscle, intestine and stomach expression 1; MSI-1: Musashi RNA binding protein 1; GCSCs: Gastric cancer stem cells; GC: Gastric cancer; EMT: Epithelial–mesenchymal transition; NANOG: nanog homeobox; Oct3/4: Octamer-binding transcription factor 3/4; SOX2: Sex determining region Y-box 2; MAPK: Mitogen-activated protein kinase; ERK: Extracellular-signal-regulated kinase; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3; EGFR: Epidermal growth factor receptor; miRNA: MicroRNA.

cells continuously produced SCs in the gastric glands of transgenic mice under normal homeostatic conditions[35]. Another group suggested increased LGR5+ cell-surface marker GCSCs during gastric tumorigenesis may play a role in the development and progression of GC[36].

Aldehyde dehydrogenase 1

Aldehyde dehydrogenase 1 (ALDH1) belongs to the aldehyde dehydrogenase family of enzymes that catalyzes the oxidation of aromatic aldehydes to carboxyl acids. Katsuno *et al*[39] found ALDH1 to be another novel GCSC-surface/intrcellular marker. The ALDH1+ cells showed higher tumorigenic potential, self-renewal, and produced heterogeneous cell populations compared with ALDH1– cells for a human GC cell line *in vitro* and *in vivo*. In addition, they used transforming growth factor- (TGF-) therapy to destroy ALDH1+ cells and their tumorigenicity through downregulation of ALDH1 and regenerating islet-derived protein 4 (REG4)[39].

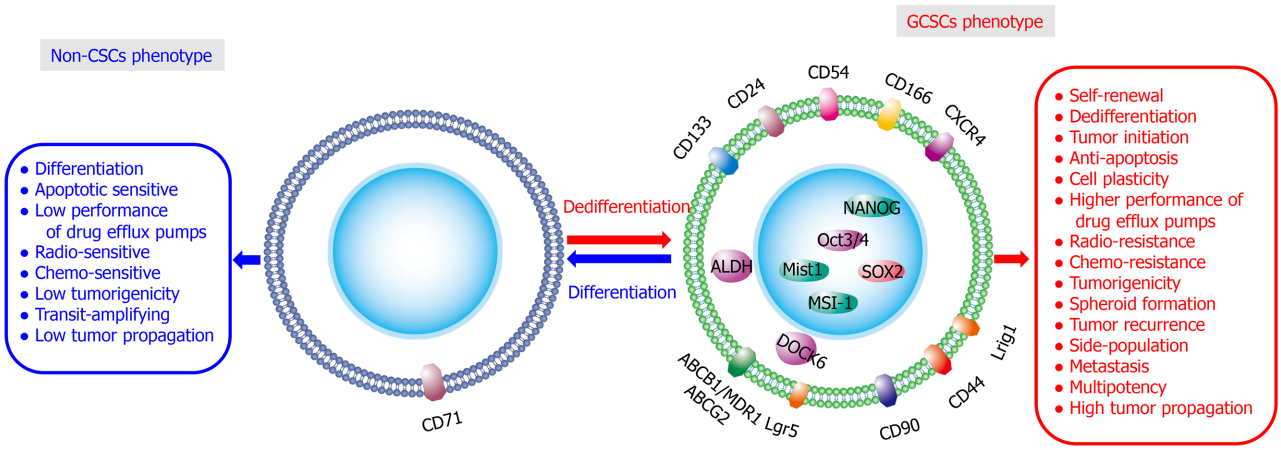

Figure 1 Molecular properties of gastric cancer stem cells. Main gastric cancer stem cells (GCSCs)-surface markers (such as CD44, CD44v, CD71, CD90, CD133, Lgr5, ALDH1, CXCR4, ABC, and Lrig1) and GCSC-intracellular markers (such as DOCK6, Mist1, MSI-1, NANOG, Oct3/4, and SOX2). GCSCs represent a subpopulation of cancer cells (non-CSCs) existing within heterogeneous tumors implicated in tumor initiation, growth, metastasis, and chemo-/radioresistance, antiapoptosis, cancer recurrence, and metastasis. However, a small number of non-CSCs can dedifferentiate and transform into GCSCs through TME-induced EMT. The newly generated GCSCs from non-CSCs, together with the intrinsic GCSCs, consequently contribute to recurrence and metastasis of cancer. CD44: cluster of differentiation 44; CD44v: CD44v: CD44 splice variant; CD71: cluster of differentiation 71; CD90: cluster of differentiation 90; CD133: cluster of differentiation 133; Lgr5: leucine-rich repeat-containing G-protein coupled receptor 5; ALDH1: aldehyde dehydrogenase 1; CXCR4: C-X-C chemokine receptor type 4; ABC: ATP-binding cassette subfamily; Lrig1: leucine rich repeats and immunoglobulin like domains protein 1; DOCK6: dedicator of cytokinesis 6; Mist1: muscle, intestine and stomach expression 1; MSI-1: musashi RNA binding protein 1; NANOG: nanog homeobox; Oct3/4: octamer-binding transcription factor 3/4; SOX2: sex determining region Y-box 2.

C-X-C chemokine receptor type 4

By using microarray analysis, studies have found that C-X-C chemokine receptor type 4+ (CXCR4+) cells can be a novel GCSC-surface marker. CXCR4+ cells can form spheroid colonies, and they have high metastatic ability and chemotherapy resistance *in vitro*. Moreover, CXCR4+ cells have tumorigenicity and GCSC generation capacity in immune-deficient mice *in vivo*. CXCR4+ cells were also found in clinical tissues[42].

Side population, Nanog homeobox, octamer-binding transcription factor 3/4, a2, a5, b3, and b5 integrins

Side population (SP) is well known as a CSC-rich population in many tumors[23,44,46,47]. GC cell lines have also isolated SP cells[23,46,65]. Furthermore, studies have confirmed that SP cells showed higher engrafted tumor formation and peritoneal metastasis with overexpression levels of adhesion molecules (such as 2, 5, 3, and 5 integrins) and CD44 compared with those of the non-SP cells in GC cell lines. Moreover, the mRNA overexpression of GCSC markers ALDH1, CD44, Nanog homeobox (NANOG), and octamer-binding transcription factor 3/4 (OCT3/4) was significant in SP cells, which are similar to CSCs[23]. Fukuda *et al*[46] described similar results and found that SP cells are more tumorigenic and chemoresistant compared with non-SP cells in GC cell lines and human GC tissues, which remain in an undifferentiated state and display a different hierarchy in malignancy.

Ehata *et al*[66] found that SP cells display greater tumorigenicity, self-renewal activity, and multipotency of SC phenotypes *in vivo* compared with non-SP cells in human diffuse-type GC cells but not intestinal-type GC cells. Another group reported that SP cells were smaller and expressed CD133 and MSI-1, which yielded SP and non-SP cells in recultivation experiments[44]. Elsewhere it was reported that SP cells also have GCSC properties in the MKN-45 GC cell line. However, SP cells did not have GCSC properties in BGC823 and other GC cell lines[21,48,67]. Nevertheless, using the SP cell assay to isolate GCSCs remains debatable.

ATP-binding cassette subfamily B member 1/multidrug resistance protein 1 and ATP-binding cassette subfamily G member 2

Jiang *et al*[33] demonstrated the overexpression of the GCSC markers ATP-binding cassette subfamily B member 1/multidrug resistance protein 1 (ABCB1/MDR1) and ATP-binding cassette subfamily G member 2 (ABCG2) of SP cells in human GC tissues and several GC cell lines. Previous studies found that ABCB1/MDR1 and ABCG2 belong to ABC transporters, which can remove toxic multidrugs extracellularly

causing chemoresistance of GCSCs. The expression of these transporters is related to the response to therapy and survival for GC patients[23,43-45].

Dedicator of cytokinesis 6

Chi *et al*[50] revealed that overexpression of dedicator of cytokinesis 6 (DOCK6) promoted chemoresistance, radioresistance, GC progression, and independent biomarkers of GC prognosis through the Rac family small GTPase 1 (Rac1) activation in the WNT/β-catenin signaling pathway. DOCK6 acts as a guanine nucleotide exchange factor (GEF) for Rac1 and CDC42. These results suggest that DOCK6 is a novel GCSC marker in GC cell lines, animal models, and clinical GC tissues[50].

Muscle, intestine, and stomach expression 1

Previous studies found that muscle, intestine, and stomach expression 1 (Mist1)+ SCs act as an initial cell for intestinal-type GC combined with K-RAS and APC mutations. On the other hand, Mist1+ SCs act as an initial cell for diffuse-type GC development that is dependent on an inflammation-mediated GCSC niche including endothelial cells secreting C-X-C motif chemokine ligand 12+ (CXCL12+) and CXCR4+ gastric innate lymphoid cells (ILCs) secreting Wnt5a to activate RhoA in the loss of E-cadherin GC cells[51].

Leucine-rich repeats and immunoglobulin-like domains protein 1

Leucine-rich repeats and immunoglobulin-like domains protein 1 (Lrig1)+ (a pan-ErbB inhibitor) GCSCs are involved in cell-cycle repression and response to oxidative damage. Loss of APC in Lrig1+ cells leads to intestinal-type GC, or genetic loss of Lrig1 resulting in higher expression of ErbB1-3[56].

Sex-determining region Y-box 2

Microarray studies have found that aberrant expression of sex-determining region Y-box 2 (SOX2) has been observed in GCs. SOX2 transcriptional activity can promote cell proliferation and migration, antiapoptosis, and chemoresistance, and suppress changes in cell cycle and tumorigenic potential in vitro and in vivo for GC development and progression[54]. These putative markers may be suitable to isolate GCSCs and offer new insights into novel approaches for GC therapy by targeting GCSCs in clinical trials. It is important to note that most published markers are not specific to GC. Moreover, different GCSCs may coexist within the same tumor mass. Thus, these putative marker GCSCs may also contain SCs and progenitor cells. Therefore, in terms of targeted therapy, identification of a single cell-intracellular marker on GCSCs may not be enough to kill all GCSCs, and thus, a combination of these GCSC markers is essential.

GCSCS REGULATE SIGNALING PATHWAYS IN THE TUMOR NICHE

In recent years, it has been discovered that it is impossible to treat a differentiated tumor without knowing exactly the true group of CSCs. However, normal cancer cells (non-CSCs) can be affected by the TME, epigenetic regulation, and other factors leading to dedifferentiation and a manifestation of CSCs. Therefore, CSCs are a dynamic balance. CSCs can be regulated by stromal cells or could differentiate into tumors with several populations. While internally differentiated tumor populations can occur, dedifferentiation under the influence of the microenvironment can cause cells to revert to CSCs. This idea has greatly affected our biological knowledge of malignant tumors. Awareness of cancer treatment, especially evaluation of treatment that is effective for CSCs, with novel methods of clinical trials should be rethought[10,68].

In addition, CSCs express specific cell-surface/intracellular antigens, and the signaling pathways also differ from non-CSCs, reflecting the characteristics of the cells. Figure 2 shows that non-CSCs can be regulated in the external environment, in signal transmission, and in epigenetic modification[69]. The ability to dedifferentiate under genetic control displays a specific characteristic of CSCs[6,70-72].

There are several signaling pathways involved in the drive and maintenance of CSCs in both normal and cancer cells (non-CSCs) (Figure 3).

Hedgehog pathway

The Hedgehog pathway has a primary role in cell differentiation and normal vertebrate embryonic development[27]. The Hedgehog pathway, which regulates

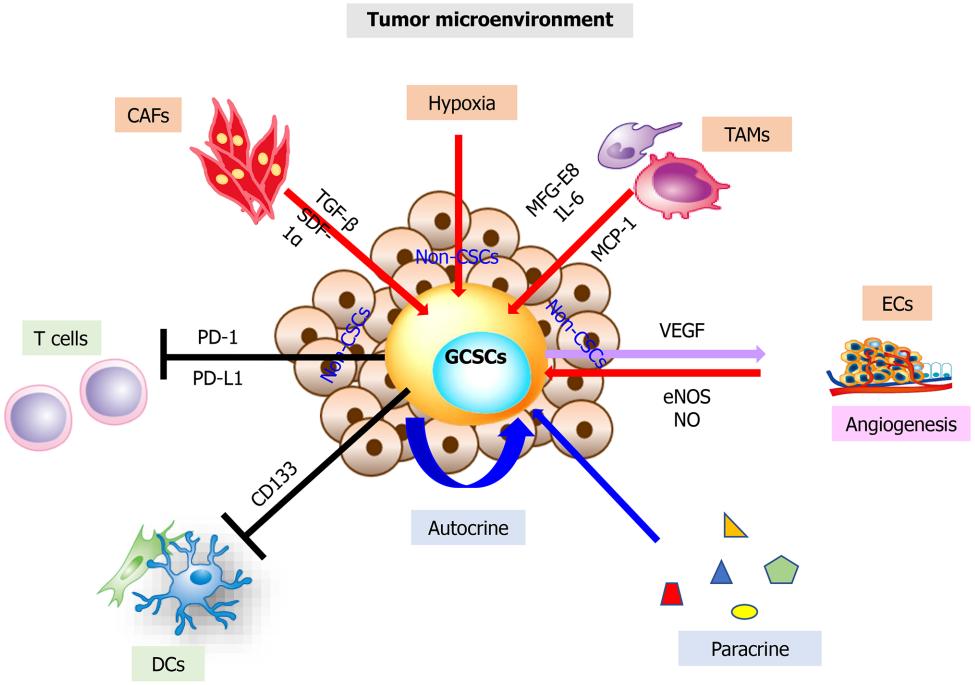

Figure 2 The roles of gastric cancer stem cells in the tumor microenvironment and activated in gastric cancer stem cells. This figure shows the dynamic regulation of the tumor niche and GCSCs. Cancer cells (non-CSCs) can dedifferentiate by regulating their intracellular signaling pathways, gene expression, and epigenetic modification through the functional connection of the tumor niche to differentiated cancer cells (non-CSCs) to obtain the GCSC phenotype. Stromal cells can support GCSCs development through various kinds of interactions. Tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), tumor vascular endothelial cells (ECs) and hypoxia not only directly enhance the CSC capabilities of GCSCs by activating the several pathways but also inhibit T cells and dendritic cells (DCs) activity. TGF-β: transforming growth factor-β; SDF-1α: stromal cell-derived factor-1α; PD-1: programmed cell death 1; PD-L1: programmed cell death 1 Ligand 1; CD133: cluster of differentiation 133; MFG-E8: milk fat globule epidermal growth factor 8; NO: nitric oxide; eNOS: endothelial NO·synthase; IL-6: interleukin 6; MCP1: monocyte chemoattractant protein 1; VEGF: vascular endothelial growth factor.

adult SCs in tissue maintenance and repair, is inactive in most adult tissues. However, activation of the Hedgehog pathway induces tumor progression and radiation resistance in several cancers to cause mutations in the patched homolog 1 (PTCH1) and smoothened (SMO; frizzled family receptor) genes in patients[31].

Notch/STAT3 pathway

In several cancers, the activation of the STAT family (such as STAT1, STAT3, STAT4, STAT5a, STAT5b, and STAT6) *via* phosphorylation of a specific tyrosine kinase residue promotes tumor growth and metastasis. Among them, STAT3 activation plays a key role in tumor progression[73]. Studies show that the action of many protein tyrosine kinases, oncogenes, IL-17, and viruses is mediated *via* activation of the downstream phosphorylated STAT3 transcription factor pathway. These actions include cellular proliferation, invasion, migration, antiapoptosis, and angiogenesis in GCSCs[74]. Additionally, STAT3 upregulated cyclin D1 and c-Myc expression, contributing to enhanced cell-cycle progression in cancer cells. Interestingly, targeting STAT3 activation suppresses tumor growth and metastasis, without affecting normal cells *in vitro* and *in vivo*, suggesting that STAT3 could be effective for GCSC-targeting therapy [75].

NANOG pathway

In embryonic SCs, signal transducer and activator of transcription 3 (STAT3) forms a complex with NANOG for maintaining SC pluripotency. Various studies propose that NANOG may act as an oncogene to be activated in several cancers in which overexpression correlates with poor survival promoting oncogenesis in patients[76].

Wnt/β-catenin pathway

Wnt ligands are produced from cells in the SC microenvironment, and the Wnt/β-catenin pathway has been identified for its role in CSC self-renewal and radiation-resistant pathways[30,77]. Wnt signaling is associated with EMT with a poor clinical outcome[78].

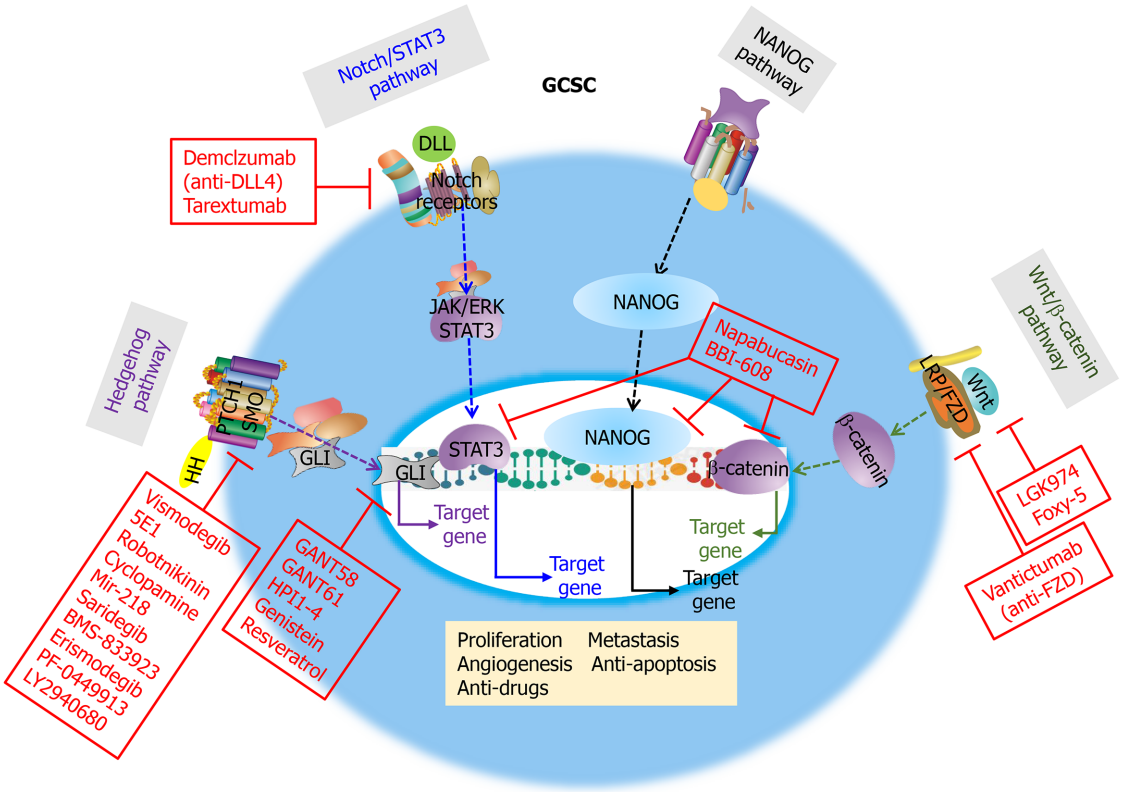

Figure 3 Four signal pathways contribute to stemness properties of cancer stem cells: Hedgehog, Notch/STAT3, NANOG, and Wnt/β-catenin pathways. Hedgehog pathway: PTCH1-induced inhibition of SMO is reversed by HH binding with PTCH1, leading to the release of the complex of GLI from microtubules, with GLI protein entering the nucleus to transcriptionally activate downstream target genes. Notch/STAT3 pathway: DLL binding-induced Notch activation causes several kinase proteins activation, further converting STAT3 to a transcriptional activator to initiate downstream gene expression. NANOG pathway: Ligand binding-induced NANOG activation causes several kinase proteins activation, further converting STAT3 to a transcriptional activator to initiate downstream gene expression. Wnt/β-catenin pathway: Wnt binds to its receptor, Frizzled to activate LRP protein. The activated LRP protein enhances the phosphorylation of the kinase (a component of the cytoplasmic complex that promotes phosphorylation of β-catenin and its degradation), which inhibits the kinase, further causing the accumulation of free and unphosphorylated β-catenin in the cytoplasm that is then translocated to the nucleus. In the nucleus, β-catenin binds to other transcriptional factors to promote downstream target gene expression.

Due to CSCs have chemotherapy resistant treatments associated with GCSC-related signaling pathways and develops CSCs targeted therapy[79,80]. The research on CSC signaling pathways is still preliminary. Their functional regulatory mechanisms are worthy of advanced discussion regarding the similarities and differences of signaling pathways between differentiated cancer cells (non-CSCs) and CSCs. A solution to these problems can help understand the resistance phenomenon caused by clinical treatment of tumors, and design approaches that could provide an important reference for anti-CSCs targeting drugs[81]. Therefore, these CSC-related pathways are commonly new targets for the development of anticancer drugs or inhibitors in various clinical trials[69,82] (Table 3)[27,28,73,75-78,83-86].

CURRENT TREATMENT OF THE POTENTIAL FOR TARGETING GCSCS

In general, most of the initial tumor appears reduced in size after treatment. However, the appearance of drug resistance after treatment is common because a subpopulation of differentiated cancer cells (non-CSCs) develops drug resistance. CSCs are more resistant to conventional chemotherapy, EMT, more antiapoptotic factors, and higher performance of drug efflux pumps than differentiated cancer cells (non-CSCs)[6,8,71,72].

The reason CSC theory has received considerable attention in recent years is because CSCs can escape the poison of chemotherapy drugs, which can explain the failure of chemotherapy.

Usually CSCs are parked in the cell cycle G_0 phase, that is, they will not enter the cell cycle. Currently, most chemotherapeutic drugs focus on inhibiting the cell cycle in a growing cell. The growth of differentiated cancer cells (non-CSCs) in the cell cycle

Table 3 Regulators of gastric cancer stem cells-related pathway stimuli in gastric cancer stem cells stemless properties and gastric cancer stem cells-targeted therapy

GCSCs pathway	Involved pathway proteins	Therapeutic inhibitor or drug	Ref.
Hedgehog (HH)	PTCH1, SMO, GLI	VismodegibE1RobotnikininGANT58GANT61HPI1-4GenisteinResveratrolCyclopamineMir-218SaridegibBMS-833923ErismodegibPF-0449913LY2940680	[27,28,83,84]
Notch/STAT3	Notch ligands (DLL), Notch receptors, JAK, ERK, STAT3	NapabucasinDemclzumab (anti-DLL4)TarextumabBBI-608	[28,76,85,86]
NANOG	NANOG, STAT3, Kinases	NapabucasinBBI-608BB1-503	[28,73,75,85]
Wnt/β-catenin	Wnt, β-catenin, LRP/FZD	Napabucasin BBI-608Vantictumab (anti-FZD)LGK974Foxy-5	[28,77,78,85,86]

GCSCs: Gastric cancer stem cells; HH: Hedgehog; NANOG: Nanog homeobox; STAT3: Signal transducer and activator of transcription 3; GLI: Glioma-associated oncogene; SMO: Smoothened; PTCH1: Patched homolog 1; ERK: Extracellular-signal-regulated kinase; JAK: Janus kinase; Wnt: Wingless/int1; LRP: Lipoprotein receptor-related proteins/low density lipoprotein receptor-related proteins; FZD: Frizzled receptors; DLL: Notch ligands.

can be prevented, but the drugs cannot kill CSCs. Once chemotherapy is over, the surviving CSCs will regenerate and proliferate, leading to the recurrence of cancer. Because some studies show that chemotherapeutic drugs may promote CSCs after treatment, it is recommended to use two-stage therapy to kill CSCs. Therefore, the first stage is to promote CSCs into the cell cycle, and the second stage uses chemotherapy drugs or targeted CSC therapy to kill CSCs[87]. Several studies have indicated that CSCs promote recurrence after chemotherapy[6].

It has also been reported that CSCs will increase tumor cell invasion, EMT, and metastatic ability[4-7]. Another group pointed out that CSCs will increase the protein level of the chemokine receptor CXCR4; thus, CSCs will follow the of its stromal cell-derived factor-1 (SDF-1; CXCR4 ligand) concentration degree of metastasis, from the original position (low levels of SDF-1) in CSCs microenvironment (tumor niche) transfer to any tissue that exhibits high levels of SDF-1. CXCR4 neutralizing antibody can effectively inhibit CSC metastasis in nude mice. These results confirm that CSC cells can indeed promote cancer EMT, metastasis, and invasion abilities[47].

Wang *et al*[88] and Ricci-Vitiani *et al*[89] found that glioma cells are derived from neuron-like glioma stem cells. They also found that the tumor tissue and the tumor inside vascular ECs (vascular endothelial cells) both have the same aberrant genes. Researchers have found that these tumor-derived vascular ECs come from CSCs, that is, CSCs can differentiate into vascular ECs to form new blood vessels. This result challenges the existing belief that the internal vascular ECs of a tumor are normal cells. It also explains why combretastatin A-4 (a vascular disrupting agent) can selectively abolish the existing tumor vascular ECs but is less harmful to normal blood vessels.

FURTHER CHALLENGES OF ANTIGCSCS THERAPY

Currently, scholars have different opinions on the origin of GCSCs. Additionally, various tumors do not have the same origin of CSCs and our understanding needs to be deepened. Importantly, GCSCs play an important role in the occurrence and development of tumors, EMT, metastasis, recurrence, and prognosis.

If only GCSCs are removed, is such treatment sufficient? Obviously, differentiated cancer cells (non-CSCs) also need targeted therapy. Moreover, both GCSCs and their differentiated cancer cells (non-CSCs) require effective targeted therapy. In the future, newly developed drugs will provide opportunities for successful cancer treatment to improve the patient's prognosis. Efficacy of these drugs must first be confirmed by clinical trials.

Given that research in GCSCs is an emerging field, many details remain unclear, and future GCSC research will need to focus on several key issues: (1) The determination of GCSC-specific molecules of cell-surface/intracellular markers; (2) The environment of the GCSC niche, the interaction and molecular mechanism between the GCSC tumor niche and GCs; (3) The molecular mechanism of malignant transformation/EMT of normal SCs; (4) The need to verify and clarify the mechanism of GCSC chemo-/radioresistance therapy; (5) Exploration and development of

therapeutic approaches for GCSCs; and (6) Overall, monitoring of the role of GCSCs to evaluate the indicators (monitoring circulating tumor cells), it means that may appear for new metastatic lesions which can be early detected. Therefore, with the evaluation of clinical trials involving GCSCs, the results of GCSC therapy will help improve anticancer treatment. GCSC-targeted drug therapy alone may not be sufficient to fight cancer, but combined treatment can improve treatment results. Finally, GCSC theory provides a new way to evaluate anticancer drugs in clinical trials and provide a framework for effective drug development. We believe that cancer can be cured, or at least, become a chronic and controllable disease; this goal will surely be achieved in the future.

Following improvements in research technology, it is hoped that a better understanding of GCSC-surface/intracellular markers and related signal pathways will help antitumor growth, early diagnosis, GCSC-targeted drug therapy, antimetastasis, anti-EMT, as well as recurrence prevention and prognosis judgment, all of which have significant therapeutic application.

CONCLUSION

In recent years, an increasing number of studies have highlighted that CSCs are indeed tumor-initiating cells in cancer tissues. These cells may not be a single homogeneous cell: different tissues or different patients have different tumor initiation cells. The more we understand these GCSCs or tumor-initiating cells, the better we can design drugs to kill them, such as using second-stage therapy to kill GCSCs[87] or designing a monoclonal Ab (antibody) or small interference RNA (siRNA) that blocks GCSC-surface/intracellular markers. For example, CD44 can promote the differentiation of GCSCs to complete the treatment. With the joint efforts of basic researchers and clinicians, the hope is that cancer can be effectively controlled in the future.

ACKNOWLEDGEMENTS

The corresponding author would like to thank the *World Journal of Stem Cells* for the opportunity and acknowledge the hard work of all supporting authors.

REFERENCES

1 **Baniak N**, Senger JL, Ahmed S, Kanthan SC, Kanthan R. Gastric biomarkers: a global review. *World J Surg Oncol* 2016; **14**: 212 [PMID: 27514667 DOI: 10.1186/s12957-016-0969-3]
2 **Kwon KJ**, Shim KN, Song EM, Choi JY, Kim SE, Jung HK, Jung SA. Clinicopathological characteristics and prognosis of signet ring cell carcinoma of the stomach. *Gastric Cancer* 2014; **17**: 43-53 [PMID: 23389081 DOI: 10.1007/s10120-013-0234-1]
3 **Bertuccio P**, Chatenoud L, Levi F, Praud D, Ferlay J, Negri E, Malvezzi M, La Vecchia C. Recent patterns in gastric cancer: a global overview. *Int J Cancer* 2009; **125**: 666-673 [PMID: 19382179 DOI: 10.1002/ijc.24290]
4 **Dalerba P**, Cho RW, Clarke MF. Cancer stem cells: models and concepts. *Annu Rev Med* 2007; **58**: 267-284 [PMID: 17002552 DOI: 10.1146/annurev.med.58.062105.204854]
5 **Visvader JE**, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008; **8**: 755-768 [PMID: 18784658 DOI: 10.1038/nrc2499]
6 **Poleszczuk J**, Hahnfeldt P, Enderling H. Evolution and phenotypic selection of cancer stem cells. *PLoS Comput Biol* 2015; **11**: e1004025 [PMID: 25742563 DOI: 10.1371/journal.pcbi.1004025]
7 **Hohenberger P**, Gretschel S. Gastric cancer. *Lancet* 2003; **362**: 305-315 [PMID: 12892963 DOI: 10.1016/s0140-6736(03)13975-x]
8 **Vermeulen L**, de Sousa e Melo F, Richel DJ, Medema JP. The developing cancer stem-cell model: clinical challenges and opportunities. *Lancet Oncol* 2012; **13**: e83-e89 [PMID: 22300863 DOI: 10.1016/S1470-2045(11)70257-1]
9 **Clarke MF**, Fuller M. Stem cells and cancer: two faces of eve. *Cell* 2006; **124**: 1111-1115 [PMID: 16564000 DOI: 10.1016/j.cell.2006.03.011]
10 **Visvader JE**, Lindeman GJ. Cancer stem cells: current status and evolving complexities. *Cell Stem Cell* 2012; **10**: 717-728 [PMID: 22704512 DOI: 10.1016/j.stem.2012.05.007]
11 **Jordan CT**, Guzman ML, Noble M. Cancer stem cells. *N Engl J Med* 2006; **355**: 1253-1261 [PMID: 16990388 DOI: 10.1056/NEJMra061808]
12 **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]

13 **Furth J**. Transmission of myeloid leukemia of mice: Its relation to myeloma. *J Exp Med* 1935; **61**: 423-446 [PMID: 19870369 DOI: 10.1084/jem.61.3.423]

14 **Lapidot T**, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**: 645-648 [PMID: 7509044 DOI: 10.1038/367645a0]

15 **Singh SK**, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003; **63**: 5821-5828 [PMID: 14522905]

16 **Al-Hajj M**, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003; **100**: 3983-3988 [PMID: 12629218 DOI: 10.1073/pnas.0530291100]

17 **Greve B**, Kelsch R, Spaniol K, Eich HT, Götte M. Flow cytometry in cancer stem cell analysis and separation. *Cytometry A* 2012; **81**: 284-293 [PMID: 22311742 DOI: 10.1002/cyto.a.22022]

18 **Xu G**, Shen J, Ou Yang X, Sasahara M, Su X. Cancer stem cells: the 'heartbeat' of gastric cancer. *J Gastroenterol* 2013; **48**: 781-797 [PMID: 23184096 DOI: 10.1007/s00535-012-0712-y]

19 **Singh SR**. Gastric cancer stem cells: a novel therapeutic target. *Cancer Lett* 2013; **338**: 110-119 [PMID: 23583679 DOI: 10.1016/j.canlet.2013.03.035]

20 **Vermeulen L**, Todaro M, de Sousa Mello F, Sprick MR, Kemper K, Perez Alea M, Richel DJ, Stassi G, Medema JP. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. *Proc Natl Acad Sci USA* 2008; **105**: 13427-13432 [PMID: 18765800 DOI: 10.1073/pnas.0805706105]

21 **Takaishi S**, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, Gordon SA, Shimada Y, Wang TC. Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells* 2009; **27**: 1006-1020 [PMID: 19415765 DOI: 10.1002/stem.30]

22 **Chen W**, Zhang X, Chu C, Cheung WL, Ng L, Lam S, Chow A, Lau T, Chen M, Li Y, Nie Y, Wong BC, Pang R. Identification of CD44+ cancer stem cells in human gastric cancer. *Hepatogastroenterology* 2013; **60**: 949-954 [PMID: 23478146 DOI: 10.5754/hge12881]

23 **Nishii T**, Yashiro M, Shinto O, Sawada T, Ohira M, Hirakawa K. Cancer stem cell-like SP cells have a high adhesion ability to the peritoneum in gastric carcinoma. *Cancer Sci* 2009; **100**: 1397-1402 [PMID: 19493275 DOI: 10.1111/j.1349-7006.2009.01211.x]

24 **Flores-Téllez TN**, Villa-Treviño S, Piña-Vázquez C. Road to stemness in hepatocellular carcinoma. *World J Gastroenterol* 2017; **23**: 6750-6776 [PMID: 29085221 DOI: 10.3748/wjg.v23.i37.6750]

25 **Ishimoto T**, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, Oshima M, Ikeda T, Asaba R, Yagi H, Masuko T, Shimizu T, Ishikawa T, Kai K, Takahashi E, Imamura Y, Baba Y, Ohmura M, Suematsu M, Baba H, Saya H. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(-) and thereby promotes tumor growth. *Cancer Cell* 2011; **19**: 387-400 [PMID: 21397861 DOI: 10.1016/j.ccr.2011.01.038]

26 **Knochelmann HM**, Dwyer CJ, Bailey SR, Amaya SM, Elston DM, Mazza-McCrann JM, Paulos CM. When worlds collide: Th17 and Treg cells in cancer and autoimmunity. *Cell Mol Immunol* 2018; **15**: 458-469 [PMID: 29563615 DOI: 10.1038/s41423-018-0004-4]

27 **Scales SJ**, de Sauvage FJ. Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. *Trends Pharmacol Sci* 2009; **30**: 303-312 [PMID: 19443052 DOI: 10.1016/j.tips.2009.03.007]

28 **Akyala AI**, Peppelenbosch MP. Gastric cancer and Hedgehog signaling pathway: emerging new paradigms. *Genes Cancer* 2018; **9**: 1-10 [PMID: 29725500 DOI: 10.18632/genesandcancer.168]

29 **Zhang C**, Li C, He F, Cai Y, Yang H. Identification of CD44+CD24+ gastric cancer stem cells. *J Cancer Res Clin Oncol* 2011; **137**: 1679-1686 [PMID: 21882047 DOI: 10.1007/s00432-011-1038-5]

30 **Chen T**, Yang K, Yu J, Meng W, Yuan D, Bi F, Liu F, Liu J, Dai B, Chen X, Wang F, Zeng F, Xu H, Hu J, Mo X. Identification and expansion of cancer stem cells in tumor tissues and peripheral blood derived from gastric adenocarcinoma patients. *Cell Res* 2012; **22**: 248-258 [PMID: 21727908 DOI: 10.1038/cr.2011.109]

31 **Han ME**, Jeon TY, Hwang SH, Lee YS, Kim HJ, Shim HE, Yoon S, Baek SY, Kim BS, Kang CD, Oh SO. Cancer spheres from gastric cancer patients provide an ideal model system for cancer stem cell research. *Cell Mol Life Sci* 2011; **68**: 3589-3605 [PMID: 21448722 DOI: 10.1007/s00018-011-0672-z]

32 **Ohkuma M**, Haraguchi N, Ishii H, Mimori K, Tanaka F, Kim HM, Shimomura M, Hirose H, Yanaga K, Mori M. Absence of CD71 transferrin receptor characterizes human gastric adenosquamous carcinoma stem cells. *Ann Surg Oncol* 2012; **19**: 1357-1364 [PMID: 21523522 DOI: 10.1245/s10434-011-1739-7]

33 **Jiang Y**, He Y, Li H, Li HN, Zhang L, Hu W, Sun YM, Chen FL, Jin XM. Expressions of putative cancer stem cell markers ABCB1, ABCG2, and CD133 are correlated with the degree of differentiation of gastric cancer. *Gastric Cancer* 2012; **15**: 440-450 [PMID: 22395309 DOI: 10.1007/s10120-012-0140-y]

34 **Wu C**, Xie Y, Gao F, Wang Y, Guo Y, Tian H, Li Y, Fan W. Lgr5 expression as stem cell marker in human gastric gland and its relatedness with other putative cancer stem cell markers. *Gene* 2013; **525**: 18-25 [PMID: 23664892 DOI: 10.1016/j.gene.2013.04.067]

35 **Barker N**, Huch M, Kujala P, van de Wetering M, Snippert HJ, van Es JH, Sato T, Stange DE, Begthel H, van den Born M, Danenberg E, van den Brink S, Korving J, Abo A, Peters PJ, Wright N, Poulsom R, Clevers H. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell* 2010; **6**: 25-36 [PMID: 20085740 DOI:

10.1016/j.stem.2009.11.013]
36 **Simon E**, Petke D, Böger C, Behrens HM, Warneke V, Ebert M, Röcken C. The spatial distribution of LGR5+ cells correlates with gastric cancer progression. *PLoS One* 2012; **7**: e35486 [PMID: 22530031 DOI: 10.1371/journal.pone.0035486]
37 **Li X**, Bu W, Meng L, Liu X, Wang S, Jiang L, Ren M, Fan Y, Sun H. CXCL12/CXCR4 pathway orchestrates CSC-like properties by CAF recruited tumor associated macrophage in OSCC. *Exp Cell Res* 2019; **378**: 131-138 [PMID: 30857971 DOI: 10.1016/j.yexcr.2019.03.013]
38 **Gong X**, Azhdarinia A, Ghosh SC, Xiong W, An Z, Liu Q, Carmon KS. LGR5-Targeted Antibody-Drug Conjugate Eradicates Gastrointestinal Tumors and Prevents Recurrence. *Mol Cancer Ther* 2016; **15**: 1580-1590 [PMID: 27207778 DOI: 10.1158/1535-7163.MCT-16-0114]
39 **Katsuno Y**, Ehata S, Yashiro M, Yanagihara K, Hirakawa K, Miyazono K. Coordinated expression of REG4 and aldehyde dehydrogenase 1 regulating tumourigenic capacity of diffuse-type gastric carcinoma-initiating cells is inhibited by TGF-β. *J Pathol* 2012; **228**: 391-404 [PMID: 22430847 DOI: 10.1002/path.4020]
40 **Nguyen PH**, Giraud J, Chambonnier L, Dubus P, Wittkop L, Belleannée G, Collet D, Soubeyran I, Evrard S, Rousseau B, Senant-Dugot N, Mégraud F, Mazurier F, Varon C. Characterization of Biomarkers of Tumorigenic and Chemoresistant Cancer Stem Cells in Human Gastric Carcinoma. *Clin Cancer Res* 2017; **23**: 1586-1597 [PMID: 27620279 DOI: 10.1158/1078-0432.CCR-15-2157]
41 **Han J**, Won M, Kim JH, Jung E, Min K, Jangili P, Kim JS. Cancer stem cell-targeted bio-imaging and chemotherapeutic perspective. *Chem Soc Rev* 2020; **49**: 7856-7878 [PMID: 32633291 DOI: 10.1039/d0cs00379d]
42 **Fujita T**, Chiwaki F, Takahashi RU, Aoyagi K, Yanagihara K, Nishimura T, Tamaoki M, Komatsu M, Komatsuzaki R, Matsusaki K, Ichikawa H, Sakamoto H, Yamada Y, Fukagawa T, Katai H, Konno H, Ochiya T, Yoshida T, Sasaki H. Identification and Characterization of CXCR4-Positive Gastric Cancer Stem Cells. *PLoS One* 2015; **10**: e0130808 [PMID: 26110809 DOI: 10.1371/journal.pone.0130808]
43 **Gottesman MM**, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002; **2**: 48-58 [PMID: 11902585 DOI: 10.1038/nrc706]
44 **Schmuck R**, Warneke V, Behrens HM, Simon E, Weichert W, Röcken C. Genotypic and phenotypic characterization of side population of gastric cancer cell lines. *Am J Pathol* 2011; **178**: 1792-1804 [PMID: 21435459 DOI: 10.1016/j.ajpath.2010.12.043]
45 **Yashiro M**, Nishii T, Hasegawa T, Matsuzaki T, Morisaki T, Fukuoka T, Hirakawa K. A c-Met inhibitor increases the chemosensitivity of cancer stem cells to the irinotecan in gastric carcinoma. *Br J Cancer* 2013; **109**: 2619-2628 [PMID: 24129235 DOI: 10.1038/bjc.2013.638]
46 **Fukuda K**, Saikawa Y, Ohashi M, Kumagai K, Kitajima M, Okano H, Matsuzaki Y, Kitagawa Y. Tumor initiating potential of side population cells in human gastric cancer. *Int J Oncol* 2009; **34**: 1201-1207 [PMID: 19360333]
47 **Haraguchi N**, Inoue H, Tanaka F, Mimori K, Utsunomiya T, Sasaki A, Mori M. Cancer stem cells in human gastrointestinal cancers. *Hum Cell* 2006; **19**: 24-29 [PMID: 16643604 DOI: 10.1111/j.1749-0774.2005.00004.x]
48 **Burkert J**, Otto WR, Wright NA. Side populations of gastrointestinal cancers are not enriched in stem cells. *J Pathol* 2008; **214**: 564-573 [PMID: 18266310 DOI: 10.1002/path.2307]
49 **Powell AE**, Wang Y, Li Y, Poulin EJ, Means AL, Washington MK, Higginbotham JN, Juchheim A, Prasad N, Levy SE, Guo Y, Shyr Y, Aronow BJ, Haigis KM, Franklin JL, Coffey RJ. The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. *Cell* 2012; **149**: 146-158 [PMID: 22464327 DOI: 10.1016/j.cell.2012.02.042]
50 **Chi HC**, Tsai CY, Wang CS, Yang HY, Lo CH, Wang WJ, Lee KF, Lai LY, Hong JH, Chang YF, Tsai MM, Yeh CT, Wu CH, Hsieh CC, Wang LH, Chen WJ, Lin KH. DOCK6 promotes chemo- and radioresistance of gastric cancer by modulating WNT/β-catenin signaling and cancer stem cell traits. *Oncogene* 2020; **39**: 5933-5949 [PMID: 32753649 DOI: 10.1038/s41388-020-01390-0]
51 **Hayakawa Y**, Ariyama H, Stancikova J, Sakitani K, Asfaha S, Renz BW, Dubeykovskaya ZA, Shibata W, Wang H, Westphalen CB, Chen X, Takemoto Y, Kim W, Khurana SS, Tailor Y, Nagar K, Tomita H, Hara A, Sepulveda AR, Setlik W, Gershon MD, Saha S, Ding L, Shen Z, Fox JG, Friedman RA, Konieczny SF, Worthley DL, Korinek V, Wang TC. Mist1 Expressing Gastric Stem Cells Maintain the Normal and Neoplastic Gastric Epithelium and Are Supported by a Perivascular Stem Cell Niche. *Cancer Cell* 2015; **28**: 800-814 [PMID: 26585400 DOI: 10.1016/j.ccell.2015.10.003]
52 **Chambers I**, Tomlinson SR. The transcriptional foundation of pluripotency. *Development* 2009; **136**: 2311-2322 [PMID: 19542351 DOI: 10.1242/dev.024398]
53 **Masui S**, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA, Ko MS, Niwa H. Pluripotency governed by Sox2 *via* regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol* 2007; **9**: 625-635 [PMID: 17515932 DOI: 10.1038/ncb1589]
54 **Hütz K**, Mejías-Luque R, Farsakova K, Ogris M, Krebs S, Anton M, Vieth M, Schüller U, Schneider MR, Blum H, Wagner E, Jung A, Gerhard M. The stem cell factor SOX2 regulates the tumorigenic potential in human gastric cancer cells. *Carcinogenesis* 2014; **35**: 942-950 [PMID: 24325912 DOI: 10.1093/carcin/bgt410]
55 **Johansson H**, Simonsson S. Core transcription factors, Oct4, Sox2 and Nanog, individually form complexes with nucleophosmin (Npm1) to control embryonic stem (ES) cell fate determination.

Aging (Albany NY) 2010; **2**: 815-822 [PMID: 21076177 DOI: 10.18632/aging.100222]

56 **Sultan M**, Coyle KM, Vidovic D, Thomas ML, Gujar S, Marcato P. Hide-and-seek: the interplay between cancer stem cells and the immune system. *Carcinogenesis* 2017; **38**: 107-118 [PMID: 27866156 DOI: 10.1093/carcin/bgw115]

57 **Bouriez D**, Giraud J, Gronnier C, Varon C. Efficiency of All-Trans Retinoic Acid on Gastric Cancer: A Narrative Literature Review. *Int J Mol Sci* 2018; **19** [PMID: 30380687 DOI: 10.3390/ijms19113388]

58 **Marhaba R**, Klingbeil P, Nuebel T, Nazarenko I, Buechler MW, Zoeller M. CD44 and EpCAM: cancer-initiating cell markers. *Curr Mol Med* 2008; **8**: 784-804 [PMID: 19075676 DOI: 10.2174/156652408786733667]

59 **Prall F**, Maletzki C, Linnebacher M. The EpCAM high/CD44 high colorectal carcinoma stem cell phenotype is not preferentially expressed in tumour buds. *Histopathology* 2010; **56**: 553-555 [PMID: 20459564 DOI: 10.1111/j.1365-2559.2010.03502.x]

60 **Masciale V**, Grisendi G, Banchelli F, D'Amico R, Maiorana A, Sighinolfi P, Stefani A, Morandi U, Dominici M, Aramini B. CD44+/EPCAM+ cells detect a subpopulation of ALDH^{high} cells in human non-small cell lung cancer: A chance for targeting cancer stem cells? *Oncotarget* 2020; **11**: 1545-1555 [PMID: 32391123 DOI: 10.18632/oncotarget.27568]

61 **Gu Q**, Chen X, Zhou L, Liu X. Exosome EpCAM promotes the metastasis of glioma by targeting the CD44 signaling molecule on the surface of glioma cells. *Adv Clin Exp Med* 2020; **29**: 1277-1282 [PMID: 33269813 DOI: 10.17219/acem/126051]

62 **Rocco A**, Liguori E, Pirozzi G, Tirino V, Compare D, Franco R, Tatangelo F, Palaia R, D'Armiento FP, Pollastrone G, Affuso A, Bottazzi EC, Masone S, Persico G, Nardone G. CD133 and CD44 cell surface markers do not identify cancer stem cells in primary human gastric tumors. *J Cell Physiol* 2012; **227**: 2686-2693 [PMID: 21898409 DOI: 10.1002/jcp.23013]

63 **Zhao P**, Li Y, Lu Y. Aberrant expression of CD133 protein correlates with Ki-67 expression and is a prognostic marker in gastric adenocarcinoma. *BMC Cancer* 2010; **10**: 218 [PMID: 20487522 DOI: 10.1186/1471-2407-10-218]

64 **Mills JC**, Shivdasani RA. Gastric epithelial stem cells. *Gastroenterology* 2011; **140**: 412-424 [PMID: 21144849 DOI: 10.1053/j.gastro.2010.12.001]

65 **Haraguchi N**, Utsunomiya T, Inoue H, Tanaka F, Mimori K, Barnard GF, Mori M. Characterization of a side population of cancer cells from human gastrointestinal system. *Stem Cells* 2006; **24**: 506-513 [PMID: 16239320 DOI: 10.1634/stemcells.2005-0282]

66 **Ehata S**, Johansson E, Katayama R, Koike S, Watanabe A, Hoshino Y, Katsuno Y, Komuro A, Koinuma D, Kano MR, Yashiro M, Hirakawa K, Aburatani H, Fujita N, Miyazono K. Transforming growth factor-β decreases the cancer-initiating cell population within diffuse-type gastric carcinoma cells. *Oncogene* 2011; **30**: 1693-1705 [PMID: 21132002 DOI: 10.1038/con.2010.546]

67 **Zhang H**, Xi H, Cai A, Xia Q, Wang XX, Lu C, Zhang Y, Song Z, Wang H, Li Q, Chen L, Guo Z. Not all side population cells contain cancer stem-like cells in human gastric cancer cell lines. *Dig Dis Sci* 2013; **58**: 132-139 [PMID: 22878917 DOI: 10.1007/s10620-012-2330-1]

68 **Tang DG**. Understanding cancer stem cell heterogeneity and plasticity. *Cell Res* 2012; **22**: 457-472 [PMID: 22357481 DOI: 10.1038/cr.2012.13]

69 **Takebe N**, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX, Ivy SP. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. *Nat Rev Clin Oncol* 2015; **12**: 445-464 [PMID: 25850553 DOI: 10.1038/nrclinonc.2015.61]

70 **Li Y**, Laterra J. Cancer stem cells: distinct entities or dynamically regulated phenotypes? *Cancer Res* 2012; **72**: 576-580 [PMID: 22298594 DOI: 10.1158/0008-5472.CAN-11-3070]

71 **Fessler E**, Dijkgraaf FE, De Sousa E Melo F, Medema JP. Cancer stem cell dynamics in tumor progression and metastasis: is the microenvironment to blame? *Cancer Lett* 2013; **341**: 97-104 [PMID: 23089245 DOI: 10.1016/j.canlet.2012.10.015]

72 **Shin K**, Lim A, Odegaard JI, Honeycutt JD, Kawano S, Hsieh MH, Beachy PA. Cellular origin of bladder neoplasia and tissue dynamics of its progression to invasive carcinoma. *Nat Cell Biol* 2014; **16**: 469-478 [PMID: 24747439 DOI: 10.1038/ncb2956]

73 **Aggarwal BB**, Kunnumakkara AB, Harikumar KB, Gupta SR, Tharakan ST, Koca C, Dey S, Sung B. Signal transducer and activator of transcription-3, inflammation, and cancer: how intimate is the relationship? *Ann N Y Acad Sci* 2009; **1171**: 59-76 [PMID: 19723038 DOI: 10.1111/j.1749-6632.2009.04911.x]

74 **Jiang YX**, Yang SW, Li PA, Luo X, Li ZY, Hao YX, Yu PW. The promotion of the transformation of quiescent gastric cancer stem cells by IL-17 and the underlying mechanisms. *Oncogene* 2017; **36**: 1256-1264 [PMID: 27524415 DOI: 10.1038/onc.2016.291]

75 **Kamran MZ**, Patil P, Gude RP. Role of STAT3 in cancer metastasis and translational advances. *Biomed Res Int* 2013; **2013**: 421821 [PMID: 24199193 DOI: 10.1155/2013/421821]

76 **Gong S**, Li Q, Jeter CR, Fan Q, Tang DG, Liu B. Regulation of NANOG in cancer cells. *Mol Carcinog* 2015; **54**: 679-687 [PMID: 26013997 DOI: 10.1002/mc.22340]

77 **Mohammed MK**, Shao C, Wang J, Wei Q, Wang X, Collier Z, Tang S, Liu H, Zhang F, Huang J, Guo D, Lu M, Liu F, Liu J, Ma C, Shi LL, Athiviraham A, He TC, Lee MJ. Wnt/β-catenin signaling plays an ever-expanding role in stem cell self-renewal, tumorigenesis and cancer chemoresistance. *Genes Dis* 2016; **3**: 11-40 [PMID: 27077077 DOI: 10.1016/j.gendis.2015.12.004]

78 **Vaiopoulos AG**, Kostakis ID, Athanasoula KCh, Papavassiliou AG. Targeting transcription factor corepressors in tumor cells. *Cell Mol Life Sci* 2012; **69**: 1745-1753 [PMID: 22527719 DOI:

10.1007/s00018-012-0986-5]

79 **Yoon C**, Cho SJ, Aksoy BA, Park DJ, Schultz N, Ryeom SW, Yoon SS. Chemotherapy Resistance in Diffuse-Type Gastric Adenocarcinoma Is Mediated by RhoA Activation in Cancer Stem-Like Cells. *Clin Cancer Res* 2016; **22**: 971-983 [PMID: 26482039 DOI: 10.1158/1078-0432.CCR-15-1356]

80 **Zhao Y**, Zhao L, Ischenko I, Bao Q, Schwarz B, Nieß H, Wang Y, Renner A, Mysliwietz J, Jauch KW, Nelson PJ, Ellwart JW, Bruns CJ, Camaj P. Antisense inhibition of microRNA-21 and microRNA-221 in tumor-initiating stem-like cells modulates tumorigenesis, metastasis, and chemotherapy resistance in pancreatic cancer. *Target Oncol* 2015; **10**: 535-548 [PMID: 25639539 DOI: 10.1007/s11523-015-0360-2]

81 **Chang L**, Graham P, Hao J, Ni J, Deng J, Bucci J, Malouf D, Gillatt D, Li Y. Cancer stem cells and signaling pathways in radioresistance. *Oncotarget* 2016; **7**: 11002-11017 [PMID: 26716904 DOI: 10.18632/oncotarget.6760]

82 **Cui J**, Li P, Liu X, Hu H, Wei W. Abnormal expression of the Notch and Wnt/β-catenin signaling pathways in stem-like $ALDH^{hi}CD44^{+}$ cells correlates highly with Ki-67 expression in breast cancer. *Oncol Lett* 2015; **9**: 1600-1606 [PMID: 25789008 DOI: 10.3892/ol.2015.2942]

83 **Balducci L**. In memory of Claude Jasmin: A prophet of Geriatric Oncology. *J Geriatr Oncol* 2018; **9**: 1-2 [PMID: 28978411 DOI: 10.1016/j.jgo.2017.09.004]

84 **Yoon C**, Park DJ, Schmidt B, Thomas NJ, Lee HJ, Kim TS, Janjigian YY, Cohen DJ, Yoon SS. CD44 expression denotes a subpopulation of gastric cancer cells in which Hedgehog signaling promotes chemotherapy resistance. *Clin Cancer Res* 2014; **20**: 3974-3988 [PMID: 24947926 DOI: 10.1158/1078-0432.CCR-14-0011]

85 **Bang YJ**, Im SA, Lee KW, Cho JY, Song EK, Lee KH, Kim YH, Park JO, Chun HG, Zang DY, Fielding A, Rowbottom J, Hodgson D, O'Connor MJ, Yin X, Kim WH. Randomized, Double-Blind Phase II Trial With Prospective Classification by ATM Protein Level to Evaluate the Efficacy and Tolerability of Olaparib Plus Paclitaxel in Patients With Recurrent or Metastatic Gastric Cancer. *J Clin Oncol* 2015; **33**: 3858-3865 [PMID: 26282658 DOI: 10.1200/JCO.2014.60.0320]

86 **Satoh T**, Xu RH, Chung HC, Sun GP, Doi T, Xu JM, Tsuji A, Omuro Y, Li J, Wang JW, Miwa H, Qin SK, Chung IJ, Yeh KH, Feng JF, Mukaiyama A, Kobayashi M, Ohtsu A, Bang YJ. Lapatinib plus paclitaxel versus paclitaxel alone in the second-line treatment of HER2-amplified advanced gastric cancer in Asian populations: TyTAN--a randomized, phase III study. *J Clin Oncol* 2014; **32**: 2039-2049 [PMID: 24868024 DOI: 10.1200/JCO.2013.53.6136]

87 **Trumpp A**, Essers M, Wilson A. Awakening dormant haematopoietic stem cells. *Nat Rev Immunol* 2010; **10**: 201-209 [PMID: 20182459 DOI: 10.1038/nri2726]

88 **Bautch VL**. Cancer: Tumour stem cells switch sides. *Nature* 2010; **468**: 770-771 [PMID: 21150987 DOI: 10.1038/468770a]

89 **Ricci-Vitiani L**, Pallini R, Biffoni M, Todaro M, Invernici G, Cenci T, Maira G, Parati EA, Stassi G, Larocca LM, De Maria R. Tumour vascularization *via* endothelial differentiation of glioblastoma stem-like cells. *Nature* 2010; **468**: 824-828 [PMID: 21102434 DOI: 10.1038/nature09557]

World Journal of Stem Cells

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

World J Stem Cells 2022 January 26; 14(1): 92-103

DOI: 10.4252/wjsc.v14.i1.92

ISSN 1948-0210 (online)

MINIREVIEWS

Epigenetic regulation by long noncoding RNAs in osteo-/adipogenic differentiation of mesenchymal stromal cells and degenerative bone diseases

Kai Xia, Li-Yuan Yu, Xin-Qi Huang, Zhi-He Zhao, Jun Liu

ORCID number: Kai Xia 0000-0003-3043-4320; Li-Yuan Yu 0000-0002-0981-0240; Xin-Qi Huang 0000-0002-2762-2438; Zhi-He Zhao 0000-0003-2955-1706; Jun Liu 0000-0001-5072-3205.

Author contributions: Xia K and Yu LY contributed equally to this work; Liu J and Zhao ZH contributed to the conception of the review; Xia K and Yu LY conducted literature research and drafted the manuscript; Huang XQ revised the manuscript critically; all authors read and approved the final version of the manuscript.

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

Supported by the National Natural Science Foundation of China, No. 81870743 and No. 81771048.

Country/Territory of origin: China

Specialty type: Biochemistry and molecular biology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Kai Xia, Li-Yuan Yu, Xin-Qi Huang, Zhi-He Zhao, Jun Liu, State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, Sichuan Province, China

Kai Xia, Li-Yuan Yu, Xin-Qi Huang, Zhi-He Zhao, Jun Liu, Department of Orthodontics, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, Sichuan Province, China

Corresponding author: Jun Liu, DDS, PhD, Professor, State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases, West China Hospital of Stomatology, Sichuan University, No. 14, 3rd Section, South Renmin Road, Chengdu 610041, Sichuan Province, China. junliu@scu.edu.cn

Abstract

Bone is a complex tissue that undergoes constant remodeling to maintain homeostasis, which requires coordinated multilineage differentiation and proper proliferation of mesenchymal stromal cells (MSCs). Mounting evidence indicates that a disturbance of bone homeostasis can trigger degenerative bone diseases, including osteoporosis and osteoarthritis. In addition to conventional genetic modifications, epigenetic modifications (*i.e.*, DNA methylation, histone modifications, and the expression of noncoding RNAs) are considered to be contributing factors that affect bone homeostasis. Long noncoding RNAs (lncRNAs) were previously regarded as 'transcriptional noise' with no biological functions. However, substantial evidence suggests that lncRNAs have roles in the epigenetic regulation of biological processes in MSCs and related diseases. In this review, we summarized the interactions between lncRNAs and epigenetic modifiers associated with osteo-/adipogenic differentiation of MSCs and the pathogenesis of degenerative bone diseases and highlighted promising lncRNA-based diagnostic and therapeutic targets for bone diseases.

Key Words: Long noncoding RNA; Epigenetics; DNA methylation; Histones; Cell differentiation; Bone diseases

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: February 26, 2021
Peer-review started: February 26, 2021
First decision: June 16, 2021
Revised: July 7, 2021
Accepted: January 5, 2022
Article in press: January 5, 2022
Published online: January 26, 2022

P-Reviewer: Oliva J
S-Editor: Gong ZM
L-Editor: A
P-Editor: Gong ZM

Core Tip: In this review, we summarized the roles of long noncoding RNAs (lncRNAs) played in mesenchymal stromal cells (MSCs) differentiation and common degenerative bone diseases through reciprocal interactions between lncRNAs and epigenetic modifiers, focusing on the most common epigenetic mechanisms: DNA methylation and histone modifications. It is our hope that this review may provide an updated summary that sheds light on the lncRNA-based precise regulation of the MSC differentiation process and highlights possible therapeutic targets of degenerative bone diseases.

Citation: Xia K, Yu LY, Huang XQ, Zhao ZH, Liu J. Epigenetic regulation by long noncoding RNAs in osteo-/adipogenic differentiation of mesenchymal stromal cells and degenerative bone diseases. *World J Stem Cells* 2022; 14(1): 92-103
URL: https://www.wjgnet.com/1948-0210/full/v14/i1/92.htm
DOI: https://dx.doi.org/10.4252/wjsc.v14.i1.92

INTRODUCTION

The skeletal system contains bones, joints, and ligaments that function together as a locomotive organ and provide structural support. Originating from mesenchymal progenitors during embryogenesis, the skeletal system undergoes modeling and remodeling throughout life[1]. Mesenchymal stromal cells (MSCs) refer to a heterogeneous unfractionated population of cells, which include fibroblasts, myofibroblasts, and progenitor cells[2,3]. MSCs are able to differentiate into chondrocytes or osteoblasts to comply with bone formation and regeneration needs[4]. It is worth mentioning that adipocytes, as well as osteoblasts, derive from the same population of MSCs. A shift in the osteoadipogenic differentiation balance may lead to bone diseases, such as osteoporosis, which typically manifests as a shift toward adipogenesis[5,6]. Likewise, osteoarthritis is usually characterized by impairment of cartilage regeneration due to the attenuated chondrogenic capacity of MSCs[7,8]. Therefore, the differentiation of MSCs, which proceeds under the control of various transcription factors, influences the pathogenesis of common bone diseases[9-11].

In addition to conventional genetic and environmental factors, epigenetic modifications can influence the bone phenotype and the development of skeletal diseases[12,13]. Epigenetic mechanisms alter gene expression patterns without changing the DNA sequence by three major mechanisms, including DNA methylation, histone modifications, and altered expression of noncoding RNAs[14]. With the rapid development of next-generation sequencing (NGS) and advanced bioinformatic tools, the crucial roles of epigenetic mechanisms in the differentiation of MSCs and the pathogenesis of bone diseases have begun to be elucidated[15-17].

Long noncoding RNAs (lncRNAs) are defined as a set of noncoding RNAs longer than 200bp that have no protein-coding ability. Evidence is rapidly accumulating on the functions of lncRNAs in epigenetic regulation in the differentiation of MSCs and the occurrence of many diseases[18-21]. In this review, we revisit the epigenetic regulatory mechanisms of lncRNAs involved in DNA methylation and histone modifications and summarize the biological functions of lncRNAs in regulation crucial differentiation- and bone disease-related genes by interacting with key epigenetic modifiers. It is our hope that this review may provide an updated summary that sheds light on the lncRNA-based precise regulation of the MSC differentiation process and highlights possible therapeutic targets of degenerative bone diseases.

DNA METHYLATION

DNA methylation functions as a regulator of osteogenesis and adipogenesis of MSCs and is involved in common bone diseases[22-24]. In humans, the majority of DNA methylation occurs at cytosines in cytosine-phospho-guanosine (CpG) dinucleotides [25,26]. Approximately 75% of all gene promoters are within CpG-rich regions, known as CpG islands, that are mostly unmethylated[27]. It is generally accepted that the methylation of these CpG islands is associated with the repression of gene expression

[28]. Nevertheless, it is worth mentioning that DNA methylation is also associated with upregulated gene expression under certain circumstances[29].

As writer enzymes, DNA methyltransferases (DNMTs) catalyze DNA methylation by transferring a methyl group onto the C5 position of a cytosine at CpG dinucleotide sites to form 5mCpG[30]. A member of the DNMT family, DNMT1, which is also called the maintenance DNMT, maintains the original methylation pattern during DNA replication, while DNMT3a and DNMT3b are involved in *de novo* methylation [30,31]. The interaction of lncRNAs with DNMTs is varied and reciprocal. For example, lncRNAs can recruit DNMTs to the promoters of target genes and regulate their expression patterns. In turn, the changes in the methylation level of specific lncRNA gene promoters can alter the expression of lncRNAs, including downstream lncRNA-regulated genes[32,33]. In MSCs, lncRNAs, as regulators of DNA methylation, have received increasing attention due to their great importance in the regulation of differentiation and bone-related diseases (Figure 1).

LncRNAs regulate DNA methylation during osteogenic differentiation

H19, a well-known lncRNA, plays a crucial role in embryo development, cell differentiation, and the occurrence and development of bone diseases[34-37]. In human dental pulp stromal cells (hDPSCs), H19 positively regulates odontogenic differentiation *via* hypomethylation of distal-less homeobox 3 (*DLX3*), a key factor in odontogenic differentiation[32]. H19 decreases SAHH and DNMT3B activity, consequently promoting the expression of *DLX3*[32]. In turn, a mutation of *DLX3* identified in dentin hypoplasia patients could increase DNMT3B activity, and the subsequently repressed H19/miR-675 axis impairs the odontoblastic differentiation of hDPSCs[38]. Similarly, in valve interstitial cells (VICs), which have a mesenchymal origin[39], the knockdown of H19 attenuated their osteogenic differentiation capacity by increasing the transcription of *NOTCH1* and decreasing the levels of RUNX2 and BMP2[40]. In mineralized aortic valve tissue, H19 was upregulated as a result of hypomethylation of CpG in its promoter region[40]. These results suggest the possibility that H19 forms a positive feedback loop with DNMTs and promotes the osteogenic differentiation of MSCs.

Another study found an inverse association between the methylation level of perinatal *CDKN2A*, which encodes the lncRNA antisense noncoding RNA in the INK4 Locus (ANRIL), and bone mass at ages 4 and 6 years[41]. Considering that transitional hypomethylation of *CDKN2A* has been identified in human bone marrow stromal cells (hBMSCs) during osteogenic differentiation[42], the authors further verified that the methylation of *CDKN2A* decreased the binding of transcription factors SMAD3/4 and consequently downregulated the expression of ANRIL[41]. In terms of the functional mechanism of ANRIL, it has been demonstrated that the knockdown of ANRIL decreased the number of live cells and induced cell apoptosis of SaOS-2 cells[41].

Given the crucial roles of *HOX* genes in development and differentiation, it is reasonable to believe that the lncRNAs encoded by the *HOX* gene cluster could also exert their function as critical biological regulators (*i.e.*, *HOTAIR* in the *HOXC* cluster and *HOTAIRM1* in the *HOXA* cluster)[43-45]. In human dental follicle stromal cells (hDFSCs), lncRNA HOTAIRM1 promoted osteogenesis by inhibiting the enrichment of DNMT1 in the *HOXA2* promoter region and subsequently maintaining two CpG islands in a hypomethylated state, which guaranteed the transcriptional activation of *HOXA2*[17].

LncRNAs regulate DNA methylation during adipogenic differentiation

lncRNA HOTAIR, encoded by the *HOXC* gene cluster as mentioned above, could also inhibit the adipogenic differentiation of hBMSCs[46]. In this process, HOTAIR probably directly interacts with DNMTs or is involved in gene regulation by triple helix formation[46].

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) and CCAAT enhancer binding protein-alpha (C/EBP-α) are key transcription factors involved in adipogenesis. They synergistically promote the transcriptional activation of genes that induce the adipocyte phenotype and maintain their expression throughout the entire differentiation process and the entire life of the adipocytes[47,48]. In mouse ST-2 cells (bone marrow stromal cells), 3T3-L1 cells (committed preadipocytes derived from MSCs), and C3H10T1/2 cells (embryonic stem cells) as well as in bone marrow stromal cells, lncRNA Plnc1 promotes adipogenesis by increasing *Ppar-γ2* transcription through reducing the DNA methylation level on its promoter[49].

Upregulation of lncRNA slincRAD is also observed in the early stages of adipocyte differentiation in 3T3-L1 cells[50]. LncRNA slincRAD guides Dnmt1 to translocate to the perinuclear region in S phase and direct Dnmt1 to the promoter of cell cycle-

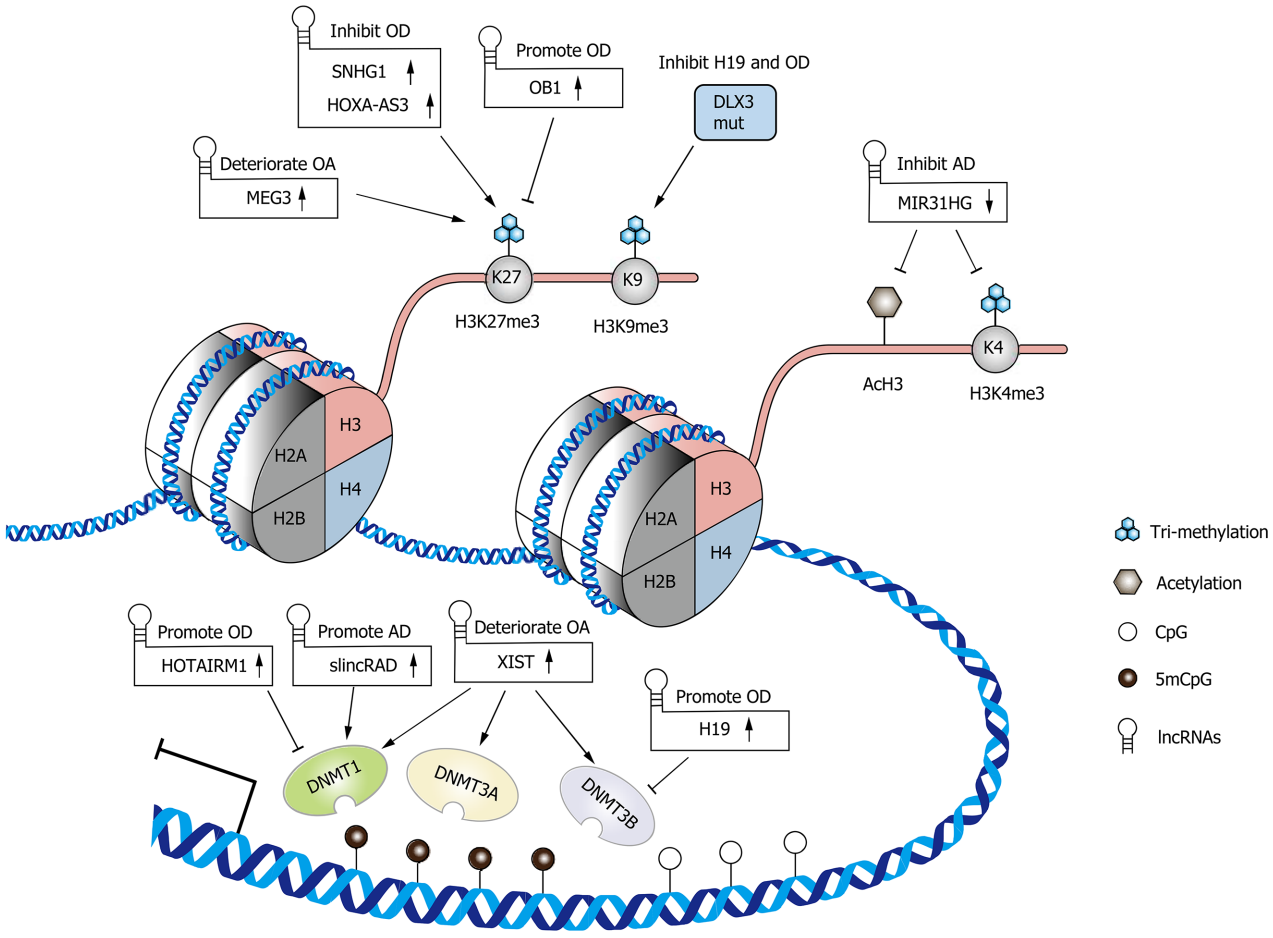

Figure 1 A brief illustration of the interactions between long noncoding RNAs and the epigenetic modification associated with osteo-/adipogenic differentiation of mesenchymal stromal cells and osteoarthritis. Histone acetylation and H3K4me3 are believed to promote transcription, whereas DNA methylation, H3K9me3 and H3K27me3 restrict gene expression. OD: Osteogenic differentiation; AD: Adipogenic differentiation; OA: Osteoarthritis.

related genes, including p21 (Cdkn1a)[50]. As p21 is a cyclin-dependent kinase inhibitor that plays an important role in the differentiation of 3T3-L1 cells, this effect facilitates the progression of differentiation[50,51].

HISTONE MODIFICATIONS

The building block of chromatin is the nucleosome, which consists of a complex of DNA and four types of core histone subunits (H2A, H2B, H3, and H4)[52]. Histone proteins are subject to a variety of modifications, with most studies focusing on methylation and acetylation. Lysine (K) residues in histone H3 are commonly modified by methylation, which is orchestrated by histone methyltransferases (HMTS) and histone demethylases (HDMs)[53,54]. Previous studies have revealed that trimethylation of H3K4 (H3K4me3) promotes transcription, whereas H3K9me3 and H3K27me3 restrict gene expression[53]. Likewise, acetylation and deacetylation of lysine residues in histones are regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. It is believed that the addition of an acetyl group to lysine residues alters the structure and folding of the nucleosome and consequently loosens the chromatin to enable transcription[55]. During cellular biological and pathologic processes, including cell differentiation, bone regeneration and disease, histone modifications are dynamically changed[53,56]. This process is at least in part mediated by lncRNAs that recruit histone-modifying enzymes to targeted gene promoters and alter histone modification enrichment (Figure 1).

Involvement of lncRNAs in osteogenic differentiation through histone modifications

As mentioned earlier, a mutation of *DLX3* identified in dentin hypoplasia patients could increase DNMT3B activity[38]. This study also reported that this mutation was

capable of repressing H19 expression by increasing the enrichment of H3K9me3 in the promoter region of the H19 gene and retarding the odontoblastic differentiation of hDPSCs[38].

Similar to RUNX2, Osterix (OSX) is considered a master transcription factor that regulates the osteogenic differentiation of MSCs and it is required for the maturation of functional osteoblasts[57]. lnc-OB1 promotes osteogenic differentiation of MSCs, probably by upregulating OSX *via* the inhibition of H3K27me3 in the OSX promoter region[58]. In human osteoblast cells, this regulation might be mediated by an interaction between lnc-OB1 and SUZ12, which is an integral component of polycomb repressive complex 2 (PRC2), responsible for H3K27me3[58,59].

Another core part of PRC2, EZH2[59], was also found to interact with lncRNAs and regulate osteogenic differentiation. It has been shown that lncRNA SNHG1 inhibits the osteogenic differentiation of human periodontal ligament stromal cells by repressing the expression of KLF2, a positive regulator of osteoblast differentiation[60], through EZH2-mediated H3K27me3 of its promoter[61]. Likewise, lncRNA HOXA-AS3 inhibits hBMSC osteogenesis, possibly *via* EZH2-dependent H3K27me3, and represses RUNX2 expression[62].

Involvement of lncRNAs in adipogenic differentiation through histone modifications

As a critical transcription factor for adipogenesis, C/EBP-α was found to be upregulated *via* the recruitment of the MLL3/4 complex to its promoter, which is guided by the binding of PA1 (a component of the MLL3/4 complex) to lncRNA ADINR during adipogenic differentiation of human adipose-derived stromal cells (hASCs)[63]. It is believed that MLL3/4 complexes are involved in the maintenance of H3K4me3 and the removal of H3K27me3, thereby regulating downstream gene expression[64,65].

Adipocyte fatty acid-binding protein (A-FABP, also known as FABP4 or aP2), a downstream target gene of PPAR-γ and C/EBP-α, is considered a marker of adipogenic differentiation[66,67]. The knockdown of lncRNA MIR31HG suppressed FABP4 expression by reducing the enrichment of acetylated histone 3 (AcH3) and H3K4me3 in the FABP4 promoter, leading to the inhibition of adipogenic differentiation of hASCs[16].

H19 and miR-675 (derived from H19) inhibited the adipogenic differentiation of hBMSCs through the miRNA-mediated repression of HDAC4, 5 and 6. In turn, the inhibition of HDACs decreased CCCTC-binding factor (CTCF) occupancy on the imprinting control region (ICR) of H19 and reduced H19 expression[68]. This evidence, combined with that mentioned in an earlier section that H19 is considered a positive regulator of osteogenic differentiation, suggests that DNA methylation and histone modifications might be linked together by H19 and shift the osteoadipogenic differentiation balance toward osteogenesis.

ROLE OF LNCRNAS IN DEGENERATIVE BONE DISEASES

More recently, epigenetic regulation of bone homeostasis has been considered as an important factor in the pathogenesis of degenerative bone diseases, such as osteoporosis, arthritis, post menopausal osteoporosis, *etc.*[69,70]. As mentioned above, lncRNAs have attracted considerable attention in the epigenetic regulation of bone homeostasis. The potential link between degenerative bone diseases and lncRNAs at the epigenetic level is also an intriguing area for exploration.

LncRNAs regulate DNA methylation in osteoarthritis and osteoporosis

Osteoarthritis (OA) is a common degenerative joint disease that is associated with the impairment of cartilage regeneration, chondrocyte apoptosis, and the degradation of the cartilage extracellular matrix (ECM)[71,72]. In this sophisticated balance between biosynthesis and degradation, lncRNAs play a role in the survival of chondrocytes and the regulation of arthritis-associated factors[73].

It has been reported that the overexpression of lncRNA CTBP1-AS2 downregulates miR-130a by increasing the methylation level of the *miR-130a* gene, which finally leads to a decreased proliferation rate of chondrocytes in OA patients[74].

As a natural inhibitor of matrix metalloproteinases (MMPs), TIMP-3 deficiency can lead to mild cartilage degeneration in patients with OA[75]. lncRNA XIST is capable of downregulating the expression of TIMP-3 through the recruitment of DNMT1, DNMT3A, and DNMT3B, which increased the methylation ratio of the CpG island in the *TIMP-3* promoter region, and consequently increased collagen degradation in OA

Table 1 Interactions between long noncoding RNAs and epigenetic modifiers during osteogenic differentiation of mesenchymal stromal cells

LncRNAs	Samples	Expression	Epigenetic regulatory mechanisms	Target genes	Effects	Ref.
H19	hDPSCs	Up	Decreasing DNMT3B activity	*DLX3*	Promote odontogenic differentiation	Zeng *et al* [32]
H19	hDPSCs	Down	H19 was inhibited by the recruitment of DNMT3B and the enrichment of H3K9me3 in its promoter	*miR-675* (derived from H19)	Inhibit odontogenic differentiation	Zeng *et al* [38]
H19	VICs	Up	H19 was upregulated by hypomethylation of its promoter	NR	Promote osteogenic differentiation	Hadji *et al*[40]
ANRIL	Umbilical cord	Down	ANRIL was inhibited by methylation of its promoter	NR	Decrease bone mass	Curtis *et al*[41]
HOTAIRM1	hDFSCs	Up	Inhibiting the recruitment of DNMT1	*HOXA2*	Promote osteogenic differentiation	Chen *et al* [17]
HOXA-AS3	hBMSCs	Up	Facilitating EZH2-mediated H3K27me3	*RUNX2*	Inhibit osteogenic differentiation	Zhu *et al* [62]
SNHG1	hPDLSCs	Up	Facilitating EZH2-mediated H3K27me3	*KLF2*	Inhibit osteogenic differentiation	Li *et al* [61]
OB1	human osteoblasts	Up	Inhibiting H3K27me3 by interacting with SUZ12 (a core part of PRC2)	*Osterix*	Promote osteogenic differentiation	Sun *et al* [58]

hDPSCs: Human dental pulp stromal cells; VICs: Valve interstitial cells; hDFSCs: Human dental follicle stromal cells; hBMSCs: Human bone marrow stromal cells; hPDLSCs: Human periodontal ligament stromal cells; NR: Not reported.

chondrocytes[76].

Increasing evidence suggests that small nucleolar RNA host gene (*SNHG*) family members are involved in the pathogenesis of OA[77-79]. The overexpression of lncRNA SNHG15 alleviated ECM degradation and promoted chondrocyte formation *via* competing endogenous RNA (ceRNA) SNHG15/miR-7/KLF4 axis[33]. In human OA cartilage tissues, however, the promoter region of lncRNA SNHG15 had a higher level of methylation than in normal cartilage tissues, and this might be a promising therapeutic target for OA[33]. Another *SNHG* family member, lncRNA SNHG9, was found to be downregulated in chondrocytes from OA patients[80]. Functional studies indicated that the overexpression of SNHG9 led to a decreased apoptotic rate through increased methylation of the *miR-34a* gene that suppressed the expression of miR-34a [80].

Osteoporosis is characterized by a loss of bone mass and microarchitectural deterioration of the skeletal structure[81]. The imbalance of bone homeostasis between osteoblastic bone formation and osteoclastic bone resorption plays a fundamental role in the pathogenesis of osteoporosis[82]. Emerging evidence suggests that epigenetic modifications are deeply involved in bone metabolism, which contributes to the development of osteoporosis.

The ERK-MAPK signaling pathway is a well-established pathway with critical roles in immune responses and embryonic development, including the regulation of bone mass *via* controlling osteoblast differentiation[83]. A previous study suggested that lncRNA H19 promoted tension-induced osteogenesis of hBMSCs through the FAK-ERK1/2-RUNX2 signaling pathway[84]. Likewise, an alteration in H19 methylation may also be involved in the disruption of bone formation in disuse osteoporosis. It has been shown that DNMT1-induced hypermethylation of the H19 promoter results in H19 downregulation and ERK-MAPK signaling inhibition, which leads to osteogenesis impairment both *in vivo* and *in vitro* (rat osteoblast/osteocyte-like UMR-106 cells)[85].

LncRNAs regulate histone modifications in osteoarthritis

An abnormality of cartilage regeneration can be related to attenuated chondrogenic differentiation of MSCs in OA patients[8]. Similar to other MSCs derived from other tissues, synovium-derived mesenchymal stromal cells (SMSCs) are multipotent but have the greatest chondrogenesis potential, representing a promising stem cell source for cartilage repair in OA patients[86]. lncRNA MEG3 was reported to have the ability to inhibit the chondrogenic differentiation of SMSCs and the expression of cartilage-associated genes (aggrecan and Col2A1) by inhibiting TRIB2 expression through EZH2-mediated H3K27me3[87].

Table 2 Interactions between long noncoding RNAs and epigenetic modifiers during adipogenic differentiation of mesenchymal stromal cells

LncRNAs	Samples	Expression	Epigenetic regulatory mechanisms	Target genes	Effects	Ref.
HOTAIR	hBMSCs	Up	Interacting with DNMTs	NR	Inhibit adipogenic differentiation	Kalwa *et al*[46]
Plnc1	BMSCs	Up	Reducing the DNA methylation level	*Ppar-γ2*	Promote adipogenic differentiation	Zhu *et al* [49]
slincRAD	3T3-L1	Up	Facilitating the recruitment of Dnmt1	*Cdkn1a*	Promote adipogenic differentiation	Yi *et al*[50]
ADINR	hASCs	Up	Facilitating the recruitment of MLL3/4 complex (involved in the maintenance of H3K4me3 and the removal of H3K27me3) by binding PA1	*C/EBP-α*	Promote adipogenic differentiation	Xiao *et al* [63]
MIR31HG	hASCs	Down	Reducing the enrichment of AcH3 and H3K4me3	*FABP4*	Inhibit adipogenic differentiation	Huang *et al*[16]
H19	hBMSCs	Up	facilitating miR-675-mediated repression of HDACs	NR	Inhibit adipogenic differentiation	Huang *et al*[68]

hBMSCs: Human bone marrow stromal cells; BMSCs: Bone marrow stromal cells; hASCs: Human adipose-derived stromal cells; NR: Not reported.

CONCLUSION

LncRNAs are extensively involved in various types of epigenetic modifications, including DNA methylation, histone modifications, and noncoding RNA interactions, during MSC differentiation and the occurrence and progression of degenerative bone diseases. Concerning the large body of available literature and comprehensive reviews on the RNA-RNA interactions of lncRNAs (*i.e.*, ceRNA mechanisms)[88,89], this topic of epigenetics is not discussed in this review, but it is worth mentioning that in some cases, ceRNA mechanisms act as mediators between lncRNAs and epigenetic modifiers. Another potential involvement of lncRNAs in epigenetics is the interaction with the key enzyme of methyl metabolism. It is known that DNMT and HMT utilize S-adenosylmethionine (SAM) as a major methyl-group donor in mammals, which is consumed and regenerated in one-carbon metabolism[90,91]. Several studies have shown that lncRNAs play a role in SAM-dependent methylation through regulating enzymes related to the metabolism[92,93]. However, similar studies on differentiation and bone diseases are lacking. Further studies are needed to assess the potential importance of lncRNAs on the methyl metabolism.

Although it seems that DNA methylation and histone modification are two different types of epigenetic modification, these two systems can be dependent on and influence one another during organism development[94]. However, the underlying molecular mechanisms are complicated and remain vague. Intriguingly, lncRNAs are capable of regulating gene expression either in a cis- or trans- manner by guiding or serving as scaffolds for transcription factors or epigenetic modifiers to specific gene loci[95]. This raises the possibility that lncRNAs could be coordinator of these processes. In this review, we summarized the roles of lncRNAs played in MSC differentiation and common degenerative bone diseases through reciprocal interactions between lncRNAs and epigenetic modifiers. A complete list of the epigenetic regulatory mechanisms of lncRNAs discussed in this review is available in Tables 1-3.

Taken in combination with previous studies[96-98], the present evidence indicates that lncRNAs could be diagnostic and prognostic biomarkers in degenerative bone diseases. Moreover, as lncRNAs can be manipulated pharmacologically to modulate epigenetic modifications[99], this also opens new avenues for future therapeutic interventions. However, multiple challenges need to be overcome before clinical applications can be achieved. Given that lncRNAs have complex secondary structures, one of the challenges that lies ahead is the off-target possibilities, as a single lncRNA is capable of binding to multiple epigenetic modifiers and targeting several genes. Therefore, more reliable bioinformatic tools in terms of *in silico* algorithms for comprehensive lncRNA interaction prediction and sequencing technologies are required. Despite these impediments, lncRNA-based epigenetic interventions have shown potential in the regulation of MSC differentiation and therapeutic strategies for bone diseases.

Table 3 Interactions between long noncoding RNAs and epigenetic modifiers in degenerative bone diseases

LncRNAs	Samples	Expression	Epigenetic regulatory mechanisms	Target genes	Effects	Ref.
CTBP1-AS2	OA chondrocytes	Up	Increasing the methylation level of target gene	*miR-130a*	Decease proliferation rate of OA chondrocytes	Zhang *et al*[74]
XIST	OA chondrocytes	Up	Facilitating the recruitment of DNMT1, DNMT3A, and DNMT3B	*TIMP-3*	Raise collagen degradation in OA chondrocytes	Chen *et al*[76]
SNHG15	OA cartilage tissues	Down	SNHG15 was inhibited by methylation of its promoter	*miR-7/KLF4*	Affect ECM homeostasis	Chen *et al*[33]
SNHG9	OA chondrocytes	Down	Altering the methylation level of target gene	*miR-34a*	Affect apoptotic rate of chondrocytes	Zhang *et al*[80]
H19	UMR-106 and bone tissues from osteoporosis rat model	Down	H19 was inhibited by DNMT1-induced hypermethylation of its promoter	ERK-MAPK signaling-related genes	Impair osteogenic differentiation	Li *et al* [85]
MEG3	SMSCs	Up	Facilitating EZH2-mediated H3K27me3	*TRIB2*	Inhibit chondrogenic differentiation	You *et al* [87]

LncRNAs: Long noncoding RNAs; OA: Osteoarthritis; SMSCs: Synovium-derived mesenchymal stromal cells.

REFERENCES

1 **Clarke B**. Normal bone anatomy and physiology. *Clin J Am Soc Nephrol* 2008; **3** Suppl 3: S131-S139 [PMID: 18988698 DOI: 10.2215/CJN.04151206]

2 **Viswanathan S**, Shi Y, Galipeau J, Krampera M, Leblanc K, Martin I, Nolta J, Phinney DG, Sensebe L. Mesenchymal stem versus stromal cells: International Society for Cell & Gene Therapy (ISCT®) Mesenchymal Stromal Cell committee position statement on nomenclature. *Cytotherapy* 2019; **21**: 1019-1024 [PMID: 31526643 DOI: 10.1016/j.jcyt.2019.08.002]

3 **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]

4 **Su P**, Tian Y, Yang C, Ma X, Wang X, Pei J, Qian A. Mesenchymal Stem Cell Migration during Bone Formation and Bone Diseases Therapy. *Int J Mol Sci* 2018; **19** [PMID: 30096908 DOI: 10.3390/ijms19082343]

5 **Qi M**, Zhang L, Ma Y, Shuai Y, Li L, Luo K, Liu W, Jin Y. Autophagy Maintains the Function of Bone Marrow Mesenchymal Stem Cells to Prevent Estrogen Deficiency-Induced Osteoporosis. *Theranostics* 2017; **7**: 4498-4516 [PMID: 29158841 DOI: 10.7150/thno.17949]

6 **Nuttall ME**, Gimble JM. Controlling the balance between osteoblastogenesis and adipogenesis and the consequent therapeutic implications. *Curr Opin Pharmacol* 2004; **4**: 290-294 [PMID: 15140422 DOI: 10.1016/j.coph.2004.03.002]

7 **Murphy JM**, Dixon K, Beck S, Fabian D, Feldman A, Barry F. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. *Arthritis Rheum* 2002; **46**: 704-713 [PMID: 11920406 DOI: 10.1002/art.10118]

8 **Rocha B**, Cillero-Pastor B, Eijkel G, Calamia V, Fernandez-Puente P, Paine MRL, Ruiz-Romero C, Heeren RMA, Blanco FJ. Integrative Metabolic Pathway Analysis Reveals Novel Therapeutic Targets in Osteoarthritis. *Mol Cell Proteomics* 2020; **19**: 574-588 [PMID: 31980557 DOI: 10.1074/mcp.RA119.001821]

9 **Jiang Y**, Tuan RS. Origin and function of cartilage stem/progenitor cells in osteoarthritis. *Nat Rev Rheumatol* 2015; **11**: 206-212 [PMID: 25536487 DOI: 10.1038/nrrheum.2014.200]

10 **Hao J**, Zhang Y, Jing D, Shen Y, Tang G, Huang S, Zhao Z. Mechanobiology of mesenchymal stem cells: Perspective into mechanical induction of MSC fate. *Acta Biomater* 2015; **20**: 1-9 [PMID: 25871537 DOI: 10.1016/j.actbio.2015.04.008]

11 **Crane JL**, Cao X. Bone marrow mesenchymal stem cells and TGF-β signaling in bone remodeling. *J Clin Invest* 2014; **124**: 466-472 [PMID: 24487640 DOI: 10.1172/JCI70050]

12 **Rice SJ**, Beier F, Young DA, Loughlin J. Interplay between genetics and epigenetics in osteoarthritis. *Nat Rev Rheumatol* 2020; **16**: 268-281 [PMID: 32273577 DOI: 10.1038/s41584-020-0407-3]

13 **Grandi FC**, Bhutani N. Epigenetic Therapies for Osteoarthritis. *Trends Pharmacol Sci* 2020; **41**: 557-569 [PMID: 32586653 DOI: 10.1016/j.tips.2020.05.008]

14 **Allis CD**, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet* 2016; **17**: 487-500 [PMID: 27346641 DOI: 10.1038/nrg.2016.59]

15 **Xin TY**, Yu TT, Yang RL. DNA methylation and demethylation link the properties of mesenchymal stem cells: Regeneration and immunomodulation. *World J Stem Cells* 2020; **12**: 351-358 [PMID: 32547683 DOI: 10.4252/wjsc.v12.i5.351]

16 **Huang Y**, Jin C, Zheng Y, Li X, Zhang S, Zhang Y, Jia L, Li W. Knockdown of lncRNA MIR31HG inhibits adipocyte differentiation of human adipose-derived stem cells *via* histone modification of FABP4. *Sci Rep* 2017; **7**: 8080 [PMID: 28808264 DOI: 10.1038/s41598-017-08131-6]

17 **Chen Z**, Zheng J, Hong H, Chen D, Deng L, Zhang X, Ling J, Wu L. lncRNA HOTAIRM1 promotes osteogenesis of hDFSCs by epigenetically regulating HOXA2 *via* DNMT1 in vitro. *J Cell Physiol* 2020; **235**: 8507-8519 [PMID: 32324272 DOI: 10.1002/jcp.29695]

18 **Chen J**, Wang Y, Wang C, Hu JF, Li W. LncRNA Functions as a New Emerging Epigenetic Factor in Determining the Fate of Stem Cells. *Front Genet* 2020; **11**: 277 [PMID: 32296461 DOI: 10.3389/fgene.2020.00277]

19 **Yang G**, Lu X, Yuan L. LncRNA: a link between RNA and cancer. *Biochim Biophys Acta* 2014; **1839**: 1097-1109 [PMID: 25159663 DOI: 10.1016/j.bbagrm.2014.08.012]

20 **Yoshioka H**, Yoshiko Y. The Roles of Long Non-Protein-Coding RNAs in Osteo-Adipogenic Lineage Commitment. *Int J Mol Sci* 2017; **18** [PMID: 28598385 DOI: 10.3390/ijms18061236]

21 **Xia K**, Cen X, Yu L, Huang X, Sun W, Zhao Z, Liu J. Long noncoding RNA expression profiles during the NEL-like 1 protein-induced osteogenic differentiation. *J Cell Physiol* 2020; **235**: 6010-6022 [PMID: 31985033 DOI: 10.1002/jcp.29526]

22 **Yu L**, Xia K, Cen X, Huang X, Sun W, Zhao Z, Liu J. DNA methylation of noncoding RNAs: new insights into osteogenesis and common bone diseases. *Stem Cell Res Ther* 2020; **11**: 109 [PMID: 32143708 DOI: 10.1186/s13287-020-01625-7]

23 **Broholm C**, Olsson AH, Perfilyev A, Gillberg L, Hansen NS, Ali A, Mortensen B, Ling C, Vaag A. Human adipogenesis is associated with genome-wide DNA methylation and gene-expression changes. *Epigenomics* 2016; **8**: 1601-1617 [PMID: 27854126 DOI: 10.2217/epi-2016-0077]

24 **Shen WC**, Lai YC, Li LH, Liao K, Lai HC, Kao SY, Wang J, Chuong CM, Hung SC. Methylation and PTEN activation in dental pulp mesenchymal stem cells promotes osteogenesis and reduces oncogenesis. *Nat Commun* 2019; **10**: 2226 [PMID: 31110221 DOI: 10.1038/s41467-019-10197-x]

25 **van Meurs JB**, Boer CG, Lopez-Delgado L, Riancho JA. Role of Epigenomics in Bone and Cartilage Disease. *J Bone Miner Res* 2019; **34**: 215-230 [PMID: 30715766 DOI: 10.1002/jbmr.3662]

26 **Ahuja N**, Sharma AR, Baylin SB. Epigenetic Therapeutics: A New Weapon in the War Against Cancer. *Annu Rev Med* 2016; **67**: 73-89 [PMID: 26768237 DOI: 10.1146/annurev-med-111314-035900]

27 **Bestor TH**, Edwards JR, Boulard M. Notes on the role of dynamic DNA methylation in mammalian development. *Proc Natl Acad Sci USA* 2015; **112**: 6796-6799 [PMID: 25368180 DOI: 10.1073/pnas.1415301111]

28 **Edwards JR**, Yarychkivska O, Boulard M, Bestor TH. DNA methylation and DNA methyltransferases. *Epigenetics Chromatin* 2017; **10**: 23 [PMID: 28503201 DOI: 10.1186/s13072-017-0130-8]

29 **Rauluseviciute I**, Drabløs F, Rye MB. DNA hypermethylation associated with upregulated gene expression in prostate cancer demonstrates the diversity of epigenetic regulation. *BMC Med Genomics* 2020; **13**: 6 [PMID: 31914996 DOI: 10.1186/s12920-020-0657-6]

30 **Moore LD**, Le T, Fan G. DNA methylation and its basic function. *Neuropsychopharmacology* 2013; **38**: 23-38 [PMID: 22781841 DOI: 10.1038/npp.2012.112]

31 **Jiang W**, Agrawal DK, Boosani CS. Non-coding RNAs as Epigenetic Gene Regulators in Cardiovascular Diseases. *Adv Exp Med Biol* 2020; **1229**: 133-148 [PMID: 32285409 DOI: 10.1007/978-981-15-1671-9_7]

32 **Zeng L**, Sun S, Han D, Liu Y, Liu H, Feng H, Wang Y. Long non-coding RNA H19/SAHH axis epigenetically regulates odontogenic differentiation of human dental pulp stem cells. *Cell Signal* 2018; **52**: 65-73 [PMID: 30165103 DOI: 10.1016/j.cellsig.2018.08.015]

33 **Chen Y**, Guo H, Li L, Bao D, Gao F, Li Q, Huang Q, Duan X, Xiang Z. Long Non-Coding RNA (lncRNA) Small Nucleolar RNA Host Gene 15 (SNHG15) Alleviates Osteoarthritis Progression by Regulation of Extracellular Matrix Homeostasis. *Med Sci Monit* 2020; **26**: e923868 [PMID: 32643707 DOI: 10.12659/MSM.923868]

34 **Zhou J**, Xu J, Zhang L, Liu S, Ma Y, Wen X, Hao J, Li Z, Ni Y, Li X, Zhou F, Li Q, Wang F, Wang X, Si Y, Zhang P, Liu C, Bartolomei M, Tang F, Liu B, Yu J, Lan Y. Combined Single-Cell Profiling of lncRNAs and Functional Screening Reveals that H19 Is Pivotal for Embryonic Hematopoietic Stem Cell Development. *Cell Stem Cell* 2019; **24**: 285-298.e5 [PMID: 30639035 DOI: 10.1016/j.stem.2018.11.023]

35 **Li Z**, Yan M, Yu Y, Wang Y, Lei G, Pan Y, Li N, Gobin R, Yu J. LncRNA H19 promotes the committed differentiation of stem cells from apical papilla *via* miR-141/SPAG9 pathway. *Cell Death Dis* 2019; **10**: 130 [PMID: 30755596 DOI: 10.1038/s41419-019-1337-3]

36 **Chan LH**, Wang W, Yeung W, Deng Y, Yuan P, Mak KK. Hedgehog signaling induces osteosarcoma development through Yap1 and H19 overexpression. *Oncogene* 2014; **33**: 4857-4866 [PMID: 24141783 DOI: 10.1038/onc.2013.433]

37 **Li Z**, Hong Z, Zheng Y, Dong Y, He W, Yuan Y, Guo J. An emerging potential therapeutic target for osteoporosis: LncRNA H19/miR-29a-3p axis. *Eur J Histochem* 2020; **64** [PMID: 33207859 DOI: 10.4081/ejh.2020.3155]

38 **Zeng L**, Sun S, Dong L, Liu Y, Liu H, Han D, Ma Z, Wang Y, Feng H. DLX3 epigenetically regulates odontoblastic differentiation of hDPCs through H19/miR-675 axis. *Arch Oral Biol* 2019; **102**: 155-163 [PMID: 31029881 DOI: 10.1016/j.archoralbio.2019.04.009]

39 **Liu AC**, Joag VR, Gotlieb AI. The emerging role of valve interstitial cell phenotypes in regulating

heart valve pathobiology. *Am J Pathol* 2007; **171**: 1407-1418 [PMID: 17823281 DOI: 10.2353/ajpath.2007.070251]

40 **Hadji F**, Boulanger MC, Guay SP, Gaudreault N, Amellah S, Mkannez G, Bouchareb R, Marchand JT, Nsaibia MJ, Guauque-Olarte S, Pibarot P, Bouchard L, Bossé Y, Mathieu P. Altered DNA Methylation of Long Noncoding RNA H19 in Calcific Aortic Valve Disease Promotes Mineralization by Silencing NOTCH1. *Circulation* 2016; **134**: 1848-1862 [PMID: 27789555 DOI: 10.1161/circulationaha.116.023116]

41 **Curtis EM**, Murray R, Titcombe P, Cook E, Clarke-Harris R, Costello P, Garratt E, Holbrook JD, Barton S, Inskip H, Godfrey KM, Bell CG, Cooper C, Lillycrop KA, Harvey NC. Perinatal DNA Methylation at CDKN2A Is Associated With Offspring Bone Mass: Findings From the Southampton Women's Survey. *J Bone Miner Res* 2017; **32**: 2030-2040 [PMID: 28419547 DOI: 10.1002/jbmr.3153]

42 **Kang MI**, Kim HS, Jung YC, Kim YH, Hong SJ, Kim MK, Baek KH, Kim CC, Rhyu MG. Transitional CpG methylation between promoters and retroelements of tissue-specific genes during human mesenchymal cell differentiation. *J Cell Biochem* 2007; **102**: 224-239 [PMID: 17352407 DOI: 10.1002/jcb.21291]

43 **Zhang X**, Lian Z, Padden C, Gerstein MB, Rozowsky J, Snyder M, Gingeras TR, Kapranov P, Weissman SM, Newburger PE. A myelopoiesis-associated regulatory intergenic noncoding RNA transcript within the human HOXA cluster. *Blood* 2009; **113**: 2526-2534 [PMID: 19144990 DOI: 10.1182/blood-2008-06-162164]

44 **Rinn JL**, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* 2007; **129**: 1311-1323 [PMID: 17604720 DOI: 10.1016/j.cell.2007.05.022]

45 **Mallo M**. Reassessing the Role of Hox Genes during Vertebrate Development and Evolution. *Trends Genet* 2018; **34**: 209-217 [PMID: 29269261 DOI: 10.1016/j.tig.2017.11.007]

46 **Kalwa M**, Hänzelmann S, Otto S, Kuo CC, Franzen J, Joussen S, Fernandez-Rebollo E, Rath B, Koch C, Hofmann A, Lee SH, Teschendorff AE, Denecke B, Lin Q, Widschwendter M, Weinhold E, Costa IG, Wagner W. The lncRNA HOTAIR impacts on mesenchymal stem cells *via* triple helix formation. *Nucleic Acids Res* 2016; **44**: 10631-10643 [PMID: 27634931 DOI: 10.1093/nar/gkw802]

47 **Chen Q**, Shou P, Zheng C, Jiang M, Cao G, Yang Q, Cao J, Xie N, Velletri T, Zhang X, Xu C, Zhang L, Yang H, Hou J, Wang Y, Shi Y. Fate decision of mesenchymal stem cells: adipocytes or osteoblasts? *Cell Death Differ* 2016; **23**: 1128-1139 [PMID: 26868907 DOI: 10.1038/cdd.2015.168]

48 **Lin FT**, Lane MD. CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc Natl Acad Sci USA* 1994; **91**: 8757-8761 [PMID: 8090719 DOI: 10.1073/pnas.91.19.8757]

49 **Zhu E**, Zhang J, Li Y, Yuan H, Zhou J, Wang B. Long noncoding RNA Plnc1 controls adipocyte differentiation by regulating peroxisome proliferator-activated receptor γ. *FASEB J* 2019; **33**: 2396-2408 [PMID: 30277818 DOI: 10.1096/fj.201800739RRR]

50 **Yi F**, Zhang P, Wang Y, Xu Y, Zhang Z, Ma W, Xu B, Xia Q, Du Q. Long non-coding RNA slincRAD functions in methylation regulation during the early stage of mouse adipogenesis. *RNA Biol* 2019; **16**: 1401-1413 [PMID: 31199203 DOI: 10.1080/15476286.2019.1631643]

51 **Tsai MS**, Su YH, Ho MC, Liang JT, Chen TP, Lai HS, Lee PH. Clinicopathological features and prognosis in resectable synchronous and metachronous colorectal liver metastasis. *Ann Surg Oncol* 2007; **14**: 786-794 [PMID: 17103254 DOI: 10.1245/s10434-006-9215-5]

52 **Luger K**, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* 1997; **389**: 251-260 [PMID: 9305837 DOI: 10.1038/38444]

53 **Greer EL**, Shi Y. Histone methylation: a dynamic mark in health, disease and inheritance. *Nat Rev Genet* 2012; **13**: 343-357 [PMID: 22473383 DOI: 10.1038/nrg3173]

54 **Turner BM**. Cellular memory and the histone code. *Cell* 2002; **111**: 285-291 [PMID: 12419240 DOI: 10.1016/s0092-8674(02)01080-2]

55 **Javaid N**, Choi S. Acetylation- and Methylation-Related Epigenetic Proteins in the Context of Their Targets. *Genes (Basel)* 2017; **8** [PMID: 28783137 DOI: 10.3390/genes8080196]

56 **Yi SJ**, Lee H, Lee J, Lee K, Kim J, Kim Y, Park JI, Kim K. Bone Remodeling: Histone Modifications as Fate Determinants of Bone Cell Differentiation. *Int J Mol Sci* 2019; **20** [PMID: 31252653 DOI: 10.3390/ijms20133147]

57 **Liu Q**, Li M, Wang S, Xiao Z, Xiong Y, Wang G. Recent Advances of Osterix Transcription Factor in Osteoblast Differentiation and Bone Formation. *Front Cell Dev Biol* 2020; **8**: 601224 [PMID: 33384998 DOI: 10.3389/fcell.2020.601224]

58 **Sun Y**, Cai M, Zhong J, Yang L, Xiao J, Jin F, Xue H, Liu X, Liu H, Zhang Y, Jiang D, Hong A, Ji X, Wang Z, Zhang G, Wang X. The long noncoding RNA lnc-ob1 facilitates bone formation by upregulating Osterix in osteoblasts. *Nat Metab* 2019; **1**: 485-496 [PMID: 32694877 DOI: 10.1038/s42255-019-0053-8]

59 **Margueron R**, Reinberg D. The Polycomb complex PRC2 and its mark in life. *Nature* 2011; **469**: 343-349 [PMID: 21248841 DOI: 10.1038/nature09784]

60 **Hou Z**, Wang Z, Tao Y, Bai J, Yu B, Shen J, Sun H, Xiao L, Xu Y, Zhou J, Geng D. KLF2 regulates osteoblast differentiation by targeting of Runx2. *Lab Invest* 2019; **99**: 271-280 [PMID: 30429507 DOI: 10.1038/s41374-018-0149-x]

61 **Li Z**, Guo X, Wu S. Epigenetic silencing of KLF2 by long non-coding RNA SNHG1 inhibits

periodontal ligament stem cell osteogenesis differentiation. *Stem Cell Res Ther* 2020; **11**: 435 [PMID: 33028420 DOI: 10.1186/s13287-020-01953-8]

62 **Zhu XX**, Yan YW, Chen D, Ai CZ, Lu X, Xu SS, Jiang S, Zhong GS, Chen DB, Jiang YZ. Long non-coding RNA HoxA-AS3 interacts with EZH2 to regulate lineage commitment of mesenchymal stem cells. *Oncotarget* 2016; **7**: 63561-63570 [PMID: 27566578 DOI: 10.18632/oncotarget.11538]

63 **Xiao T**, Liu L, Li H, Sun Y, Luo H, Li T, Wang S, Dalton S, Zhao RC, Chen R. Long Noncoding RNA ADINR Regulates Adipogenesis by Transcriptionally Activating C/EBPα. *Stem Cell Reports* 2015; **5**: 856-865 [PMID: 26489893 DOI: 10.1016/j.stemcr.2015.09.007]

64 **Mohan M**, Herz HM, Shilatifard A. SnapShot: Histone lysine methylase complexes. *Cell* 2012; **149**: 498-498.e1 [PMID: 22500810 DOI: 10.1016/j.cell.2012.03.025]

65 **Agger K**, Cloos PA, Christensen J, Pasini D, Rose S, Rappsilber J, Issaeva I, Canaani E, Salcini AE, Helin K. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature* 2007; **449**: 731-734 [PMID: 17713478 DOI: 10.1038/nature06145]

66 **Lefterova MI**, Zhang Y, Steger DJ, Schupp M, Schug J, Cristancho A, Feng D, Zhuo D, Stoeckert CJ Jr, Liu XS, Lazar MA. PPARgamma and C/EBP factors orchestrate adipocyte biology *via* adjacent binding on a genome-wide scale. *Genes Dev* 2008; **22**: 2941-2952 [PMID: 18981473 DOI: 10.1101/gad.1709008]

67 **Tchoukalova YD**, Sarr MG, Jensen MD. Measuring committed preadipocytes in human adipose tissue from severely obese patients by using adipocyte fatty acid binding protein. *Am J Physiol Regul Integr Comp Physiol* 2004; **287**: R1132-R1140 [PMID: 15284082 DOI: 10.1152/ajpregu.00337.2004]

68 **Huang Y**, Zheng Y, Jin C, Li X, Jia L, Li W. Long Non-coding RNA H19 Inhibits Adipocyte Differentiation of Bone Marrow Mesenchymal Stem Cells through Epigenetic Modulation of Histone Deacetylases. *Sci Rep* 2016; **6**: 28897 [PMID: 27349231 DOI: 10.1038/srep28897]

69 **Huang T**, Peng X, Li Z, Zhou Q, Huang S, Wang Y, Li J, Song Y. Epigenetics and bone diseases. *Genet Res (Camb)* 2018; **100**: e6 [PMID: 30047344 DOI: 10.1017/S0016672318000034]

70 **Yang S**, Duan X. Epigenetics, Bone Remodeling and Osteoporosis. *Curr Stem Cell Res Ther* 2016 [PMID: 28002993 DOI: 10.2174/1574888X11666161221125656]

71 **Zhao X**, Petursson F, Viollet B, Lotz M, Terkeltaub R, Liu-Bryan R. Peroxisome proliferator-activated receptor γ coactivator 1α and FoxO3A mediate chondroprotection by AMP-activated protein kinase. *Arthritis Rheumatol* 2014; **66**: 3073-3082 [PMID: 25047750 DOI: 10.1002/art.38791]

72 **Maneiro E**, Martín MA, de Andres MC, López-Armada MJ, Fernández-Sueiro JL, del Hoyo P, Galdo F, Arenas J, Blanco FJ. Mitochondrial respiratory activity is altered in osteoarthritic human articular chondrocytes. *Arthritis Rheum* 2003; **48**: 700-708 [PMID: 12632423 DOI: 10.1002/art.10837]

73 **Cen X**, Huang XQ, Sun WT, Liu Q, Liu J. Long noncoding RNAs: a new regulatory code in osteoarthritis. *Am J Transl Res* 2017; **9**: 4747-4755 [PMID: 29218077]

74 **Zhang H**, Li J, Shao W, Shen N. LncRNA CTBP1-AS2 is upregulated in osteoarthritis and increases the methylation of miR-130a gene to inhibit chondrocyte proliferation. *Clin Rheumatol* 2020; **39**: 3473-3478 [PMID: 32388751 DOI: 10.1007/s10067-020-05113-4]

75 **Sahebjam S**, Khokha R, Mort JS. Increased collagen and aggrecan degradation with age in the joints of Timp3(-/-) mice. *Arthritis Rheum* 2007; **56**: 905-909 [PMID: 17328064 DOI: 10.1002/art.22427]

76 **Chen H**, Yang S, Shao R. Long non-coding XIST raises methylation of TIMP-3 promoter to regulate collagen degradation in osteoarthritic chondrocytes after tibial plateau fracture. *Arthritis Res Ther* 2019; **21**: 271 [PMID: 31815654 DOI: 10.1186/s13075-019-2033-5]

77 **Xiao K**, Yang Y, Bian Y, Feng B, Li Z, Wu Z, Qiu G, Weng X. Identification of differentially expressed long noncoding RNAs in human knee osteoarthritis. *J Cell Biochem* 2019; **120**: 4620-4633 [PMID: 30302799 DOI: 10.1002/jcb.27750]

78 **Lei J**, Fu Y, Zhuang Y, Zhang K, Lu D. LncRNA SNHG1 alleviates IL-1β-induced osteoarthritis by inhibiting miR-16-5p-mediated p38 MAPK and NF-κB signaling pathways. *Biosci Rep* 2019; **39** [PMID: 31383786 DOI: 10.1042/bsr20191523]

79 **Shen H**, Wang Y, Shi W, Sun G, Hong L, Zhang Y. LncRNA SNHG5/miR-26a/SOX2 signal axis enhances proliferation of chondrocyte in osteoarthritis. *Acta Biochim Biophys Sin (Shanghai)* 2018; **50**: 191-198 [PMID: 29409014 DOI: 10.1093/abbs/gmx141]

80 **Zhang H**, Li J, Shao W, Shen N. LncRNA SNHG9 is downregulated in osteoarthritis and inhibits chondrocyte apoptosis by downregulating miR-34a through methylation. *BMC Musculoskelet Disord* 2020; **21**: 511 [PMID: 32738890 DOI: 10.1186/s12891-020-03497-7]

81 **Raisz LG**. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *J Clin Invest* 2005; **115**: 3318-3325 [PMID: 16322775 DOI: 10.1172/jci27071]

82 **Yang TL**, Shen H, Liu A, Dong SS, Zhang L, Deng FY, Zhao Q, Deng HW. A road map for understanding molecular and genetic determinants of osteoporosis. *Nat Rev Endocrinol* 2020; **16**: 91-103 [PMID: 31792439 DOI: 10.1038/s41574-019-0282-7]

83 **Greenblatt MB**, Shim JH, Glimcher LH. Mitogen-activated protein kinase pathways in osteoblasts. *Annu Rev Cell Dev Biol* 2013; **29**: 63-79 [PMID: 23725048 DOI: 10.1146/annurev-cellbio-101512-122347]

84 **Wu J**, Zhao J, Sun L, Pan Y, Wang H, Zhang WB. Long non-coding RNA H19 mediates mechanical tension-induced osteogenesis of bone marrow mesenchymal stem cells *via* FAK by sponging miR-138. *Bone* 2018; **108**: 62-70 [PMID: 29253550 DOI: 10.1016/j.bone.2017.12.013]

85 **Li B**, Zhao J, Ma JX, Li GM, Zhang Y, Xing GS, Liu J, Ma XL. Overexpression of DNMT1 leads to hypermethylation of H19 promoter and inhibition of Erk signaling pathway in disuse osteoporosis.

Bone 2018; **111**: 82-91 [PMID: 29555308 DOI: 10.1016/j.bone.2018.03.017]

86 **de Sousa EB**, Casado PL, Moura Neto V, Duarte ME, Aguiar DP. Synovial fluid and synovial membrane mesenchymal stem cells: latest discoveries and therapeutic perspectives. *Stem Cell Res Ther* 2014; **5**: 112 [PMID: 25688673 DOI: 10.1186/scrt501]

87 **You D**, Yang C, Huang J, Gong H, Yan M, Ni J. Long non-coding RNA MEG3 inhibits chondrogenic differentiation of synovium-derived mesenchymal stem cells by epigenetically inhibiting TRIB2 *via* methyltransferase EZH2. *Cell Signal* 2019; **63**: 109379 [PMID: 31376524 DOI: 10.1016/j.cellsig.2019.109379]

88 **Ratneswaran A**, Kapoor M. Osteoarthritis year in review: genetics, genomics, epigenetics. *Osteoarthritis Cartilage* 2021; **29**: 151-160 [PMID: 33227439 DOI: 10.1016/j.joca.2020.11.003]

89 **Ju C**, Liu R, Zhang YW, Zhang Y, Zhou R, Sun J, Lv XB, Zhang Z. Mesenchymal stem cell-associated lncRNA in osteogenic differentiation. *Biomed Pharmacother* 2019; **115**: 108912 [PMID: 31048188 DOI: 10.1016/j.biopha.2019.108912]

90 **Johnson C**, Warmoes MO, Shen X, Locasale JW. Epigenetics and cancer metabolism. *Cancer Lett* 2015; **356**: 309-314 [PMID: 24125862 DOI: 10.1016/j.canlet.2013.09.043]

91 **Mentch SJ**, Locasale JW. One-carbon metabolism and epigenetics: understanding the specificity. *Ann N Y Acad Sci* 2016; **1363**: 91-98 [PMID: 26647078 DOI: 10.1111/nyas.12956]

92 **Guo T**, Gong C, Wu P, Battaglia-Hsu SF, Feng J, Liu P, Wang H, Guo D, Yao Y, Chen B, Xiao Y, Liu Z, Li Z. LINC00662 promotes hepatocellular carcinoma progression *via* altering genomic methylation profiles. *Cell Death Differ* 2020; **27**: 2191-2205 [PMID: 31959915 DOI: 10.1038/s41418-020-0494-3]

93 **Zhou J**, Yang L, Zhong T, Mueller M, Men Y, Zhang N, Xie J, Giang K, Chung H, Sun X, Lu L, Carmichael GG, Taylor HS, Huang Y. H19 lncRNA alters DNA methylation genome wide by regulating S-adenosylhomocysteine hydrolase. *Nat Commun* 2015; **6**: 10221 [PMID: 26687445 DOI: 10.1038/ncomms10221]

94 **Cedar H**, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet* 2009; **10**: 295-304 [PMID: 19308066 DOI: 10.1038/nrg2540]

95 **Rinn JL**, Chang HY. Genome regulation by long noncoding RNAs. *Annu Rev Biochem* 2012; **81**: 145-166 [PMID: 22663078 DOI: 10.1146/annurev-biochem-051410-092902]

96 **Chen Y**, Lin Y, Bai Y, Cheng D, Bi Z. A Long Noncoding RNA (lncRNA)-Associated Competing Endogenous RNA (ceRNA) Network Identifies Eight lncRNA Biomarkers in Patients with Osteoarthritis of the Knee. *Med Sci Monit* 2019; **25**: 2058-2065 [PMID: 30890688 DOI: 10.12659/MSM.915555]

97 **Zhao Y**, Xu J. Synovial fluid-derived exosomal lncRNA PCGEM1 as biomarker for the different stages of osteoarthritis. *Int Orthop* 2018; **42**: 2865-2872 [PMID: 30128669 DOI: 10.1007/s00264-018-4093-6]

98 **Silva AM**, Moura SR, Teixeira JH, Barbosa MA, Santos SG, Almeida MI. Long noncoding RNAs: a missing link in osteoporosis. *Bone Res* 2019; **7**: 10 [PMID: 30937214 DOI: 10.1038/s41413-019-0048-9]

99 **Prabhakar B**, Zhong XB, Rasmussen TP. Exploiting Long Noncoding RNAs as Pharmacological Targets to Modulate Epigenetic Diseases. *Yale J Biol Med* 2017; **90**: 73-86 [PMID: 28356895]

World Journal of Stem Cells

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

World J Stem Cells 2022 January 26; 14(1): 104-116

DOI: 10.4252/wjsc.v14.i1.104

ISSN 1948-0210 (online)

ORIGINAL ARTICLE

Basic Study

Therapeutic effects of menstrual blood-derived endometrial stem cells on mouse models of streptozotocin-induced type 1 diabetes

Yu-Liang Sun, Ling-Rui Shang, Rui-Hong Liu, Xin-Yi Li, Sheng-Hui Zhang, Ya-Kun Ren, Kang Fu, Hong-Bin Cheng, Badrul Hisham Yahaya, Yan-Li Liu, Jun-Tang Lin

ORCID number: Yu-Liang Sun 0000-0002-6338-0077; Ling-Rui Shang 0000-0001-5007-0646; Rui-Hong Liu 0000-0003-2449-161X; Xin-Yi Li 0000-0001-7578-3183; Sheng-Hui Zhang 0000-0002-2760-6550; Ya-Kun Ren 0000-0002-7961-8430; Kang Fu 0000-0002-9228-3161; Hong-Bin Cheng 0000-0001-8963-8702; Badrul Hisham Yahaya 0000-0002-3295-9676; Yan-Li Liu 0000-0003-0886-8907; Jun-Tang Lin 0000-0001-9300-1771.

Author contributions: Sun YL and Shang LR contributed equally to this work; Cheng HB, Yahaya BH, Liu YL and Lin JT conceived the idea and designed the study; Sun YL, Liu RH, Li XY, Zhang SH, Ren YK, and Fu K contributed to the literature review and integrated the materials; Sun YL and Shang LR prepared the draft; Liu YL and Lin JT revised the manuscript and approved the final version as the corresponding authors; all the authors read and approved the final manuscript.

Institutional animal care and use committee statement: The research was reviewed and approved by Xinxiang Medical University.

Conflict-of-interest statement: The author declares that there is no conflict of interest at the time of

Yu-Liang Sun, Ling-Rui Shang, Xin-Yi Li, Yan-Li Liu, Jun-Tang Lin, Stem Cell and Biotherapy Technology Research Center, Xinxiang Medical University, Xinxiang 453000, Henan Province, China

Yu-Liang Sun, Badrul Hisham Yahaya, Regenerative Medicine Cluster, Advanced Medical and Dental Institute (IPPT), Universiti Sains Malaysia, Kepala Batas 13200, Penang, Malaysia

Rui-Hong Liu, Hong-Bin Cheng, Jun-Tang Lin, College of Biomedical Engineering, Xinxiang Medical University, Xinxiang 453000, Henan Province, China

Sheng-Hui Zhang, The Third Affiliated Hospital of Xinxiang Medical University, Xinxiang Medical University, Xinxiang 453000, Henan Province, China

Ya-Kun Ren, Henan Key Laboratory of Medical Tissue Regeneration, Xinxiang Medical University, Xinxiang 453000, Henan Province, China

Kang Fu, Department of Technical, Henan Intercell Biotechnology co. LTD, Xinxiang 453000, Henan Province, China

Corresponding author: Jun-Tang Lin, PhD, Professor, Stem Cell and Biotherapy Technology Research Center, Xinxiang Medical University, East of JinSui Road, Xinxiang 453000, Henan Province, China. linjt@xxmu.edu.cn

Abstract

BACKGROUND

Type 1 diabetes (T1D), a chronic metabolic and autoimmune disease, seriously endangers human health. In recent years, mesenchymal stem cell (MSC) transplantation has become an effective treatment for diabetes. Menstrual blood-derived endometrial stem cells (MenSC), a novel MSC type derived from the decidual endometrium during menstruation, are expected to become promising seeding cells for diabetes treatment because of their noninvasive collection procedure, high proliferation rate and high immunomodulation capacity.

AIM

To comprehensively compare the effects of MenSC and umbilical cord-derived MSC (UcMSC) transplantation on T1D treatment, to further explore the potential mechanism of MSC-based therapies in T1D, and to provide support for the

publishing this article.

Data sharing statement: No additional data is available.

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

Supported by Henan Province Foundation of China, No. 202300410307 and No. 212102310611; Xinxiang City Foundation of China, No. GG2020009.

Country/Territory of origin: China

Specialty type: Cell and tissue engineering

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B, B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

Received: May 5, 2021
Peer-review started: May 5, 2021
First decision: June 23, 2021
Revised: July 20, 2021
Accepted: December 25, 2021
Article in press: December 25, 2021
Published online: January 26, 2022

clinical application of MSC in diabetes treatment.

METHODS

A conventional streptozotocin-induced T1D mouse model was established, and the effects of MenSC and UcMSC transplantation on their blood glucose and serum insulin levels were detected. The morphological and functional changes in the pancreas, liver, kidney, and spleen were analyzed by routine histological and immunohistochemical examinations. Changes in the serum cytokine levels in the model mice were assessed by protein arrays. The expression of target proteins related to pancreatic regeneration and apoptosis was examined by western blot.

RESULTS

MenSC and UcMSC transplantation significantly improved the blood glucose and serum insulin levels in T1D model mice. Immunofluorescence analysis revealed that the numbers of insulin$^+$ and CD31$^+$ cells in the pancreas were significantly increased in MSC-treated mice compared with control mice. Subsequent western blot analysis also showed that vascular endothelial growth factor (VEGF), Bcl2, Bcl-xL and Proliferating cell nuclear antigen in pancreatic tissue was significantly upregulated in MSC-treated mice compared with control mice. Additionally, protein arrays indicated that MenSC and UcMSC transplantation significantly downregulated the serum levels of interferon γ and tumor necrosis factor α and upregulated the serum levels of interleukin-6 and VEGF in the model mice. Additionally, histological and immunohistochemical analyses revealed that MSC transplantation systematically improved the morphologies and functions of the liver, kidney, and spleen in T1D model mice.

CONCLUSION

MenSC transplantation significantly improves the symptoms in T1D model mice and exerts protective effects on their main organs. Moreover, MSC-mediated angiogenesis, antiapoptotic effects and immunomodulation likely contribute to the above improvements. Thus, MenSC are expected to become promising seeding cells for clinical diabetes treatment due to their advantages mentioned above.

Key Words: Type 1 diabetes; Menstrual blood-derived endometrial stem cell; Umbilical cord mesenchymal stem cells; Improvement; Complication

Core Tip: Mesenchymal stem cell (MSC)-based therapies have resulted in promising improvements for patients with type 1 diabetes (T1D). Menstrual blood-derived endometrial stem cells (MenSC) transplantation has therapeutic effects equal to those of umbilical cord-derived MSC, which can significantly improve the symptoms of streptozotocin-induced T1D mice and exert protective effects on their main organs; MSC-induced angiogenesis, antiapoptotic effects and immunomodulation contribute to these protective effects. The results of this study showed that MenSC are expected to become a promising alternative for clinical diabetes treatment due to their advantages, including their regular and noninvasive collection protocol, abundant availability, and superior proliferative capacity.

Citation: Sun YL, Shang LR, Liu RH, Li XY, Zhang SH, Ren YK, Fu K, Cheng HB, Yahaya BH, Liu YL, Lin JT. Therapeutic effects of menstrual blood-derived endometrial stem cells on mouse models of streptozotocin-induced type 1 diabetes. *World J Stem Cells* 2022; 14(1): 104-116
URL: https://www.wjgnet.com/1948-0210/full/v14/i1/104.htm
DOI: https://dx.doi.org/10.4252/wjsc.v14.i1.104

P-Reviewer: Al-Hadhrami R, Chrcanovic BR, Hamad ARA, Herold Z
S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

INTRODUCTION

Diabetes is a common metabolic disease characterized by hyperglycemia, and diabetic patients are prone to chronic damage or dysfunction of their eyes, kidneys, heart, blood vessels, and nerves, as well as other complications, severely affecting their quality of life[1,2]. The loss of functional β-cell mass is the key pathogenesis leading to both type 1 diabetes (T1D) and T2D. T1D is a chronic autoimmune disease resulting from a complete insulin deficiency caused by the attack and destruction of pancreatic islet β cells by the autoimmune system. On the other hand, T2D manifests as the progressive loss of β-cell insulin secretion and frequently occurs in the background of insulin resistance[3,4]. Generally, exogenous insulin supplementation, which serves as the main treatment for diabetes, can control blood glucose and delay the occurrence of diabetic complications. However, the long-term use of exogenous insulin leads to insulin resistance and an increased risk of severe hypoglycemia and does not fundamentally inhibit the pathological development of diabetes or potential diabetic complications. Therefore, to improve the symptoms of diabetes and prevent the occurrence and deterioration of diabetes complications, novel effective treatments for diabetes are required. Mesenchymal stem cell (MSC), which have self-renewal and multilineage differentiation abilities as well as low immunogenicity, have exhibited promising therapeutic potential for various diseases in the clinic, and previous studies have also demonstrated that MSC transplantation is safe and effective in diabetes treatment[5-8].

Umbilical cord-derived MSC (UcMSC), as the main source of MSC for diabetes treatment in the clinic, not only enhance the antiapoptotic capacity of islet β cells and stimulate their regeneration but also differentiate into islet β cells to compensate for insufficient insulin secretion[9-11]. Generally, UcMSC are transplanted into the body through intravenous injection, intrapancreatic artery injection or both, and the improvements after UcMSC transplantation have been extensively confirmed in clinical practice. After 1 year of UcMSC therapy, the mean HbA1c% values were consistently decreased in diabetic patients compared with the baseline levels; the mean C-peptide levels were consistently elevated, and the daily insulin requirement was uniformly decreased compared with their respective baseline[12-14]. In addition to the extensive application of UcMSC in diabetes treatment, menstrual blood-derived endometrial stem cells (MenSC), harvested from the menstrual blood of women of reproductive age, have recently attracted increased attention in the field of regenerative medicine. MenSC can be collected continuously every month, and the noninvasive collection method (menstrual cup) does not impose physiological or psychological burdens on the donor. Furthermore, regarding the richness of stem cells, primary MenSC account for approximately 3%-5% of all karyocytes in menstrual blood samples, quantitatively supporting their extensive application in the clinic[15]. Therefore, guaranteeing the quantity of MenSC satisfies the requirement for not only autologous transplantation but also for the family members of patients. Additionally, nude mice exhibited no tumorigenicity after MenSC transplantation, and no obvious adverse effects were observed after MenSC transplantation in clinical trials on multiple sclerosis[16], congestive heart failure[17], and lower limb ischemia[18]. These results guarantee the safety of MenSC in clinical applications[19]. Additionally, in 2014, Wu *et al*[20] preliminarily demonstrated the positive effect of MenSC transplantation on streptozotocin (STZ)-induced T1D mice, suggesting that MenSC are promising for clinical diabetes treatment[20].

Therefore, in this study, T1D animal models were generated by the injection of STZ, a strategy that effectively and specifically damages pancreatic β cells and inhibits insulin secretion, resulting in elevated blood glucose levels and the induction of T1D symptoms[21]. This study aimed to evaluate the therapeutic effects of MenSC and UcMSC transplantation on STZ-induced T1D mice, focusing on the improvements in T1D-related symptoms and the underlying mechanism, as well as the influences on the main organs of model mice. We believe that both the therapeutic effect and mechanistic studies of MenSC in T1D will provide support for and accelerate the clinical application of these cells in diabetes treatment strategies.

MATERIALS AND METHODS

Cells and animals

Both the MenSC and UcMSC used in this study were provided by the Zhongyuan Stem Cell Research Institute of Xinxiang High-tech Zone, and informed consent was

provided by all donors who agreed to use their UcMSC and MenSC in scientific research. All the experimental procedures in this study were approved by the Ethics Committee of Xinxiang Medical University and were performed in accordance with the approved guidelines. Male C57BL/6N mice (18-25 g) aged 6 to 8 wk were purchased from Vital River Laboratories [Beijing, China; license no. SCXK (Beijing) 2012-0001]. The mice were randomly grouped in separate cages and housed in a specific pathogen-free environment maintained at a temperature of 25 ℃, a humidity of 50 ± 5% and a 12-h light-dark cycle. The mouse handling and laboratory procedures were performed in accordance with the guidelines of the Animal Health Committee of Xinxiang Medical University.

Establishment of the T1D model and MSC transplantation

C57BL/6N mice (n = 50) were adaptively fed for one week and randomly divided into 2 groups: The normal group (n = 10) and the STZ-induced group (n = 40). The mice in the STZ-induced group were intraperitoneally injected with STZ (Sigma, United States; 45 mg/kg/d in citric acid buffer at pH 4.3) each day after fasting overnight for 5 consecutive days. The mice in the control group received equal amounts of citric acid buffer (pH 4.3). The first day of STZ injection was labeled as day-13; after the last STZ injection (day-9), the physiological statuses of the model mice were observed for 1 wk. After that, the model mice with fasting blood glucose (FBG) levels greater than 16 mmol/L were diagnosed with diabetes and randomly divided into 3 groups (n = 10): The PBS, UcMSC group, and MenSC treatment groups. The mice in the MSC treatment group were injected with 1 × 10^6 cells (suspended in 200 μL of saline) *via* their tail vein on days 0, day 7, and day 14. The mice in the PBS treatment group received an equal amount of saline. The mice were weighed, and their FBG levels and food intake were monitored every week during the experimental period. At the end of the experiment (day 63), all the mice were sacrificed, and their sera, pancreases, livers, spleens, and kidneys were harvested for further examination.

Biochemical assays

The isolated serum samples were sent to Xinxiang Assegai Medical Laboratory Center (Xinxiang, China) within 4 h. Routine liver and kidney functional indexes, including alanine transaminase (ALT), aspartic acid transaminase (AST) and alkaline phosphatase (ALP), albumin (ALB), globulin (GLB), total protein (TP), UREA, uric acid (UA), and creatinine (CRE), were assayed using a Chemray240 automated biochemical analyzer (Rayto, Shenzhen, China). The serum insulin levels in the mice were determined using an ELISA Kit (D721159; Sangon Biotech) according to the manufacturer's instructions.

Protein array assays

The isolated serum samples were kept on dry ice and sent to RayBiotech (Guangzhou, China). A protein array (AAH-CUST-G1; Norcross, GA, United States) was used according to the manufacturer's instructions to measure the expression levels of 20 cytokines in the samples (Figure 1A). Positive signals were captured on glass chips using a laser scanner (InnoScan 300 Microarray Scanner; Innopsys, Carbonne, France), and the observed fluorescence intensities were normalized to those of the internal positive controls.

Hematoxylin and eosin staining

The pancreas, kidney, liver, and spleen samples were routinely fixed with 4% phosphate-buffered paraformaldehyde, embedded in paraffin, and sliced into 5-μm-thick sections for conventional hematoxylin and eosin (HE) staining. Next, the morphologies of the targeted organs were observed and imaged under a microscope (DMi8; Leica, Wetzlar, Germany).

Glycogen staining (PAS)

Dewaxed liver sections were successively immersed in 95% and 75% ethanol solutions for 10 min and then rinsed with water for 10 min. After removing the excess water in a fume hood, the samples were stained with a sodium periodate solution for 10 min and rinsed with running water for 10 min. Next, the sections were incubated with Schiff solution at 37 ℃ in the dark for 15-25 min. After removing the excessive Schiff's solution, the sections were washed with sodium sulfite solution for 5 min and then with running water for another 10 min. The sections were soaked in 95% ethanol and xylene for 1-2 min and conventionally sealed after air drying in a fume hood. Finally, the sections were observed and imaged under a microscope.

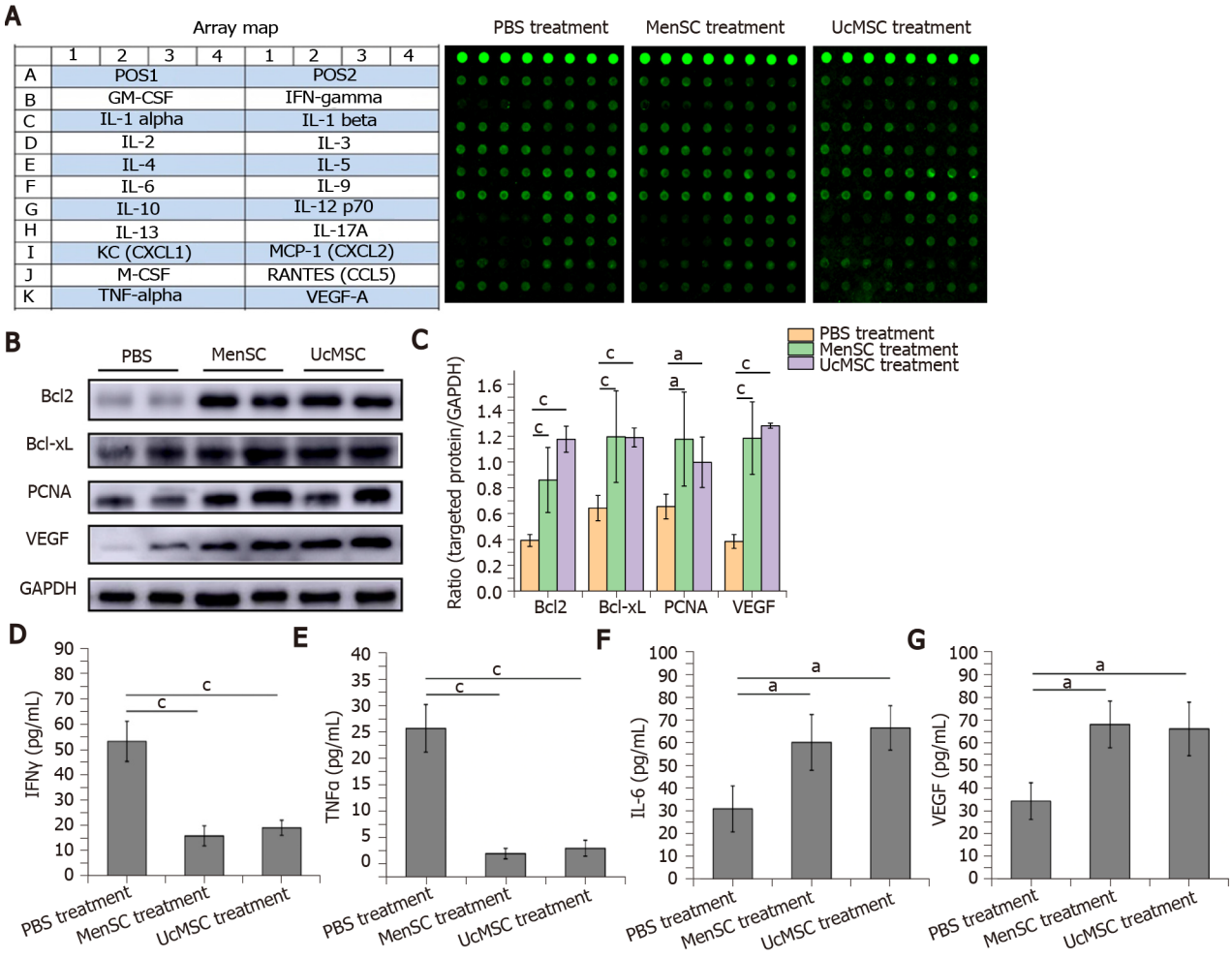

Figure 1 Mesenchymal stem cell transplantation promotes pancreatic regeneration and regulates immune homeostasis in streptozotocin-induced type 1 diabetes mice. A: The cytokines examined and representative array images are shown; B and C: The expression levels of vascular endothelial growth factor, Bcl2, BCL-xL, and proliferating cell nuclear antigen in the mouse pancreas were detected by WB, and the relative expression of targeted proteins was quantitated using ImageJ software; D-G: The concentrations of significantly changed cytokines were quantified based on the fluorescence intensities. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. MSC: Mesenchymal stem cell; UcMSC: Umbilical cord-derived MSC; MenSC: Menstrual blood-derived endometrial stem cells; IFN: Interferon; TNF: Tumor necrosis factor; IL: Interleukin; VEGF: Vascular endothelial growth factor; PCNA: Proliferating cell nuclear antigen.

Immunofluorescence

Dewaxed pancreatic tissue sections were immersed in antigenic repair solution at 90 ℃-100 ℃, incubated in water at 100 ℃ for 20 min, cooled naturally to room temperature, and washed with PBS three times. Subsequently, the sections were permeabilized with 0.5% Triton X-100 at room temperature for 25 min and washed with PBS three times. After blocking with 10% goat serum for 2 h, the samples were incubated with mouse anti-mouse insulin (GB13121; Servicebio) and goat anti-mouse CD31 (GB13063; Servicebio) primary antibodies at 4 ℃ overnight in a wet box. The sections were incubated with FITC-labeled goat anti-mouse IgG and CY3-labeled donkey anti-goat IgG secondary antibodies at 37 ℃ for 1 h, and nuclei were stained with DAPI. Finally, the sections were observed and imaged under a fluorescence microscope (Leica, Germany), and the percentages of insulin^{+} (green fluorescence) and CD31^{+} (red fluorescence) cells from at least 10 areas *per* slide were quantified using IPP software (Image-Pro Plus 6.0 Software).

Western blotting

TP was extracted from pancreatic tissue using RIPA buffer and a protease inhibitor (Beyotime, China), and the protein concentration was determined by the BCA method. The protein samples were denatured in a metal bath at 95 ℃, separated by SDS-PAGE and transferred onto PVDF membranes. The samples were subsequently blocked with 5% nonfat milk in PBS for 1 h and then incubated with the following primary antibodies overnight at 4 ℃: Rabbit-derived Bcl2 (ab182858; Abcam), Bcl-xL (ab32370; Abcam), proliferating cell nuclear antigen (PCNA) (ab92552; Abcam), and vascular

endothelial growth factor (VEGF) (ab32152; Abcam). The membranes were then incubated with goat anti-rabbit HRP-conjugated secondary antibodies at room temperature for 2 h. GAPDH was used as the internal control. Immunoreactions were detected using enhanced an chemiluminescence reagent and an Amersham Imager 600 system (GE Healthcare Life Sciences), and the grayscale values of the bands representing the targeted proteins were quantitated using ImageJ software.

Statistical analysis

The results are presented as the mean ± SD and were analyzed using Statistical Package for GraphPad Prism 8.0. The nonparametric Mann-Whitney *U* test was used for comparisons between two independent samples, and one-way ANOVA followed by Dunnett's test was used for comparisons among ≥ 3 groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

MenSC transplantation significantly improves diabetic symptoms in T1D mice

The characteristics of the MenSC and UcMSC used in this study were consistent with the typical characteristics of MSC, which express CD73, CD90, and CD105 but not CD34 and CD29. After MSC transplantation, the FBG levels in MSC-treated mice were significantly decreased starting on day 42 (Figure 2A; $P < 0.05$), and the body weights of UcMSC-treated mice showed a significantly increasing trend starting on day 28 (Figure 2B; $P < 0.05$), which was positively related to food consumption (Figure 2C). Subsequently, both the area of islets and the number of insulin$^+$ cells in the pancreas were significantly increased after MSC transplantation (Figure 2D-F). Consistent with the above findings, the serum insulin levels were also upregulated (Figure 2G), but the levels were not significantly different between MenSC- and UcMSC-treated mice. Additionally, the number of CD31$^+$ cells in the pancreas was significantly increased in MSC-treated mice compared with PBS-treated mice (Figure 2E and H), and the expression of VEGF in the pancreas was upregulated (Figure 1B and C). Additionally, the final WB results confirmed that the antiapoptotic markers (Bcl2 and Bcl-xL) and PCNA in the pancreas were significantly upregulated in MSC-treated mice compared with PBS-treated mice (Figure 1B and C).

MenSC transplantation effectively improves inflammation and angiogenesis in T1D mice

A protein array was used to detect the expression levels of 20 cytokines in the sera of STZ-treated mice treated with or without MSC. The serum expression levels of interferon (IFN) γ and tumor necrosis factor (TNF) α were significantly downregulated in MSC-treated mice compared with PBS-treated mice (Figure 1A and D-G; $P < 0.05$), suggesting that the inflammatory response was relieved in vivo. Simultaneously, the serum expression levels of IL-6 and VEGF in the MSC-treated mice were significantly upregulated ($P < 0.05$).

MenSC transplantation significantly improves the liver micromorphology and glycogen synthesis in T1D mice

The liver is the main target organ of insulin and is highly likely to be damaged by diabetes-induced chronic complications[22]. After treatment with MenSC and UcMSC, liver lobule destruction and pseudolobule formation were improved, and liver injury was relieved (Figure 3). MenSC and UcMSC transplantation increased the liver weights and liver indexes of T1D mice, but these parameters were not significantly different from those of the PBS-treated mice (Figure 3B and C). Subsequent PAS staining showed that the rose-red plaque areas in the liver tissues of MSC-treated mice were significantly increased (Figure 3A), suggesting that both MenSC and UcMSC transplantation improved the ability of T1D mice to synthesize glycogen in their livers. Additionally, the liver functional indexes of mice treated with MSC were similar to those of the control mice, and no significant differences were observed between the two groups (Figure 3D-K).

MenSC transplantation partially improves the renal biochemical indexes in T1D mice

The kidney is another organ commonly affected by diabetes, and early diagnosis and prevention can delay the onset and development of diabetic nephropathy, which is critical for improving the survival rate and quality of life of patients[23]. No marked

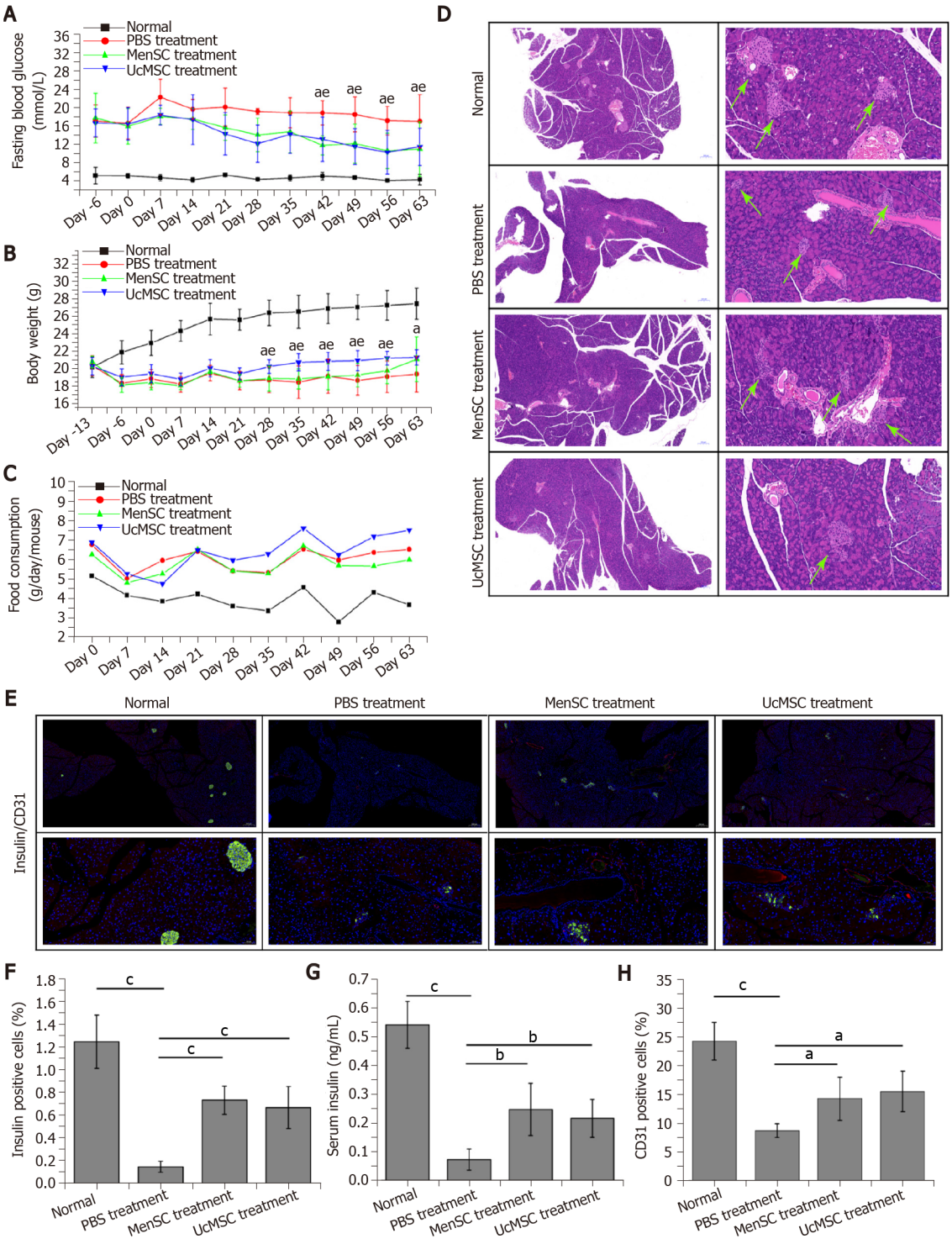

Figure 2 Menstrual blood-derived endometrial stem cells transplantation significantly improves the morphology and function of the pancreas in streptozotocin-induced type 1 diabetes mice. A: Both Menstrual blood-derived endometrial stem cells Menstrual blood-derived endometrial stem cells (MenSC) and Umbilical cord-derived mesenchymal stem cell (UcMSC) treatments significantly improved the fasting blood glucose levels. $^{a}P < 0.05$ UcMSC treatment *vs* PBS treatment; $^{e}P < 0.05$ MenSC treatment *vs* PBS treatment; B and C: UcMSC treatment significantly inhibited the body weight loss and increased the food intake in streptozotocin-induced type 1 diabetes mice. $^{a}P < 0.05$ UcMSC treatment *vs* PBS treatment; $^{e}P < 0.05$ UcMSC treatment *vs* MenSC treatment; D: Morphological changes in the pancreases of mice were examined by hematoxylin and eosin staining, and the islets are indicated by green arrows. The images in the left column provide a full view of the pancreas, and the images in the right column are locally magnified; E, F and H: Insulin^{+} (green fluorescence) and CD31^{+} (red fluorescence) cells in the mouse pancreas were detected by immunofluorescence and quantified by IPP software. The images in the top row provide a full view of the pancreases, and the images in the bottom row are locally magnified; G: The serum insulin levels in the mice were determined by ELISA. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. MSC: Mesenchymal stem cell; UcMSC: Umbilical cord-derived MSC; MenSC: Menstrual blood-derived endometrial stem cells.

morphological differences were observed in the kidneys of normal mice and STZ-induced T1D mice treated with or without MSC (Figure 4). Although the kidney weight of MSC-treated mice was slightly increased compared with that of PBS-treated

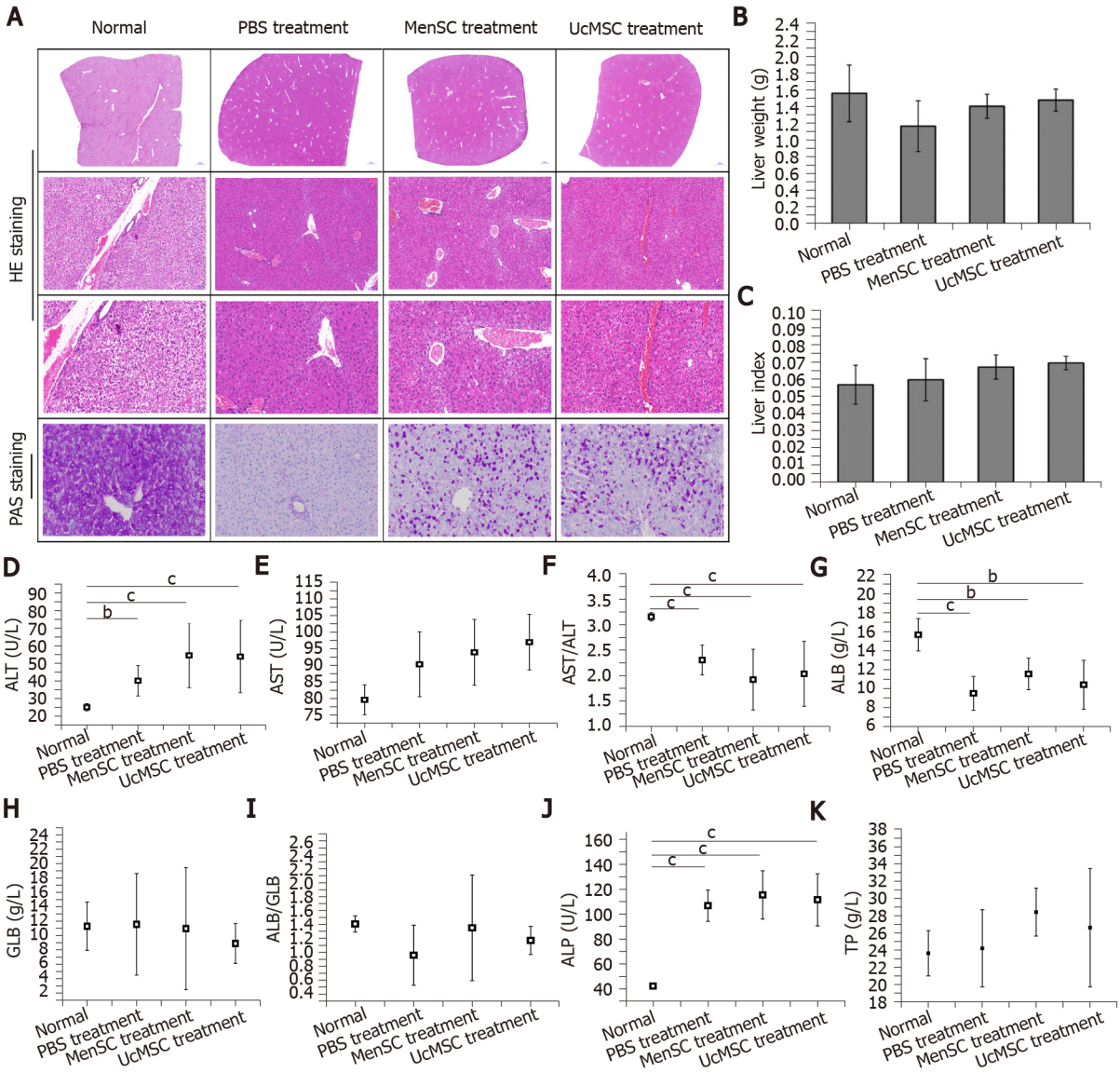

Figure 3 Morphological and functional improvements in the livers of streptozotocin-induced type 1 diabetes mice after mesenchymal stem cell transplantation. A: Morphological changes in the livers of mice were examined by hematoxylin and eosin staining (the images in the top row provide a full view of the livers, and the images in the middle row are locally magnified), and the capacity for glycogen synthesis and storage in the livers was determined by Periodic Acid-Schiff staining; B and C: Mesenchymal stem cell treatment had no obvious effect on the liver weights or liver indexes in streptozotocin-induced type 1 diabetes mice; D-K: The activities of alanine transaminase and aspartic acid transaminase in the mouse serum were examined, and the concentrations of albumin, globulin, total protein, and alkaline phosphatase in the serum were measured. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. MSC: Mesenchymal stem cell; UcMSC: Umbilical cord-derived MSC; MenSC: Menstrual blood-derived endometrial stem cells; UA: Uric acid; CRE: Creatinine; HE: Hematoxylin and eosin; PAS: Periodic Acid-Schiff; ALT: Alanine transaminase; AST: Aspartic acid transaminase; ALP: Alkaline phosphatase; ALB: Albumin; GLB: Globulin; TP: Total protein.

mice, the difference was not significant (Figure 4B and C). Additionally, subsequent biochemical assays showed that the UREA and UA levels in MSC-treated mice were significantly downregulated (Figure 4D and E).

MenSC transplantation partially ameliorates morphological spleen injuries in T1D mice

Dysfunction of the spleen, an important immune organ, is closely related to T1D[24]. In the present study, the spleen weights and spleen indexes of STZ-induced T1D mice were significantly reduced compared with those of control mice, and MSC treatment increased the mouse spleen weight; however, the spleen weights were not significantly different between the two groups ($P > 0.05$; Figure 5A and B). Subsequent HE staining (Figure 5C) indicated that the white pulp of the spleen in normal mice was well developed, the splenic corpuscles were round or oval, the germinal center was obvious, the periarterial lymphatic sheath was located beside the splenic corpuscle, the proportion of white pulp and red pulp was reasonable, and the boundary was clear.

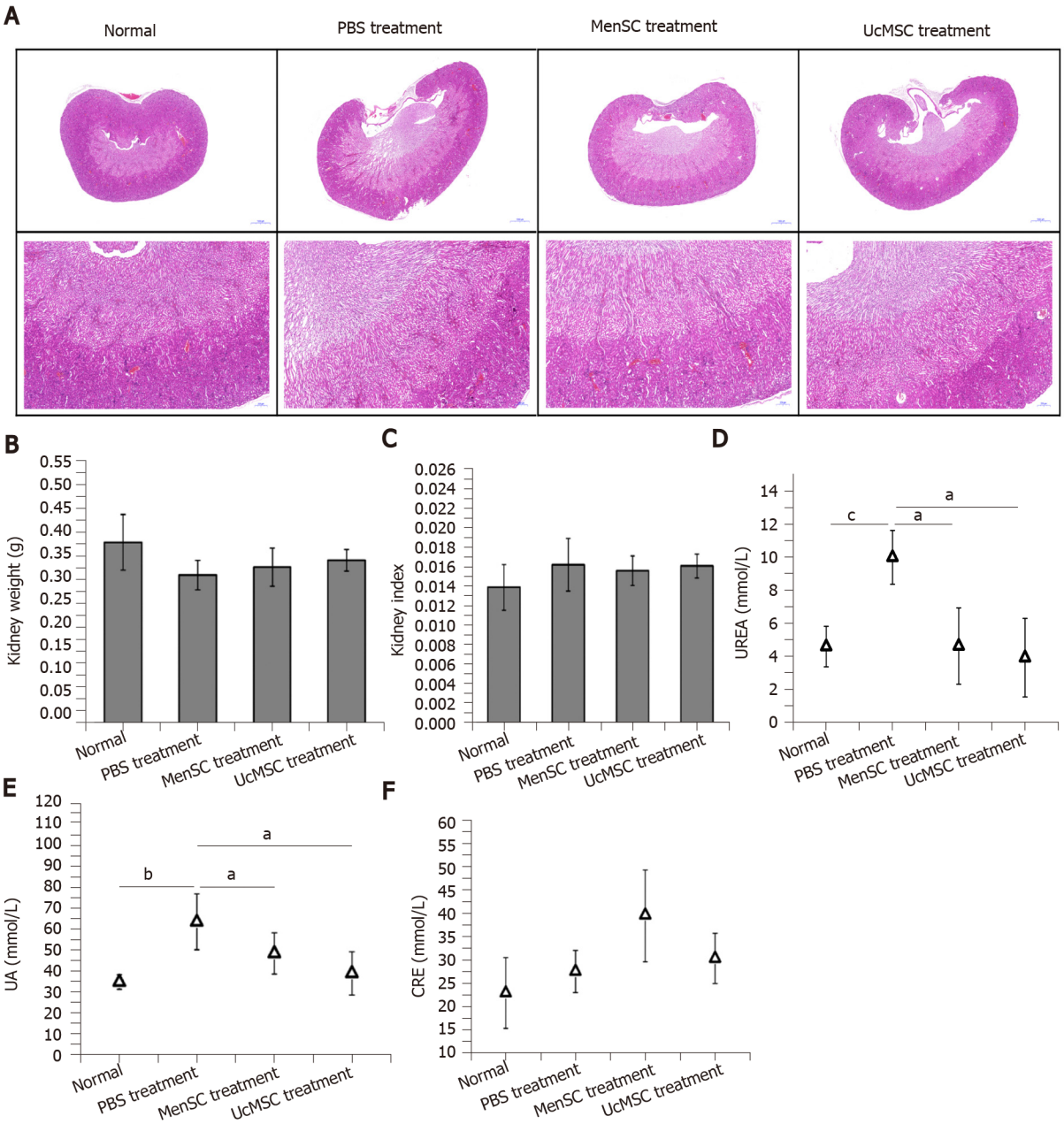

Figure 4 Morphological and functional differences in the kidneys of streptozotocin-induced type 1 diabetes mice after mesenchymal stem cell transplantation. A: Morphological changes in the kidneys of mice were examined by hematoxylin and eosin staining. The images in the top row provide a full view of the kidneys, and the images in the bottom row are locally magnified; B and C: Mesenchymal stem cell treatment had no obvious effect on the kidney weights or kidney indexes of streptozotocin-induced type 1 diabetes mice; D-F: The serum concentrations of UREA, uric acid, and creatinine in the mice were measured. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. MSC: Mesenchymal stem cell; UcMSC: Umbilical cord-derived MSC; MenSC: Menstrual blood-derived endometrial stem cells; UA: Uric acid; CRE: Creatinine.

However, the area of white pulp in the spleens of STZ-induced T1D mice was significantly reduced and had a disordered structure, and the lymphatic follicles were atrophied. Although the spleen weights and spleen indexes of the STZ-induced T1D mice were not significantly increased after MSC treatment, the degree of lymphatic follicle atrophy in MSC-treated mice was improved, and the white pulp area was increased.

DISCUSSION

Diabetes, a common metabolic disease, is characterized by long-term high blood sugar levels and hormone disruption, which can cause severe complications in the eyes, liver, kidneys, and immune system. Currently, approximately 451 million patients

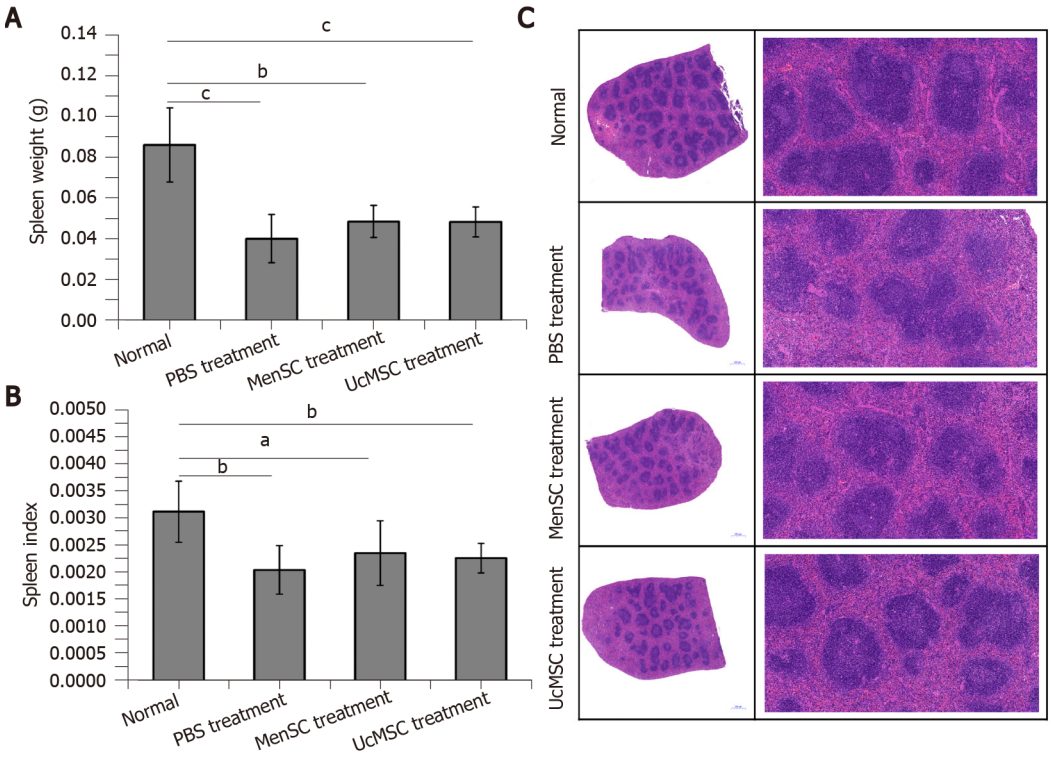

Figure 5 Morphological differences in the spleens of streptozotocin-induced type 1 diabetes mice after mesenchymal stem cell transplantation. A and B: Mesenchymal stem cell treatment did not significantly increase the spleen weights or spleen indexes in streptozotocin-induced type 1 diabetes mice; C: Morphological changes in the spleens of mice were examined by hematoxylin and eosin staining. The images in the left column provide a full view of spleens, and the images in the right column are locally magnified. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. MSC: Mesenchymal stem cell; UcMSC: Umbilical cord-derived MSC; MenSC: Menstrual blood-derived endometrial stem cells.

have diabetes worldwide, and that number is estimated to reach 693 million by 2045 [25]; diabetes causes at least five million deaths and costs at least $850 billion in health care costs annually. Additionally, MSC transplantation is considered to be an effective treatment for improving diabetes-derived symptoms and complications when considering the limitations of conventional diabetes treatments, and the efficacy and safety of MSC transplantation to improve diabetic symptoms have been confirmed by many preclinical and clinical studies[15,18,26-28]. In addition to the frequently used UcMSC, MenSC have attracted notable attention as a promising alternative stem cell-based therapy for various diseases based on their wide-ranging advantages, such as their regular and noninvasive collection method, abundant availability, superior proliferative capacity and autologous transplantation potential. Furthermore, published clinical trials on MenSC-based therapies have reported no adverse events for any of the enrolled patients. Medistem Inc. launched a phase II clinical trial on MenSC treatment for patients with congestive heart failure, and the follow-up results revealed no adverse events associated with 17 patients who received MenSC stem cell transplantation[17]. A subsequent clinical report also confirmed the therapeutic effect and safety of autologous MenSC transplantation for patients with Asherman's syndrome[29]. Additionally, six clinical studies on MenSC-based therapies have been officially approved by the National Health Commission of the People's Republic of China due to the superior biological characteristics of MenSC.

Based on published reports, the therapeutic mechanism of MSC in patients with diabetes is mainly focused on angiogenesis promotion, immunomodulation, islet β cell regeneration and islet β cell apoptosis inhibition[30-32]. Consistent with published results, MenSC treatment exerted similar positive effects on STZ-induced T1D mice and significantly reduced their FBG levels. Subsequent histological examinations revealed that MSC transplantation significantly increased the number of insulin^{+} islet β cells in the T1D mouse pancreas, and the enhancement of angiogenesis, antiapoptotic effects and regeneration likely contributed to these effects. Furthermore, subsequent protein assays confirmed the presence of systemically reduced inflammation (downregulation of IL-1β and TNFα) and enhanced angiogenesis potential (upregulation of VEGF) in MSC-treated mice. Additionally, MSC treatment was shown to upregulate the expression of IL-6 in the sera of T1D mice, and a low dose of IL-6

counteracted the cytotoxicity of IL-1β on islet β cells and stimulated insulin secretion by islet β cells[33,34].

Furthermore, because of the severe diabetes-derived complications in other organs, attention should be paid to not only the improvement of pancreatic function but also the therapeutic effects of MSC on these other organs. Additionally, the prevention of diabetes-derived complications could effectively increase the survival and improve the quality of life of patients with diabetes. Our previous studies revealed that MenSC resided in the livers and kidneys of mice after injection *via* the tail vein, providing direct evidence of the MSC-mediated improvement in the functions of these organs. Therefore, the morphologies and functions of the livers and kidneys (highly subject to diabetic complications) of STZ-induced T1D mice were examined after MSC transplantation. As expected, MenSC treatment partially improved the functions of the liver and kidney, and no visible morphological abnormalities were observed in these organs. Additionally, spleen dysfunction plays a role in the onset and development of T1D, and a reasonable ratio of white pulp to red pulp in the spleen plays a critical role in the maintenance of immune homeostasis. Our results indicated that MSC treatment did not significantly increase the spleen weight in STZ-induced T1D mice but did improve the degree of lymphatic follicle atrophy and the ratio of white pulp to red pulp, which contribute to re-establishing immune homeostasis.

CONCLUSION

In conclusion, the therapeutic effects of MenSC transplantation are equal to those of UcMSC and can significantly improve the symptoms of T1D mice and exert protective effects on their main organs. Moreover, MSC-induced angiogenesis, antiapoptotic effects and immunomodulation contribute to these protective effects. Additionally, MenSC are expected to become a promising alternative for diabetes treatment in the clinic due to their advantages, including their regular and noninvasive collection method, abundant availability, and superior proliferative capacity.

ARTICLE HIGHLIGHTS

Research background

Type 1 diabetes (T1D), a chronic metabolic disease that lacks an effective cure, seriously endangers human health. In recent years, mesenchymal stem cell (MSC) transplantation has become an effective treatment for diabetes. Menstrual blood-derived endometrial stem cells (MenSC), a novel MSC type derived from the decidual endometrium during menstruation, are expected to become promising seeding cells for diabetes treatment due to their therapeutic effects on many diseases.

Research motivation

T1D is a highly prevalent disease and lacks an effective treatment. MenSC are expected to become promising seeding cells for diabetes treatment in the clinic.

Research objectives

The objective of our study was to evaluate the therapeutic effects of MenSC on a T1D mouse model.

Research methods

Streptozotocin (STZ) was used to induce the T1D mouse model. Then, improvements in the blood glucose levels and biochemical indexes of the mice were detected after the injection of MenSC *via* their tail vein. Moreover, the morphological and functional improvements in the livers, spleens and kidneys of MenSC-treated T1D model mice were examined.

Research results

In the STZ-induced T1D model, MenSC transplantation significantly improved the symptoms of T1D mice. Immunofluorescence and western blot analyses revealed that the numbers of insulin$^+$ cells and CD31$^+$ cells in the pancreas were significantly increased in MenSC-treated mice compared with control mice and inhibited the apoptosis of pancreatic cells. Additionally, protein arrays showed that MenSC

transplantation significantly downregulated the serum levels of interferon γ and tumor necrosis factor α and upregulated the serum levels of interleukin-6 and vascular endothelial growth factor in the model mice. Subsequent histological and immunohistochemical analyses demonstrated that MSC transplantation systematically improved the morphologies and functions of the liver, kidneys, and spleen in the T1D model mice and effectively alleviated the complications of T1D.

Research conclusions

The therapeutic effects of MenSC transplantation are equal to those of umbilical cord-derived MSC and can significantly improve the symptoms of T1D mice and exert protective effects on their main organs. MenSC are expected to become promising seeding cells for the treatment of T1D.

Research perspectives

In the STZ-induced T1D mouse model, MenSC can effectively improve the symptoms and complications of T1D and lay a foundation for the clinical use of MenSC in the treatment of T1D.

ACKNOWLEDGEMENTS

The authors would like to thank Ya-Nan He for her assistance with the stem cell cultures.

REFERENCES

1 **Cole JB**, Florez JC. Genetics of diabetes mellitus and diabetes complications. *Nat Rev Nephrol* 2020; **16**: 377-390 [PMID: 32398868 DOI: 10.1038/s41581-020-0278-5]
2 **Forbes JM**, Cooper ME. Mechanisms of diabetic complications. *Physiol Rev* 2013; **93**: 137-188 [PMID: 23303908 DOI: 10.1152/physrev.00045.2011]
3 **Eizirik DL**, Pasquali L, Cnop M. Pancreatic β-cells in type 1 and type 2 diabetes mellitus: different pathways to failure. *Nat Rev Endocrinol* 2020; **16**: 349-362 [PMID: 32398822 DOI: 10.1038/s41574-020-0355-7]
4 **Janghorbani M**, Van Dam RM, Willett WC, Hu FB. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. *Am J Epidemiol* 2007; **166**: 495-505 [PMID: 17575306 DOI: 10.1093/aje/kwm106]
5 **Song N**, Scholtemeijer M, Shah K. Mesenchymal Stem Cell Immunomodulation: Mechanisms and Therapeutic Potential. *Trends Pharmacol Sci* 2020; **41**: 653-664 [PMID: 32709406 DOI: 10.1016/j.tips.2020.06.009]
6 **Fan XL**, Zhang Y, Li X, Fu QL. Mechanisms underlying the protective effects of mesenchymal stem cell-based therapy. *Cell Mol Life Sci* 2020; **77**: 2771-2794 [PMID: 31965214 DOI: 10.1007/s00018-020-03454-6]
7 **Mishra VK**, Shih HH, Parveen F, Lenzen D, Ito E, Chan TF, Ke LY. Identifying the Therapeutic Significance of Mesenchymal Stem Cells. *Cells* 2020; **9** [PMID: 32384763 DOI: 10.3390/cells9051145]
8 **Ghoneim MA**, Refaie AF, Elbassiouny BL, Gabr MM, Zakaria MM. From Mesenchymal Stromal/Stem Cells to Insulin-Producing Cells: Progress and Challenges. *Stem Cell Rev Rep* 2020; **16**: 1156-1172 [PMID: 32880857 DOI: 10.1007/s12015-020-10036-3]
9 **Stiner R**, Alexander M, Liu G, Liao W, Liu Y, Yu J, Pone EJ, Zhao W, Lakey JRT. Transplantation of stem cells from umbilical cord blood as therapy for type I diabetes. *Cell Tissue Res* 2019; **378**: 155-162 [PMID: 31209568 DOI: 10.1007/s00441-019-03046-2]
10 **Chandravanshi B**, Bhonde RR. Human Umbilical Cord-Derived Stem Cells: Isolation, Characterization, Differentiation, and Application in Treating Diabetes. *Crit Rev Biomed Eng* 2018; **46**: 399-412 [PMID: 30806260 DOI: 10.1615/CritRevBiomedEng.2018027377]
11 **Choi KS**, Shin JS, Lee JJ, Kim YS, Kim SB, Kim CW. In vitro trans-differentiation of rat mesenchymal cells into insulin-producing cells by rat pancreatic extract. *Biochem Biophys Res Commun* 2005; **330**: 1299-1305 [PMID: 15823584 DOI: 10.1016/j.bbrc.2005.03.111]
12 **Chen J**, Zheng CX, Jin Y, Hu CH. Mesenchymal stromal cell-mediated immune regulation: A promising remedy in the therapy of type 2 diabetes mellitus. *Stem Cells* 2021; **39**: 838-852 [PMID: 33621403 DOI: 10.1002/stem.3357]
13 **Li Y**, Wang F, Liang H, Tang D, Huang M, Zhao J, Yang X, Liu Y, Shu L, Wang J, He Z. Efficacy of mesenchymal stem cell transplantation therapy for type 1 and type 2 diabetes mellitus: a meta-analysis. *Stem Cell Res Ther* 2021; **12**: 273 [PMID: 33957998 DOI: 10.1186/s13287-021-02342-5]
14 **Hsiao CY**, Chen TH, Huang BS, Chen PH, Su CH, Shyu JF, Tsai PJ. Comparison between the therapeutic effects of differentiated and undifferentiated Wharton's jelly mesenchymal stem cells in

rats with streptozotocin-induced diabetes. *World J Stem Cells* 2020; **12**: 139-151 [PMID: 32184938 DOI: 10.4252/wjsc.v12.i2.139]

15 **Chen JY**, Mou XZ, Du XC, Xiang C. Comparative analysis of biological characteristics of adult mesenchymal stem cells with different tissue origins. *Asian Pac J Trop Med* 2015; **8**: 739-746 [PMID: 26433660 DOI: 10.1016/j.apjtm.2015.07.022]

16 **Riordan NH**, Morales I, Fernández G, Allen N, Fearnot NE, Leckrone ME, Markovich DJ, Mansfield D, Avila D, Patel AN, Kesari S, Paz Rodriguez J. Clinical feasibility of umbilical cord tissue-derived mesenchymal stem cells in the treatment of multiple sclerosis. *J Transl Med* 2018; **16**: 57 [PMID: 29523171 DOI: 10.1186/s12967-018-1433-7]

17 **Bockeria L**, Bogin V, Bockeria O, Le T, Alekyan B, Woods EJ, Brown AA, Ichim TE, Patel AN. Endometrial regenerative cells for treatment of heart failure: a new stem cell enters the clinic. *J Transl Med* 2013; **11**: 56 [PMID: 23510656 DOI: 10.1186/1479-5876-11-56]

18 **Murphy MP**, Wang H, Patel AN, Kambhampati S, Angle N, Chan K, Marleau AM, Pyszniak A, Carrier E, Ichim TE, Riordan NH. Allogeneic endometrial regenerative cells: an "Off the shelf solution" for critical limb ischemia? *J Transl Med* 2008; **6**: 45 [PMID: 18713449 DOI: 10.1186/1479-5876-6-45]

19 **Liu Y**, Niu R, Yang F, Yan Y, Liang S, Sun Y, Shen P, Lin J. Biological characteristics of human menstrual blood-derived endometrial stem cells. *J Cell Mol Med* 2018; **22**: 1627-1639 [PMID: 29278305 DOI: 10.1111/jcmm.13437]

20 **Wu X**, Luo Y, Chen J, Pan R, Xiang B, Du X, Xiang L, Shao J, Xiang C. Transplantation of human menstrual blood progenitor cells improves hyperglycemia by promoting endogenous progenitor differentiation in type 1 diabetic mice. *Stem Cells Dev* 2014; **23**: 1245-1257 [PMID: 24499421 DOI: 10.1089/scd.2013.0390]

21 **Goyal SN**, Reddy NM, Patil KR, Nakhate KT, Ojha S, Patil CR, Agrawal YO. Challenges and issues with streptozotocin-induced diabetes - A clinically relevant animal model to understand the diabetes pathogenesis and evaluate therapeutics. *Chem Biol Interact* 2016; **244**: 49-63 [PMID: 26656244 DOI: 10.1016/j.cbi.2015.11.032]

22 **Dewidar B**, Kahl S, Pafili K, Roden M. Metabolic liver disease in diabetes - From mechanisms to clinical trials. *Metabolism* 2020; **111S**: 154299 [PMID: 32569680 DOI: 10.1016/j.metabol.2020.154299]

23 **Nistala R**, Savin V. Diabetes, hypertension, and chronic kidney disease progression: role of DPP4. *Am J Physiol Renal Physiol* 2017; **312**: F661-F670 [PMID: 28122713 DOI: 10.1152/ajprenal.00316.2016]

24 **Bellinger DL**, Lorton D. Sympathetic Nerve Hyperactivity in the Spleen: Causal for Nonpathogenic-Driven Chronic Immune-Mediated Inflammatory Diseases (IMIDs)? *Int J Mol Sci* 2018; **19** [PMID: 29652832 DOI: 10.3390/ijms19041188]

25 **Haddad D**, Al Madhoun A, Nizam R, Al-Mulla F. Role of Caveolin-1 in Diabetes and Its Complications. *Oxid Med Cell Longev* 2020; **2020**: 9761539 [PMID: 32082483 DOI: 10.1155/2020/9761539]

26 **Bozorgmehr M**, Gurung S, Darzi S, Nikoo S, Kazemnejad S, Zarnani AH, Gargett CE. Endometrial and Menstrual Blood Mesenchymal Stem/Stromal Cells: Biological Properties and Clinical Application. *Front Cell Dev Biol* 2020; **8**: 497 [PMID: 32742977 DOI: 10.3389/fcell.2020.00497]

27 **Rodrigues MC**, Lippert T, Nguyen H, Kaelber S, Sanberg PR, Borlongan CV. Menstrual Blood-Derived Stem Cells: In Vitro and In Vivo Characterization of Functional Effects. *Adv Exp Med Biol* 2016; **951**: 111-121 [PMID: 27837558 DOI: 10.1007/978-3-319-45457-3_9]

28 **Garrido R**, Castillo L, Hernández G, Abarca J, Montes JM, Monsalve V, Espinoza R, Valenzuela S, Pérez G. [Systemic leptospirosis as a cause of multiple organ failure. Report of a case]. *Rev Med Chil* 1996; **124**: 359-362 [PMID: 9008950 DOI: 10.1089/hum.2010.173]

29 **Tan J**, Li P, Wang Q, Li Y, Li X, Zhao D, Xu X, Kong L. Autologous menstrual blood-derived stromal cells transplantation for severe Asherman's syndrome. *Hum Reprod* 2016; **31**: 2723-2729 [PMID: 27664218 DOI: 10.1093/humrep/dew235]

30 **Ohnishi S**, Yasuda T, Kitamura S, Nagaya N. Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. *Stem Cells* 2007; **25**: 1166-1177 [PMID: 17289933 DOI: 10.1634/stemcells.2006-0347]

31 **Aguayo-Mazzucato C**, Bonner-Weir S. Pancreatic β Cell Regeneration as a Possible Therapy for Diabetes. *Cell Metab* 2018; **27**: 57-67 [PMID: 28889951 DOI: 10.1016/j.cmet.2017.08.007]

32 **Chen J**, Chen J, Cheng Y, Fu Y, Zhao H, Tang M, Lin N, Shi X, Lei Y, Wang S, Huang L, Wu W, Tan J. Mesenchymal stem cell-derived exosomes protect beta cells against hypoxia-induced apoptosis *via* miR-21 by alleviating ER stress and inhibiting p38 MAPK phosphorylation. *Stem Cell Res Ther* 2020; **11**: 97 [PMID: 32127037 DOI: 10.1186/s13287-020-01610-0]

33 **Buschard K**, Aaen K, Horn T, Van Damme J, Bendtzen K. Interleukin 6: a functional and structural *in vitro* modulator of beta-cells from islets of Langerhans. *Autoimmunity* 1990; **5**: 185-194 [PMID: 2129751 DOI: 10.3109/08916939009002977]

34 **Shimizu H**, Sato N, Tanaka Y, Ohtani K, Fukatsu A, Mori M. Interleukin-6 stimulates insulin secretion in HIT-T 15 cells. *Horm Metab Res* 1995; **27**: 37-38 [PMID: 7729791 DOI: 10.1055/s-2007-979904]

World Journal of Stem Cells

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

World J Stem Cells 2022 January 26; 14(1): 117-141

DOI: 10.4252/wjsc.v14.i1.117

ISSN 1948-0210 (online)

SYSTEMATIC REVIEWS

Stem cell therapy applied for digestive anastomosis: Current state and future perspectives

Jacobo Trébol, Tihomir Georgiev-Hristov, Isabel Pascual-Miguelañez, Hector Guadalajara, Mariano García-Arranz, Damian García-Olmo

ORCID number: Jacobo Trébol 0000-0002-6579-533X; Tihomir Georgiev-Hristov 0000-0002-4428-6413; Isabel Pascual-Miguelañez 0000-0003-2088-9030; Hector Guadalajara 0000-0001-6297-9347; Mariano García-Arranz 0000-0002-6266-9055; Damian García-Olmo 0000-0002-9369-2338.

Author contributions: All authors equally contributed to this paper with drafting and critical revision. Trebol J and Georgiev-Hristov T performed literature review and analysis; Georgiev-Hristov T, Pascual I and Guadalajara H revised language editing; Trebol J wrote the paper; all authors reviewed the paper and gave their final approval of manuscript.

Conflict-of-interest statement: García-Olmo D is a member of the Advisory Board of Tigenix S.A.U. García-Olmo D and García-Arranz M co-hold patent rights for patents related to this study entitled Biomaterial for suture/suturing (WO2006035083A1), Identification and isolation of multipotent cells from non-osteochondral mesenchymal tissue (WO2006037649A1) and about Use of adipose tissue-derived stromal stem cells in treating fistula (WO2006136244A2). García-Olmo D and García-Arranz M are shareholders of Biosurgery, an

Jacobo Trébol, Servicio de Cirugía General y del Aparato Digestivo, Complejo Asistencial Universitario de Salamanca, Salamanca 37007, Spain

Jacobo Trébol, Departamento de Anatomía e Histología Humanas, Universidad de Salamanca, Salamanca 37007, Spain

Tihomir Georgiev-Hristov, Servicio de Cirugía General y del Aparato Digestivo, Hospital General Universitario de Villalba, Madrid 28400, Spain

Isabel Pascual-Miguelañez, Servicio de Cirugía General y del Aparato Digestivo, Hospital Universitario La Paz, Madrid 28046, Spain

Hector Guadalajara, Servicio de Cirugía General y del Aparato Digestivo, Hospital Universitario Fundación Jiménez Díaz, Madrid 28040, Spain

Mariano García-Arranz, Grupo de Investigación en Nuevas Terapias, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz, Madrid 28040, Spain

Mariano García-Arranz, Damian García-Olmo, Departamento de Cirugía, Universidad Autónoma de Madrid, Madrid 28029, Spain

Damian García-Olmo, Servicio de Cirugía General y del Aparato Digestivo, Hospital Universitario Fundación Jiménez Díaz y Grupo Quiron-Salud Madrid, Madrid 28040, Spain

Corresponding author: Jacobo Trébol, MD, PhD, Adjunct Professor, Surgeon, Surgical Oncologist, Servicio de Cirugía General y del Aparato Digestivo, Complejo Asistencial Universitario de Salamanca, Paseo de San Vicente, No. 58-182, Salamanca 37007, Spain.
jtrebol@saludcastillayleon.es

Abstract

BACKGROUND

Digestive tract resections are usually followed by an anastomosis. Anastomotic leakage, normally due to failed healing, is the most feared complication in digestive surgery because it is associated with high morbidity and mortality. Despite technical and technological advances and focused research, its rates have remained almost unchanged the last decades. In the last two decades, stem cells (SCs) have been shown to enhance healing in animal and human studies; hence, SCs have emerged since 2008 as an alternative to improve anastomoses outcomes.

educational company providing services to Takeda. Other authors disclosed no potential conflicts of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist statement, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist statement.

Country/Territory of origin: Spain

Specialty type: Surgery

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

Received: March 24, 2021
Peer-review started: March 24, 2021
First decision: June 5, 2021
Revised: June 21, 2021
Accepted: December 31, 2021
Article in press: December 31, 2021
Published online: January 26, 2022

P-Reviewer: Grawish ME
S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

AIM
To summarise the published knowledge of SC utilisation as a preventative tool for hollow digestive viscera anastomotic or suture leaks.

METHODS
PubMed, Science Direct, Scopus and Cochrane searches were performed using the key words "anastomosis", "colorectal/colonic anastomoses", "anastomotic leak", "stem cells", "progenitor cells", "cellular therapy" and "cell therapy" in order to identify relevant articles published in English and Spanish during the years of 2000 to 2021. Studies employing SCs, performing digestive anastomoses in hollow viscera or digestive perforation sutures and monitoring healing were finally included. Reference lists from the selected articles were reviewed to identify additional pertinent articles.

METHODS
Given the great variability in the study designs, anastomotic models, interventions (SCs, doses and vehicles) and outcome measures, performing a reliable meta-analysis was considered impossible, so we present the studies, their results and limitations.

RESULTS
Eighteen preclinical studies and three review papers were identified; no clinical studies have been published and there are no registered clinical trials. Experimental studies, mainly in rat and porcine models and occasionally in very adverse conditions such as ischaemia or colitis, have been demonstrated SCs as safe and have shown some encouraging morphological, functional and even clinical results. Mesenchymal SCs are mostly employed, and delivery routes are mainly local injections and cell sheets followed by biosutures (sutures coated by SCs) or purely topical. As potential weaknesses, animal models need to be improved to make them more comparable and equivalent to clinical practice, and the SC isolation processes need to be standardised. There is notable heterogeneity in the studies, making them difficult to compare. Further investigations are needed to establish the indications, the administration system, potential adjuvants, the final efficacy and to confirm safety and exclude definitively oncological concerns.

CONCLUSION
The future role of SC therapy to induce healing processes in digestive anastomoses/sutures still needs to be determined and seems to be currently far from clinical use.

Key Words: Surgical anastomosis; Anastomotic leak; Digestive system surgical procedure; Cell transplantation; Cell therapy; Stem cells; Tissue engineering

Core Tip: Digestive anastomoses leakages reflect impaired healing, are frequent and are associated with severe consequences. Despite technical and technological advancements, leakage rates have remained stable in the last decades. Stem cells (SCs) could improve anastomotic healing, as they have in other altered healing conditions. We present a descriptive review of the published literature about digestive anastomoses and sutures and SCs, analyzing the results and discussing their limitations and concerns. Eighteen preclinical studies have confirmed the feasibility and safety and have shown interesting results, however, with some limitations and high heterogenicity. Additional studies and better models are needed prior to human testing.

Citation: Trébol J, Georgiev-Hristov T, Pascual-Miguelañez I, Guadalajara H, García-Arranz M, García-Olmo D. Stem cell therapy applied for digestive anastomosis: Current state and future perspectives. *World J Stem Cells* 2022; 14(1): 117-141
URL: https://www.wjgnet.com/1948-0210/full/v14/i1/117.htm
DOI: https://dx.doi.org/10.4252/wjsc.v14.i1.117

INTRODUCTION

Despite all technical and technological advancements, digestive anastomotic leakages (DAL) occur and are the most feared complications in digestive surgery because they lead to significant morbidity and represent the principal surgical complication for mortality. All regions from the oesophagus to the anus and the biliary and pancreatic ducts can be affected.

There is no generally accepted definition of DAL and multiple descriptions have been proposed combining clinical aspects, analytical parameters, radiological findings and treatment consequences. There are also multiple grading systems. The United Kingdom Surgical Infection Study Group introduced one of the first definitions: 'a leak of luminal contents from a surgical join between two hollow viscera that emerge either through the wound or at the drain site, or that may collect near the anastomosis'[1]. A systematic review published by Bruce *et al* in 2001 found 56 definitions for DAL. Many efforts have been made to define colorectal anastomotic leakage (CAL), due to the high frequency of colorectal resections. The International Study Group of Rectal Cancer proposed the definition 'a defect of the intestinal wall integrity at the colorectal or colo-anal anastomotic site leading to a communication between the intra- and extraluminal compartments' and a grading system[2], recommended recently by an international expert panel[3]. A similar definition may be extended to other digestive anastomoses. The lack of a uniform definition for each anastomotic site, has a clear impact on the reported incidence rates.

The DAL incidence varies widely depending on the organ and anastomosis studied, as well as on the definition and diagnostic criteria employed. As examples, we highlight three surgical areas. A systematic review on oesophagectomy including 174 studies and 74226 patients found an overall pooled AL rate of 11% (range 0%-49%)[4]. An international multicentre snapshot audit, conducted in 2015 by the European Society of Coloproctology, included 3208 right hemicolectomies or ileo-caecal resections; the overall AL rate was 8.1%[5]. A meta-analysis including 18 studies and 18039 curative rectal cancer resections found an overall AL rate of 9.8% (range 2.5%-14.8%)[6].

DAL are associated with severe adverse outcomes, including nosocomial and organ-space infections (as mediastinitis or peritonitis); systemic inflammatory response; sepsis; other organ complications or failures (including multi-organ dysfunction); reoperations; need for intestinal stomas; increased re-admission rates, length of stay, hospital and health care costs and in-hospital mortality; and could impact quality of life and delay the start of adjuvant therapy[4,7]. DAL after cancer surgery could negatively impact cancer-specific outcomes and could be considered an independent negative prognostic factor. For example, in rectal cancer, AL are significantly associated with an increased risk of local recurrence, worse overall survival and decreased disease-free and cancer-specific survival, but not with distant recurrence and overall recurrence excluding 30-day mortality[6].

DAL incidences have remained stable over the last decades. Great efforts have been made in the following areas trying to decrease them: (1) Risk factor identification: risk factors could be local or general and modifiable (target to reduce AL rates) or non-modifiable[2,4]. Identifying high risk patients enables better perioperative planning and patient counselling; and (2) Technical development: with a focus on manual or mechanical suture material, endoluminal anastomotic or protective devices and robotic surgery. There are many expectations for operative perfusion assessment with indocyanine green fluorescence angiography. Based on a meta-analysis[8], it seems to reduce CAL; however, this was not the case in a recently published randomised controlled trial[9].

Anastomotic strictures, frequently associated with a previous AL, could also be an important complication in some anastomoses, such as biliary anastomosis during liver transplantation, which is associated with considerable morbidity and costs. In a systematic review including 14359 liver transplants, the overall incidence was 12% among deceased donor liver transplantation patients and 19% among living donor recipients[10]. Its gold standard treatment – balloon dilatation and stent placement – has a success rate of approximately 50% and usually requires multiple procedures[11], so preventive measures or better therapies are also needed.

Stem cell (SC) therapy has been demonstrated as safe and has shown promising results in a wide variety of clinical and experimental settings: haematological, cardiovascular[12], neurological, digestive[13], traumatological[14], endocrine and renal conditions are some examples. The most commonly used are haematopoietic SCs [15], mesenchymal SCs (MSCs)[16,17] and adipose-derived SCs (ASCs)[15,18,19]. Some SCs play crucial roles in the healing process by different mechanisms, including

increasing angiogenesis, local blood flow, fibroblast activity and collagen synthesis, coordinating the repair response by recruiting other host cells and secreting growth factors and matrix proteins, among others[20]. ASCs have been applied in environments that are particularly unfavourable for wound healing, such as experimental colitis[21], sepsis[22], anal and other digestive fistula[23-27], Crohn's disease[28,29], faecal incontinence[30] and tracheal anastomoses[31], with favourable outcomes.

With these promising results, it was only a matter of time before SCs would be applied in digestive anastomoses; indeed, members of our group published the pioneer paper in 2008[32]. Based on our group's experience using ASCs in experimental and clinical settings (conducting or participating in more than 13 clinical trials) and in digestive surgery, our aim was to review the published literature related to SC use for digestive anastomoses and registered clinical trials. To the best of our knowledge, Caziuc *et al*[33] published the only review focused on this field, including studies published prior to September 2014, and other reviews have dedicated brief sections to SCs, such as those by Foppa *et al*[34] and Reischl *et al*[35].

METHODS AND MATERIALS

Literature search

We performed an exhaustive search of the published literature in the electronic databases from the United States National Library of Medicine (PubMed), Elsevier's Science Direct and Scopus and Cochrane. The United States National Library of Medicine official registry of clinical trials, ClinicalTrials.gov (www.clinicaltrials.gov), and the European Union Clinical Trials Register (www.clinicaltrialsregister.eu) were also searched to identify ongoing or finished registered clinical trials.

The following terms were used: 'anastomosis', 'digestive anastomosis', 'colorectal/colonic anastomoses', 'anastomotic leak', 'stem cells', 'progenitor cells', 'cellular therapy' and 'cell therapy'. Secondary searches were performed with the terms 'biosutures' and 'sutures coated by stem cells' in an attempt to find more publications.

Papers published in indexed peer-reviewed journals in English or Spanish with access to full text since 2000 were included. The last search was run on 10 February 2021.

Eligibility criteria

Only studies employing SCs, performing digestive anastomoses in hollow viscera or digestive perforation sutures and monitoring healing or evolution were finally included.

Study selection

All titles and abstracts were scanned independently in an unblinded standardised manner by two of the reviewers. The 'Similar articles' list in PubMed and bibliographies of the selected studies were also analyzed to find more potentially includable articles. Disagreements between reviewers were solved by consensus.

The full text of selected references was reviewed. The minimal information that must be presented in the study to definitively consider it for this review included at least seven of the following: (1) SCs source; (2) SC characterization; (3) Mode of administration; (4) SCs dosage; (5) Anastomosis technique; (6) The periods of healing assessment; (7) Healing or functional parameters considered to assess the anastomoses; (8) Anastomotic leakage (AL) or rupture frequency; and (9) Whether there is a control group.

Statistical analysis

There is a great variability in the study designs, anastomotic models, interventions (SCs, doses and vehicles) and outcome measures in the selected published literature. Also, many studies do not provide the absolute or relative value of some variables (*i.e.*, anastomotic leaks or dehiscence in each experimental group), so we are not able to aggregate the data to estimate the potential benefit. That is the reason we consider impossible to perform a reliable meta-analysis, so we will focus on describing the studies, their results and limitations, presenting a descriptive or narrative review. We are going to expound data and statistics provided by each publication.

RESULTS

Study selection

Finally, 18 primary references and 3 review articles were eligible for a deeper analysis. PubMed was the fundamental publication source; Science Direct and Scopus did not contain any articles not found previously in PubMed. Moreover, no systematic review has been published in Cochrane.

Briefly, primary PubMed searches provided 272 references; an initial analysis applying eligibility criteria to the titles and abstracts reduced it to 49. Deep abstract content review served to exclude another 20, and duplicate removal left 16 primary sources and 2 review articles. The selected studies' reference lists served to identify another original paper and another review. Secondary term searches allowed us to find the last original research. See Figure 1 for an overview of the search.

The pioneering report in the field was published by Pascual *et al*[32] in 2008. All the primary references are preclinical studies on animal models. There have been neither reported experiences on humans nor registered clinical trials nor publications combining bioengineering and SCs in digestive anastomoses or suturing.

Studies global characteristics

Analyzed anastomoses/digestive sutures: Ten studies are related to colon and colorectal anastomoses (one provides a more detailed description of the methodology of a previous one), 3 to gastric perforations, 2 to small bowel anastomoses, 2 to biliary anastomosis and 1 to oesophageal anastomosis fistula.

Methods of SC therapy and anastomoses/digestive sutures: Related to SCs (Table 1), all the studies, except one employing allogeneic myoblasts, used MSCs harvested from adipose tissue (13) or from bone marrow (3). SCs were identified mostly based on flow cytometry and/or the differentiation ability. The cell transplant was autologous in 8 studies, allogeneic in 7 and xenogeneic in 2 (human). The systems utilised to apply SCs are local injection, cell sheets, biosutures (sutures coated by ASCs), topical, systemic injection, gelatine sponge and luminal stent plus mesh (see Table 2). The employed animals (see Table 3) are mostly rats (12 studies), followed by pigs (4) and rabbits (1).

Three anastomotic models have been described: conventional (4 studies), high risk of AL (8) and insufficient (2). The high-risk models were obtained through 4% icodextrin (1), chemical colitis (1), ischaemia (5), radiation (1) and a cytotoxic (mitomycin C, 1). The study employing mitomycin C applied it simultaneously to inducing ischaemia. Oesophageal insufficient anastomosis was combined with a trans-defect plastic tube for 1 wk to establish the fistula tract. Gastric perforation models either included (2 studies) or did not include (1) repair.

The anastomoses performed were conventional end-to-end in all the publications except one, with usual sutures in a running or an interrupted fashion mostly in a single layer. One study created a functional end-to-end small bowel anastomosis with a high-energy sealing device (this anastomosis is not performed in humans). Gastric perforation suturing was also either running or interrupted.

Outcome measures: Although the maximum follow-up of the subjects was 8 wk, the most frequent evaluation periods were in the first week (9 studies) or during the first month (5).

All the studies evaluated macroscopically the abdominal cavity and/or anastomosis, looking for signs of AL or dehiscence, stenosis, dilatation, peritonitis, *etc*. Some monitored the severity of local or general adherence syndrome. One study employed cholangiogram and another used cervical magnetic resonance imaging (MRI) to look for stenosis or leaks. A colorectal anastomosis study investigated macroscopic inflammation with positron emission tomography (PET) and mucosal changes with colonoscopy. All studies analyzed healing histopathology, with a focus on inflammation, necrosis, collagen deposition, angiogenesis and signs of regeneration.

It is assumed that all the subjects were observed during the postoperative period until the scheduled sacrifice date to detect abnormalities (weight loss, pain, *etc*). Three studies also analyzed blood or serum chemistries and one of them examined the composition of peritoneal lavage fluid.

Eleven out of 17 studies analyzed anastomotic or suture strength with bursting pressure (ABP) evaluation. Briefly, this approach comprises injecting saline (with or without a dye) or air through the sutured segment while monitoring pressure. Bursting pressure is defined as the maximum pressure achieved before leakage is noted at any site. Derived measures are medium bursting pressure (MBP) or bursting

Table 1 Kind of stem cells employed in published studies classified by their origin and type of transplant

Kind of stem cells employed					
Myoblasts	**Bone marrow MSCs**	**ASCs**	**Autologous/syngeneic**	**Allogeneic**	**Xenogeneic**
ALLOG: 1	AUT: 1; ALLO: 2	AUT: 7; ALLOG: 4; XENOG: 2	8	7	2

Numbers indicate the number of published studies. MSCs: Mesenchymal stem cells; ASCs: Adipose-derived stem cells; AUT: Autologous; ALLOG: Allogeneic; XENOG: Xenogeneic.

Table 2 Systems utilized to apply stem cells

SCs delivery system							
Biosutures	**Local injection**	**Systemic injection**	**Local + systemic injection**	**Topical**	**Cell sheets**	**Gelatin sponge**	**Stent + mesh**
2	7[1,2,3]	1	1	2[4]	4	1	1[4]

[1]One study associated thrombin and fibrin.
[2]One study compared local injection with and without fibrin glue.
[3]One study applied stem cells (SCs) in fibrin glue into a stablished fistula.
[4]One study compared topical versus stent and mesh with SCs.
SCs: Stem cells. Numbers indicate the number of published studies.

Table 3 Animal species employed in published preclinical studies

Type of animals		
Rats	**Pigs**	**Rabbits**
12 (9 colorectal, 3 gastric perforation)	4 (small bowel and biliary anastomoses)	1 (esophageal fistula)

Numbers indicate the number of published studies.

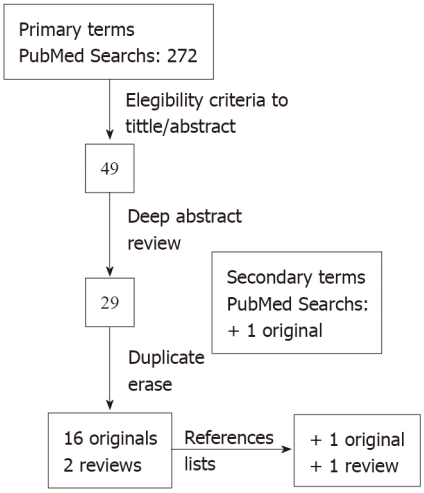

Figure 1 Steps for study selection and final inclusion, presented in a flow diagram.

tension calculated using Laplace's law, in order to identify differences between tissues of different sizes.

Finally, most of the publications, analyzed free and nuclear proteins, surface markers and/or RNA – using immunohistochemistry (IHQ), immunofluorescence (IF), reverse transcriptase polymerase chain reaction (rtPCR), RNA arrays or western blotting – to assess inflammation, angiogenesis, proliferation, fibrosis and cytokine production, among other processes. Some of them also studied SC tracing, proliferation and differentiation capacities.

Results of individual studies

We will summarise the publications ordered by the implicated digestive viscera and by the publication date. A brief overview of the studies is presented in Table 4.

Colon and colorectal anastomoses

The first report was from Pascual *et al*[32] in 2008 and described for the first time SC-coated sutures (named biosutures). Syngeneic (equivalent to autologous) ASCs were obtained from two male BDIX rats. Thirty-centimetre braided polyglactin 910 sutures were cultured with 1.5×10^6 ASCs; ASCs almost completely coated the suture after 24 h and each thread was used for only two stitches. Forty BDIX rats were divided in four groups depending on sacrifice date (4, 7, 14 and 21 d post-anastomosis). Five animals in each group received anastomosis with biosutures and 5 with conventional sutures. Anastomoses consisted of right colon section and end-to-end manual anastomosis with six monoplane interrupted stitches. The authors analyzed colon dehiscence, dilatation or obstruction; an adhesion index; ABP and bursting tension; and histology. Biosutures did not modify the incidence of dehiscence, dilatation, obstruction, the pattern of inflammation and ABP or bursting tension at any time point compared with control sutures. Only the adhesion index was significantly lower with biosutures at day 4 ($P = 0.025$) and 7 ($P = 0.006$), but not at later times.

Going further, the same group published a related study in 2010[36]. First, they modelled a higher leakage risk colonic anastomosis, keeping it adhesion free by intraperitoneal instillation of icodextrin 4%. Biosutures and anastomoses were as described in their previous study[32]. Six BDIX rats receiving biosuture anastomoses and icodextrin were compared to 12 Sprague-Dawley (SD) rats with conventional anastomoses, 6 with and 6 without icodextrin. Animals were sacrificed on postoperative (PO) day 4, and dehiscence, the adhesion index and ABP were analyzed. No significant differences appeared in dehiscence. With conventional sutures and icodextrin 4%, a decrease in the adhesion index ($P = 0.01$) and a lower ABP ($P = 0.15$) were observed compared with no icodextrin. When adhesion-free (icodextrin 4%) anastomoses were compared, those with biosutures had a higher ABP ($P = 0.008$) with a similar adhesion index ($P = 0.48$). In conclusion, biosutures could improve the strength of adhesion-free anastomoses.

In 2011, Adas *et al*[37] analysed local allogeneic bone marrow-derived MSCs (BM-MSCs) in left colonic anastomoses in male Wistar rats. BM-MSCs were isolated from donor animals and marked with bromodeoxyuridine. The left colon was sectioned 3 cm proximal to the peritoneal reflection and mesocolon vessels 2 cm proximal and 2 cm distal to the section were ligated to establish ischaemia. End-to-end anastomoses were made with eight interrupted inverted 6/0 polypropylene stitches. Twenty animals received 5×10^5 injected BM-MSCs around the anastomosis and 20 received saline solution. Ten animals per group were sacrificed on PO days 4 and 7. ABP, hydroxyproline, histological (necrosis, epithelialisation, inflammatory processes, fibroblastic activity and neovascularisation) and cell tracing analyses were performed. Proliferating cells with the added markers appeared at both postoperative times. The MBP (two times) and hydroxyproline levels were significantly ($P < 0.01$) higher in the presence of BM-MSCs at both time points. No leakage or peritonitis appeared in any animal. At PO day 4, necrosis, epithelialisation, collagen deposition, fibroblast activity and angiogenesis and at PO day 7, necrosis, collagen deposition and fibroblast activity were significantly favourable for healing with BM-MSCs. The authors attributed the favourable observed effects mainly to fibroblastic and angiogenic activities.

The following publication was from Yoo *et al*[38] in 2012, with another model of rat ischaemic colonic anastomoses controlled with Doppler flowmetry. Colon division and ischaemia were identical to the previous study[37]. Anastomoses were performed in a single layer, termino-terminal fashion with 6-0 polypropylene sutures (the authors did not describe whether they were running or discontinuous). Blood flow around it was measured using Doppler; further marginal vessel ligation was made until it decreased to < 50% of the normal level. The authors employed male SD rats: some to obtain allogeneic subcutaneous ASCs and 60 to receive ischaemic anastomoses (30 animals) or ischaemic anastomoses plus ASCs (30). A total of 1×10^6 ASCs within a mixture of fibrinogen and thrombin were injected at 4-5 points around the anastomosis. Rats were sacrificed on PO day 7. Anastomosis healing was assessed by measuring weight loss, wound infection, AL, mortality, adhesions, ileus, anastomotic stricture, the ABP, histopathology and the microvascular density. No significant differences in wound infection, AL, mortality, adhesions, or ulcer size between the groups were observed. The ASC group had significantly more favourable anastomotic healing and less ischaemic colitis manifestations, including less weight loss ($P < 0.001$) and earlier

Table 4 Overview and concise review of different published studies related to digestive anastomosis/perforations and stem cell therapy in animal models

Ref.	Animal	N	Randomized	Anast/perf model and repair	SC doses and type	SC treatment	Compared to	Effect measure	Follow up	Principal results	Security concerns
Colon and colorectal anastomoses											
Pascual *et al* [32]	Rats (BDIX)	40	No	Right colon section. Interrupted end-to-end	1.5×10^6 SYNG ASCs	20 biosutures	Conventional suture	Surgical evaluation (dehiscence, dilatation, obstruction, adherences). Bursting pressure; Histology	4, 7, 14, 21 d	Lower adhesion index at 4 d ($P = 0.025$) and 7 d ($P = 0.006$). No differences in the other outcome measures	No
Pascual *et al* [36]	Rats (BDIX + SD)	18	No	Identical to high risk: icodextrin. Identical	SYNG ASCs	6 biosutures + icodextrin	Conventional suture +/- icodextrin	Surgical evaluation (dehiscence, adhesion). Bursting pressure	4 d	No differences in dehiscence. Conventional sutures: icodextrin ↓ adhesion and MBP. Icodextrin: Biosuture ↑ MBP with equal adherences	No
Adas *et al* [37]	Rats (WI)	40	No	Ischemic: Left colon section + 4 cm vessel ligation. Interrupted end-to-end.	5×10^5 ALLOG BM-MSCs	20 local injection	Saline solution	Surgical evaluation. Bursting pressure. Hydroxyproline. Histology. SC tracing	4 and 7 d	No leakages, peritonitis, mortality. SCs ↑ MBP (2×) and hydroxyproline. Histology favourable for healing at both timelines. SC survive and proliferate	No
Yoo *et al* [38]	Rats (SD)	60	No	Ischemic: Left colon section + vessel ligation until > 50% flow reduction. End-to-end PLP.	1×10^6 ALLO ASCs	30 local injection + fibrinogen & thrombin	Ischemic anastomoses	Clinical follow-up: Surgical evaluationABP. Histology	7 d	ASCs: ↓ weight loss and earlier weight recovery; ↓ ileus, ulcers and strictures. ↑ MBP. Histology: SCs ↓ inflammation and ↑ collagen and microvascular density.	No
Adas *et al* [39]	Rats (WI)	40	No	Ischemic: Left colon section + 4 cm vessel ligation. End-to-end interrupted	1×10^6 ALLOG BM-MSCs	20 systemic injection	Saline solution	Surgical evaluation. Bursting pressure. Hydroxyproline. Histology. SC tracing	4 and 7 d	No leakages, peritonitis, mortality. SCs ↑ MBP (43%) at 4^{th} but not 7^{th} day. SCs ↑ SS hydroxyproline. Histology SS favourable for healing (4, 7d). SC Survive and proliferate	No
Sukho *et al* [40]	Rats (WI)	60	Yes	Partial right colectomy. Insufficient end-to-end (5 stitches).	XENOG human ASCs	30 sheets wrapping anastomosis	Insufficient anastomosis	Follow-up: Macroscopic evaluation. ABP. Histology	3 and 7 d	ASCs ↓ dehiscence (14% *vs* 71%) at 3 d, abscesses at 7 d and abdominal adhesions at 3 d. ABP ↑ 3 to 7 d, but NSS differences between groups. Labelled cells detected at both periods. Histol: SCs ↑ CD3+ and maintain CD163+ cells at 7 d.	No
Van de Putte *et al* [43]	Rats (SD)	24	No	IrradiatedColon section. Interrupted end-to-end	5×10^6 IV and 2.5×10^6 local. ALLOG ASCs	10 local injection + IV - 7, 10, 20 d	Conventional anastomosis. Irradiation + anast + PBS	PET. Colonoscopy. Histology	4 wk	PET: preop IV ASCs ↓ activity to non-irradiated level. No differences at 4 wk. Colonoscopy: ASCs ↓ necrotic tissue and fibrin and bleeding (??*P*). Histology: SS ASCs ↓ ulcerated area and ↑ number vessels. ↑ M2 macrophages (??*P*).	0/3/3 deaths. No ASCs related
Alvarenga *et al*[44]	Rats (WI)	61	Yes	TNBS colitis. Left colon section. Ent-to-end interrupted	2×10^6 ALLOG ASCs	15 instillation over anastomosis	G1, TNBS colitis. G2, Laparotomy. G3, colitis + anast. G5, colitis + anast + CS	Follow-up: Macroscopic. Histology, IHQ, RNA	7 d	ASCs ↓ mortality to 0% compared to G3/G5 and local complications to 0%. ASCs: ↓ inflammation, tissue damage, myeloperoxidase activity, CD4+ and ED1+ macrophages, apoptosis; and ↑ epithelization (*vs* G5). ASCs: ↓ IFN-γ. TGFβ, IL-17, TNF-α, and MMPs are not ↑ (as in G5), NSS, and equal to G2/G3.	No

Morgan *et al*[45]	Rats (WI)	48	No	Ischemic: Left colon resection + Vessel ligation. End-to-end interrupted. Air checked	1×10^6 XENOG human ASCs	16 ASCs Gelatin sponge wrapping	Anastomosis. Anastomosis + gelatin sponge wrapping	Follow-up: Macroscopic. MBP in situ. Histology, IF, rtPCR. SC tracing	3 and 7 d	No mortality/complications. ASCs: ↓ AL and abscesses (3, 7 d); ↓ adhesions (3 d). No changes in MBP. ASCs ↑ collagen and microvascular density. Labelled cells in submucosa and muscularis. No SS differences in rtPCR.	No
Small bowel anastomoses											
Maruya *et al*[46]	Pigs	7	Yes (anast)	High risk: vessel ligation + local mitomycin C. 8/animal. Multilayer end-to-end	AUT ASCs	28 anastomoses wrapped with 3 ASCs sheets	Anastomosis without sheets	MBP, histology and hydroxyproline (5, 7d). mRNA (1, 7d)	1, 5, 7 d	ASCs: MBP ↑ at 7 d, similar to normal healing. ASCs ↑ hydroxyproline at 7 d. ASCs ↑ submucosal collagen 7 d (??*P*). ASCs: ↑ FGF2, COL1A1 and COL3A1 day 1 and COL1A1 and COL3A1 day 7.	No
Pan *et al*[47]	Pigs	16	No	5/animal. Section. Functional end-to-end (energy sealing device)	0.5×10^6 ALLO ASCs	8 × 5 anastomoses. Local injection	Anastomosis without ASCs	Follow-up: Macroscopic. MBP. Histology, IHQ, IF, western, PCR arrays. SC tracing	7, 14 d	NSS in complications/leakage and MBP. ASCs: Reepithelialization and ↑ collagen at 7 d (??*P*). ASCs ↑ proliferation, and ↓ CDH1, SMAD3, STAT3, TGF-α, VEGFA. Labelled cells in mucosa.	1 death in ASCs (ileus)
Digestive (gastric) perforations											
Komiyama *et al*[48]	Rats (WI)	40	No	Greater curvature incision. Block continuous suture	1×10^7 AUT ASCs	20 local injection	PBS local injection	Histology day 7 (n = 5), day 28 MBP, day 7 (n = 5) SC tracing	7 and 28 d	Labelled cells at 7, 28 d without differentiation. ASCs ↑ neovascularity and connective tissue at 7 d and ↓ connective tissue at 30 d. MBP ↑ 7 d with ASCs.	No
Liu *et al*[49]	Rats (SD)	108	No	2 cm body incision. Interrupted suture	5×10^6 AUT ASCs	24 local injection. 24 topical in fibrin glue	Sham operated. PBS injection. Topical fibrin glue	Macroscopic. Histology. IHQ, IF, western. SC tracing	3, 5, 7 d	Injected ASCs ↓ severe adhesions (3, 5, 7 d), dehiscence (3 d), abscesses (7 d). 20% total healing at 7 d (*vs* 0%). ASCs ↑ MBP (5, 7 d). Injection the highest values (comparable to sham operated at 5 d). ASCs ↓ inflammation and ↑ granulation (5, 7 d, ??*P*), more with injections. Injected ASCs ↓ IL-6 (day 5, 7) and ↑ TGFβ1 (day 3, 5). Label+ cells submucosa/granulation, differentiation+.	No
Tanaka *et al* [50]	Rats (SD)	30	N	5 mm incision. No suture	ALLO myoblasts sheet	15 sheet placed with shifter	No suture	Macroscopic (adhesion). Blood and ascites. Histology. SC tracing	3, 5, 10, 20 d	Sheets ↓ adhesions in all periods. Histology: sheets regenerated mucosa and muscle; control connective tissue (??*P*). Myoblast in gastric wall. ↓ SS peritoneal fluid hyaluronic acid (??*P*) all periods.	No
Oesophageal anastomotic leakage/fistula											
Xue *et al* [51]	Rabbits	21	No	Transection, incomplete anast, tube during 7 d.	2×10^6 AUT MSCs	12 MSCs in fibrin sealant in fistula	9 fibrin sealant	Cervical MRI (5 wk). Macroscopic, histology, IF, cytokine at 8 wk. SC tracing	5, 8 wk	MRI: ↓ inflammatory reaction MSCs. Macroscopic: ↑ closure and ↓ infection MSCs. Histology/IF: MSCs survive & differentiate. Milder inflammation and less collagen (??*P*) with MSCs. MSCs: ↑ IL-10, MMP-9 and ↓ TNF-α, TGF-β.	5/9 control, 3/12 MSCs died (NSS)
Biliary anastomoses leakage/stenosis											
Zhang *et al* [52]	Pigs	9	No	CBD transection. Running sutures	4×10^6 AUT ASCs	3/3 stent + mesh with ASCs. Topical ASCs	3 plastic stent + vycril mesh	Serum BQ (0, 7, 30 d). Cholangiogram 30 d. Histology, IHQ and IF 30 d	0, 7, 30 d	No clinical/laboratory suggesting cholestasis. No leaks/stenosis on cholangiogram (??*P*). Topical ASCs ↑ SS CD44, CD34 (MSCs) and CD31 (angiogenesis) and ↓ fibrosis and inflammation (??*P*).	1 death (ASCs + mesh) – cholangitis
Hara *et al* [53]	Pigs	11	No	Hepatic conduct section. End-to-end running (post)/interrupted (ant)	AUT ASCs	6 ASCs sheets around anastomosis	5 anastomosis without sheets	Blood (0, 7, 14 d). Macroscopic, histology at 14 d	0, 7, 14 d	No leakages, abscesses, mortality, lab cholestasis. Macroscopic: CBD diameter higher in controls due to wall thickening. Histology: ↓ inflammation, collagen and ↑	No

	small vessels with ASCs (??*P*)

Only statistically significant or highly relevant results are shown, the last remarking their statistical value. SCs: Stem cells; MSCs: Mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; ASCs: Adipose-derived stem cells; IV: Intravenous; MBP: Medium bursting pressure; IHQ: Immunohistochemistry; IF: Immunofluorescence; PCR: Polymerase chain reaction; N: Number; anast: Anastomosis; Perf: Perforation; AUT: Autologous; ALLOG: Allogeneic; SYNG: Syngeneic; XENOG: Xenogeneic; SD: Sprague-Dawley; WI: Wistar; SSF: Saline solution; NSS: Non-statistically significant; SS: Statistically significant; ??*P*: No statistics provided; PLP: Polypropylene; CS: Culture solution.

weight recovery, less ileus (P < 0.05) and fewer ulcers and strictures (P < 0.05). ASCs augmented the ABP (153.92 ± 46.13 mmHg *vs* 121.31 ± 35.99 mmHg, P < 0.01). The histological analysis revealed that the ASC group had less inflammation (P < 0.01) and more collagen deposition (P < 0.05) and microvascular density (P < 0.05). The authors considered angiogenesis as the principal explanation for their positive findings.

In 2013, Adas *et al*[39] published a study with an identical methodology to their previous one[37]; the only change is that 1 × 10^6 BM-MSCs were injected very slowly into the vena cava and control groups received physiological saline. Viable and proliferating cells with the added labelling appeared at both postoperative times. The MBP was significantly (P < 0.01) higher with BM-MSCs at PO day 4 (48.5 *vs* 69, a 43% increase) but not significantly at PO day 7. Hydroxyproline levels were significantly higher in the SC group at both time points (P < 0.01). No leakage, peritonitis or mortality appeared. The histological findings are almost superposable to their previous publication[37]: at PO day 4, necrosis, epithelialisation, collagen deposition, fibroblasts activity and angiogenesis, and at PO day 7, necrosis (less) and collagen deposition (more) were significantly favourable for healing with BM-MSCs. The authors attributed the results mainly to paracrine effects and angiogenesis.

Sukho *et al*[40] published in 2017 a study with ASC sheets in a model of CAL. Human ASCs were isolated from subcutaneous abdominal fat, creating a sheet from each donor. Sixty male Wistar rats were randomly allocated to four groups with 15 animals each: two groups received ASC sheets and two were not reinforced. The authors employed the CAL experimental model from Wu *et al*[41], consisting of a partial colectomy near the caecum and an insufficient end-to-end suturing with five one-layer inverting interrupted stitches with 8/0 polyamide. In the therapeutic groups, one ASC sheet was wrapped around the anastomosis. Two groups were sacrificed after 3 d and the others after 7 d. Evaluation consisted of *in vivo* follow-up (weight and wellness score), macroscopic observation [peritonitis, adhesions, abscesses and anastomosis (stricture, disruption, adhesion, abscess)], air ABP and histology. No differences between groups appeared during *in vivo* observation. In intra-abdominal evaluation, there were significant differences in anastomotic disruption favourable to ASCs (14% *vs* 71%, P = 0.002) at PO day 3 but not at PO day 7. Significantly more rats in the control group had anastomoses abscesses at PO day 7 (P = 0.04) and the abscess scores were lower with ASCs at PO day 7 (P = 0.048). There were also fewer intra-abdominal adhesions at PO day 3 (P = 0.043). The ABP increased between PO days 3 and 7, but there were no significant differences; on day 7, bursting occurred predom-

inantly in the anastomosis in controls (66%), whereas in the ASC group bursting appeared mostly (57%) out of it. Labelled cells were detected at PO days 3 and 7. Regarding histology, there were no differences in vessel density and collagen deposition between the groups and no endothelial cells with human markers appeared in the ASC groups. A significantly higher (P = 0.001) number of CD3+ cells appeared in the ASC group at PO day 7, and the level of CD163+ (M2 macrophages) did not decline between PO days 3 and 7 compared with controls. The authors defended sheets as a cell delivery system and postulated paracrine healing promotion as the principal mechanism of action. They published later a more detailed explanation of ASC sheet creation and surgical protocol[42].

In 2017, Van de Putte *et al*[43] published an evaluation of allogeneic subcutaneous ASCs on colonic anastomoses after high-dose irradiation in rats. Thirty-two SD males received 27 Gy irradiation of the colorectal region. Four weeks later, the damaged zone was identified, the colon was cut just above it and end-to-end anastomosis was performed with interrupted 6/0 polydioxanone stitches leaving knots outside. Three experimental groups were defined: G1, control/sham (n = 4), anastomosis after sham irradiation; G2, phosphate-buffered saline (PBS) (n = 10), irradiation, anastomosis and PBS injections; and G3, ASCs (n = 10): irradiation, 5 × 10^6 intravenous (IV) ASCs 1 wk before anastomosis, intraoperative injection of 5 × 10^6 ASCs around anastomosis and two other IV doses on PO days 10 and 20. In G2 and G3, 3 animals died postoperatively. 18F-fluorodeoxyglucose PET scans were taken just before surgery (4 wk) and PET and colonoscopy were performed at 8 wk when animals were sacrificed to obtain samples for histology. With colonoscopy, G2 anastomoses presented large amounts of necrotic tissue and fibrin, which were less frequent in G3; bleeding appeared in 0% G1, 57% G2 and 14% G3 animals (no P value provided). Regarding *histology*, the ulcerated area was statistically smaller in G3 compared to G2 (P < 0.05). For PET scans, isolated anastomoses (G1) did not generate a significant activity change; irradiation increased it 65%; and IV ASCs prior to anastomoses reduced activity by 21%, making it similar to G1. While G2 had greater values than G1 (P = 0.03), there was no difference between G2 and G3 at 8 wk. At 8 wk, G3 had the highest percentage of M2 macrophages compared with G2 and G1 (no p value provided) and the G3 vessel number was significantly increased (P = 0.007) compared with G2, reaching a value even higher than that of G1. The authors proposed that the observed benefits are probably due to the stimulation of endogenous cells.

Alvarenga *et al*[44] (2019) investigated topical allogeneic ASCs in high-risk colonic anastomosis in Wistar rats randomly assigned to the following groups: G1, 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis (n = 11); G2, laparotomy (n = 11); G3, laparotomy and anastomosis (n = 14); G4, TNBS-colitis followed by anastomosis and ASCs (n = 15); and G5, TNBS-colitis, anastomosis and acellular culture solution (CS, n = 15). Endoscopic colitis was required at 7 d to receive ASCs or CS. The descending colon 4 cm over the rectum was transected without ligating vessels, and then an end-to-end anastomosis was performed using 6-0 polypropylene interrupted stitches. Immediately after, a solution with 2 × 10^6 ASCs or CS was applied onto the external surface of the anastomosis. One week later, anastomotic area macroscopic, histologic, IHQ and RNA analyses were performed. No postoperative deaths occurred in G4 compared with 27% (G5) and 7% (G3), (P = 0.028). No local complications (fistula, abscess, peritonitis) appeared in G4 compared with G5 (53%) and G3 (14%), P = 0.012. In G4, an overall decrease in the histological score, including inflammation improvement, less tissue damage and clear epithelialisation, was observed compared with G5 (P = 0.011). ASC application decreased collagen deposition (P = 0.003) and preserved goblet cells (P = 0.033) compared with G1; it also decreased myeloperoxidase activity to G3 Levels (P = 0.012), CD4+ T-cells (P = 0.014) and macrophages ED1+ (P = 0.011) in the lamina propria, apoptotic cells (P = 0.008) and NF-κB activation (P = 0.036), all compared to G5. For mRNA expression, there was only a significant difference between G4 (lower) and G5 in IFN-γ levels (P = 0.02), but the significant (P < 0.05) overexpression in G5 of TGFβ, IL-17, TNF-α, IFN-γ and metalloproteinases compared with G2 decreased in G4 to G2 or G3 values. These favourable results for clinically relevant variables (mortality and complications) need to be highlighted.

Morgan *et al*[45] in 2020 evaluated xenogeneic ASCs on ischaemic colonic anastomoses in male Wistar rats. ASCs were isolated from subcutaneous fat of healthy human donors. Rats underwent a 1 cm colectomy 2 cm proximal to the peritoneal reflection. End-to-end anastomosis with interrupted 6/0 polypropylene suture and an air-liquid leak checking was performed and mesocolon vessels 2 cm proximal and distal were ligated. Three groups were created: control (only anastomosis), vehicle-only [anastomosis was wrapped with an absorbable gelatine sponge (gelfoam)]; and ASC (gelfoam containing 1 × 10^6 ASCs). Each group was subdivided in two (n = 8 per

subgroup) depending on the sacrifice date (3 or 7 d). After sacrifice, the abdomen was explored, looking for leakage and assessing abscess and adhesion severity with scales; ABP *in situ* was determined; and the anastomotic site was resected for histology, IHQ, IF and quantitative rtPCR for genes associated with angiogenesis, inflammation and proliferation. There was no mortality or relevant complications during the follow-up. In macroscopic evaluation, ASCs significantly decreased AL compared with the control group at PO days 3 and 7 (25.0% *vs* 100% and 25% *vs* 87.5% respectively; $P = 0.02$ for both) and with the vehicle-only group (87.5% at both time points, $P < 0.01$); and also abscess scores compared with the control and gelfoam groups (PO days 3 and 7) and adherence scores (PO day 3). ASCs increased without significance the *MBP* compared with controls. Regarding histological evaluation, ASCs significantly increased microvascular density and collagen compared with the control and vehicle-only groups ($P < 0.01$) at both time points. IHQ showed that the endothelial marker CD31 was markedly increased at both time points with ASCs (no *P* value). Labelled cells were identified in the submucosa and muscularis. For quantitative rtPCR, although treatment with ASCs markedly increased the expression of VEGF and CD31 and decreased TNFα and IL-1, none of these changed reached statistical significance. The authors attributed the enhanced healing to angiogenesis and did not recommend gelfoam as a vehicle because it produced an undesirable inflammatory reaction.

Small bowel anastomoses

In 2017, Maruya *et al*[46] analysed autologous ASC sheets in a model of high-risk small bowel anastomoses comprising terminal vessel ligation and serosal mitomycin C injection in 7 miniature female pigs. Each animal received eight 2-cm incisions in the anti-mesenteric border of ligated vessels, closed with a layer-to-layer anastomosis with five 5–0 polyglactin 910 sutures. These eight anastomoses were divided randomly into two groups: ASC [each anastomosis was wrapped with three ASC sheets (dosage not clearly defined)] and untreated. One pig was euthanised on PO day 1, two on PO day 5 and 4 on PO day 7. ABP, histology and hydroxyproline at PO days 5 and 7 and mRNA expression of FGF2, TGFβ1, COL1A1 and COL3A1 at PO days 1 and 7 were analyzed. The ABP in the ASC group was higher at PO day 5 (118.5 ± 85.9 mmHg *vs* 146.5 ± 58.8 mmHg, $P > 0.05$) and at PO day 7 (226 ± 87.7 mmHg *vs* 267 ± 49.1 mmHg, $P < 0.05$) making ABP similar to normal healing conditions. Hydroxyproline was significantly higher ($P < 0.01$) in the ASC group at PO day 7 but not at PO day 5. Regarding histology, more submucosal collagen appeared with ASC at PO day 7. ASCs significantly increased the mRNA levels of FGF2, COL1A1 and COL3A1 at PO day 1 and of COL1A1 and COL3A1 at PO day 7. The authors attributed the effects to paracrine-enhanced collagen synthesis.

Pan *et al*[47] combined tissue fusion technology with allogenic ASCs in their 2020 publication. Sixteen pigs were divided in two groups related to the sacrifice date (7 or 14 d) and each group was subdivided in an ASC-treated or a control subgroup ($n = 4$ each). Five anastomoses were created per animal using LigaSure ForceTriad (Covidien, MA, United States) in a functional end-to-end format. Five subserosal injections at each anastomotic site containing vehicle solution with or without 5.0×10^5 ASCs were added. Daily vigilance, the surgical site, the abdominal cavity and the anastomoses were checked; an abscess or dense adhesion was considered AL signs. ABP, histology, IHQ, IF, western blot and PCR arrays (only at PO day 7) were analysed in each anastomosis. Only one animal died (from ASC group) due to ileus and there were no significant differences in postoperative complications and AL (1 and 1 in the ASC group and 1 and 2 in the control group at 7 and 14 d, respectively) between groups. The *MBP* was not significantly different among the groups. Regarding histology, total re-epithelialisation and more connective tissue appeared in the ASC group (no p value provided), with no differences in neovascularisation, inflammatory cell infiltration and arrangement of collagen fibres. Proliferating cell nuclear antigen (PCNA) was significantly higher in the ASC group ($P = 0.021$). Labelled cells were found in the mucosal layer, and in the muscularis mucosae exhibited smooth muscle cell characteristics. Western blotting showed that ASCs did not influence CD31, VEGF and FGF2 expression. Eighty-four key genes critical for wound healing were assessed in 3 animals per group with *PCR* arrays; compared with the control group, 10 were upregulated and 75 were downregulated in the ASC group. Five of these changes were statistically significant ($P < 0.05$): CDH1, SMAD3, STAT3, TGFα and VEGFA. The authors attributed the observed effects to paracrine activity and also highlighted ASC migration, differentiation and safety even in the thermally fused tissues.

Digestive (gastric) perforations

These digestive sutures or defects are also prone to leakages, modelling AL.

In 2013, Komiyama *et al*[48] published a study with an incision in the gastric greater curvature of 40 male Wistar rats closed with a single-layer continuous 6/0 polypropylene suture. Twenty animals received 1.0 × 10^7 autologous ASCs injected in the submucosa around the suture and the other 20 received PBS. Ten animals in each group were sacrificed at PO days 7 and 28. Histological evaluation included assessment of necrosis, epithelialisation, inflammation, neovascularisation and fibroblastic activity; the BP was measured in 50% of the animals sacrificed at PO day 7. Labelled ASCs were detected at PO days 7 and 28 in the submucosa, but no differentiation was observed. For histology, at PO day 7 neovascularity and connective tissue were significantly denser ($P < 0.01$) in the ASC-treated animals. By contrast, at PO day 28, connective tissue was significantly reduced ($P < 0.01$). The MBP was higher with ASC treatment (291 ± 14.8 *vs* 121 ± 30 mmHg, $P < 0.01$). The authors proposed that paracrine mechanisms explain the enhanced healing with accelerated angiogenesis and fibrosis (early period) and the excessive fibrosis prevention (late period).

In 2015, Liu *et al*[49] explored local autologous ASCs in female SD rats that received a 2-cm vertical incision at the gastric body closed with five 5/0 interrupted non-absorbable sutures. Four groups of 24 animals were created receiving: G1, 5 × 10^6 ASCs injected in the submucosa around the suture; G2, the same SC dosage on fibrin glue and applied topically; G3, submucosal injection of PBS; and G4, topical fibrin glue. A sham-operated group (only laparotomy, $n = 12$) was also employed. Animals were sacrificed at PO days 3, 5 and 7, and macroscopy, histology, BP, re-epithelialisation, angiogenesis and inflammation (IL-6 and TGFβ1) were assessed. Injected ASCs promoted healing: severe adhesions decreased significantly at the three time points, dehiscence decreased (significantly at PO day 3); no abscesses appeared at any time point (significant at PO day 7); and 20% of the G1 animals appeared completely healed at PO day 7, but none in the other groups. G1 achieved the highest pneumatic ABP at PO days 3 and 5, with significant differences in favour of G1 and G2 compared with G3 and G4; G1 had similar values to the sham-operated group at 5 d. Regarding histology, the ASC groups displayed reduced inflammation (less neutrophils) and increased granulation and re-epithelialisation at PO days 5 and 7, being better in G1. G1 showed significantly decreased IL-6 (PO days 5 and 7) and increased TGFβ1 (PO days 3 and 5). No differences appeared in angiogenesis, VEGF and COX-2. Transplanted ASCs were detected in submucosa and granulation tissue at PO days 3, 5 and 7. At PO days 14 and 21, their morphology changed and they expressed smooth muscle cell markers at PO day 21. In conclusion, ASC injection was more effective than topical administration, and the anti-inflammatory role of ASCs and the earlier onset of granulation enhanced healing.

Tanaka *et al*[50] in 2017 established a new perforation model (5 mm incision in the anterior gastric wall) and evaluated the capacity of allogeneic myoblast cell sheets to contain the leakage. They evaluated 30 male SD rats, 15 receiving a cell sheet and 15 (controls) in which the gastrotomy was not treated at all. The number of implanted myoblasts is not specified. Animals were killed on PO days 3, 5, 10 and 20. Outcome measures were an adhesion severity score (from 0 to 4) to measure peritonitis, blood and ascites fluid exams and histology. Related to adhesions, at all PO time points, cell sheet group had significantly lower score (1-1.5 points difference), and the area with adherences were also lower. Regarding histology, in therapeutic group a regenerated mucosa lined with muscle was found whereas in controls dense connective tissue and discontinuity in all layers appeared; transplanted cells were detected at the gastrotomy site. No differences were found in serum C reactive protein but, in contrast, hyaluronic acid (an inflammatory marker) levels in the peritoneal washing lavage were significantly lower at every time point in the cell sheet group (no *P* value provided). The authors speculated that the effects might be due to paracrine factors and partly to the physical coating effect of the sheet.

Oesophageal AL/fistula

In 2019, Xue *et al*[51] evaluated autologous BM-MSCs in subacute AL in New Zealand rabbits. The AL model comprised cervical oesophagus transection, incomplete anastomosis leaving 2 mm without suturing and a polyethylene tube through the wound and anastomosis defect, maintained for 1 wk. 2 × 10^6 MSCs in 0.2 mL fibrin sealant were injected onto the fistula of 12 animals; 9 animals received only fibrin sealant. The evaluation included cervical MRI at 5 wk by a blinded radiologist and anastomosis macroscopy, histology, IF and cytokine expression at 8 wk. MRI revealed decreased inflammation with MSCs (25% *vs* 88.9% infection/abscess, $P = 0.008$). For

macroscopic evaluation, the MSC group presented a higher closure rate (83.3% *vs* 11.1%, $P = 0.02$) and lower infection rate (33.3% *vs* 88.9%, $P = 0.02$). Although there were no significant differences, 5/9 animals in the control group and 3/12 in the MSC group died of sepsis. Histology and IF showed that MSCs persisted in the fistula tract and submucosa and they expressed myofibroblast markers; less inflammation and collagen (but better organized) were observed in the MSC group (no *P* value provided). Cytokine analyses revealed significant increases in IL-10 and MMP-9 whereas TNF-α and TGF-β decreased significantly in the MSC group (all $P < 0.05$). These findings suggest paracrine suppressing effects on inflammatory response and fibrosis.

Biliary anastomoses leakage/stenosis

Two studies were published in 2020 with autologous ASCs. The first one is from Zhang *et al*[52]. Nine domestic white pigs were divided in three groups: G1 (control) received plastic biliary stents wrapped with Vicryl (polyglactin 910) mesh; in G2, 4×10^6 ASCs were added to the mesh; and G3 received non-wrapped stents and 4×10^6 ASCs applied topically. Surgery involved common bile duct (CBD) transection, posterior wall suturing with a running 7/0 PDS suture, stent luminal insertion and anterior wall closure in a similar fashion; fascia around the CBD was closed with a running 1/0 PDS suture. In G3, CBD stumps were immersed for 10 min in ASC suspension and after suturing, additional ASC suspension was placed in a pocket created in CBD fascia. Serum was collected on PO days 0, 7, and 30 for biochemistry. On PO day 30, cholangiograms and anastomotic specimens were obtained for histology, IHQ and IF. One pig in G2 died on PO day 3 due to acute cholangitis; the others had no complications. The surviving animals had no symptoms or abnormal liver biochemistries suggesting clinical biliary strictures. Cholangiography demonstrated no leaks or stenoses and minimal luminal narrowing (3/3, 1/3, 2/3 in G1, G2 and G3, respectively, no *P* value provided). G3 showed greater CD44 and CD34, indicating ASC engraftment and significantly ($P < 0.05$) reduced fibrosis compared with G1/G2 and enhanced neo-angiogenesis (higher CD31 compared with G1/G2). Other proinflammatory and fibrotic cytokines were also reduced (no *P* value provided). ASC engraftment correlated with fibrosis and inflammation reduction and increased neo-angiogenic areas. Extraluminal immersion seems safer than ASC-coated stents.

Hara *et al*[53] used autologous ASC (2.6×10^6/dish) sheets in pigs. The CBD proximal to the cystic duct was sectioned and anastomosis was performed with 6/0 absorbable monofilament, the posterior wall in a running fashion and the anterior with interrupted suturing. Six animals received one ASC sheet wrapping anastomosis and 5 were controls. Blood samples were obtained on surgery day and after 7 and 14 d; anastomosis areas were collected at PO day 14. Macroscopic changes, inflammatory cells and collagen content were evaluated. Labelled ASCs remained around the CBD wall ($n = 1$). For macroscopic evaluation, there were no leakages or abscesses; adhesions around the liver hilum were more severe in controls (grade ≥ 2: 80% *vs* 17%, but $P = 0.07$). The CBD diameter was larger in the control group ($P = 0.02$) due to thickening of the wall ($P = 0.02$). No laboratory cholestasis appeared in either group. Regarding histology, more inflammatory cells and collagen fibres thickening the wall appeared in the control group, while the ASC group showed fewer inflammatory cells and many small vessels (without statistical analysis). Thus, ASC sheet reduced hypertrophic changes at PO day 14, but long-term follow-up is required to know if this could prevent strictures.

Brief analysis of these publications

There is an important heterogeneity in the anastomosed/sutured viscera and in anastomotic models (high risk, conventional, insufficient) and employed materials; however, the procedures are technically similar (mostly manual end-to-end). The follow-up was sufficient to include the vast majority of clinical AL, but more studies assessing late leakages are needed. Random assignation of treatments was applied only in 3 publications and blinded evaluations were scarce; these factors represent important sources of biases and confounding factors.

Regarding SCs, the variability appears in the SC delivery system – the most frequent are local injection (7) and cell sheets (4) – and dosage (5×10^5 to 1×10^7).

All investigations confirmed the safety and absence of relevant adverse events attributable to SCs. It must be highlighted the relatively low severe complications rate and the very low mortality reported (mortality appears principally in oesophageal fistula and radiated colorectal anastomoses studies), probably due to the animals employed: they are less sensitive to AL-related sepsis than humans.

In general, good and encouraging morphological (mainly histological, nearly all the studies), functional (based on the MBP, 8 studies positive and 3 without effect) and even clinical results have been observed as well as some data suggesting regeneration. Clinically, five studies[40,44,45,49,51] reported significant lower AL incidence, five[32,40,45,49,50] reported fewer adhesions, four[40,45,49,51] fewer abscesses and one less mortality[44]. Eight studies[37,39,45,47-51], analyzed SC labelling and confirmed SC survival in this potentially septic area.

DISCUSION

Despite technical advancements and focused research for decades, anastomotic healing still fails much more than is desirable, producing ALs. Anastomotic healing is classically divided in three phases that overlap: (1) Inflammatory: the haemostatic clot forms a matrix that fills the gaps between the edges and the inflammatory infiltrate arrives. A timed shift from pro- to anti-inflammatory signalling, comprising a phenotypical switch of immune cells, is important to restrict inflammation to a physiological limit; (2) Proliferative: fibroblasts migrate to the focus, proliferate and produce collagen that stabilises the anastomosis, so the suture begins to stop being the fundamental support; and (3) Maturation or remodelling: full mechanical resistance is restored by remodelling the collagen type and fibres.

Growth factors, cytokines and cell-to-cell connections mediate communication between immune and matrix-forming cells. Collagen degrading enzymes, or MMPs, are highly active during early healing and must be tightly regulated[54]. In the colorectal area, the microbiome is also a relevant component: certain microbial stems directly increase MMP activity while other populations seem to have protective functions[55].

Besides this knowledge, AL in certain cases is not yet clearly understood. Classical surgical principles for successful digestive anastomosis are a well-nourished patient with no systemic illness, no faecal or purulent contamination, adequate exposure and access, gentle tissue handling, absence of tension and distal obstruction, approximation of well vascularised bowel ends and meticulous surgical technique. However, even if all these are accomplished, AL could appear. The early healing phase is the most dangerous because AL most often occurs during the first week.

Based in the anastomotic healing physiology, many approaches are currently at different stages of the translational research process attempting to reduce AL.

(1) In preclinical stage: Selective inhibitors of MMPs[56]; hyperbaric oxygen therapy [56,57] and induction of the hypoxic adaptive response (with erythropoietin and VEGF)[56] for perfusion deficits; administration of growth factors (the most studied IGF-1 and GH)[56]; and anti-inflammatory therapies are being explored. Individualised bowel preparation, also called bowel preparation 2.0, to reduce selectively certain detrimental flora could become an interesting approach[58]. Finally, as we have seen, SC therapy is also in this stage.

(2) With published clinical application: approaches aiming to seal the suture line and/or avoid microbiome or faecal contact. Among them, gluing[59,60], additional attachment of laminar biomaterials[61] and seaming the staple line (*i.e.* with bioabsorbable laminae or bovine pericardium)[62] have shown promise but have not yet demonstrated positive effects. Temporary intraluminal tubes, such as a transanal tube, seem to lower AL after rectal resection in the published literature[63]; however, more trials are needed and its use is very low. Anastomoses performed with compression devices have shown equivalent AL rates to conventional anastomoses, although the former are associated with more bowel obstructions[64] and require more research.

(3) Currently applied or under evaluation: These approaches include established protective stoma to avoid faecal passage as well as the virtual or ghost ileostomy, a bowel preparation for easy formation of a stoma in the case of AL, under evaluation [65]. Intraoperative anastomosis quality control with fluorescence angiography and flexible endoscopy are also in this stage.

Related to animal models and outcome measures, the principal publications refer to colorectal anastomoses. In their 2011 systematic review, Pommergaard *et al*[66] recommended using mice because they best mimic clinical CAL and rats are relatively resistant to intra-abdominal infections; however, mice use did not increase. A 2015 systematic review including 1342 studies concluded that animal research on AL is of poor quality, explaining the difficult translation to humans[67]. To solve this, an international consensus on the most appropriate animal models and outcome

measures in lower gastrointestinal tract anastomoses research was developed in 2015 based on Delphi analysis; there is no similar consensus on the upper tract. We highlight some of its items[68]: (1) Animal model: Mouse, rat and pig are considered appropriate and rabbit and dog are not validated; rats are preferred to mice; (2) Location and type of surgery: The small intestine should not be used; resection is appropriate, but there is no consensus on transection; all types of sutures or staplers are appropriate; (3) Macroscopic outcomes: AL should always be analyzed; adhesions to the anastomotic site are relevant, but not abdominal cavity adhesions; (4) Histology: Is mandatory in healing studies; there are no specific scores; (5) Mechanical assays: ABP and tensile strength are appropriate; they are comparable within one publication, but often not between different ones; (6) Biochemistry: This technique provides additional information; and (7) Animal testing and welfare: The methodology should be deeply described; randomisation and blinding should be used and the ARRIVE[69] guidelines should be followed as much as possible.

Analyzing the included publications on colorectal and small bowel anastomoses, most of them accomplished the aforementioned recommendations. The animals employed were rats (9) or pigs (2). Two evaluated small bowel anastomoses, which are not considered appropriate due to inherent physiology, the easy healing in animals, the different immunobiology and blood supply compared with humans and the low clinical relevance[68]. All studies included macroscopic and histological evaluation and all except 2[43,44] ABP. Related to randomisation, blinding and ARRIVE guidelines compliance, we found frequent methodological weakness in almost all the studies.

It is important to analyze the highly heterogeneous anastomoses, perforation and fistula models and their clinical correlations. From a technical point of view, the described anastomoses simulate the usual surgical practice, except for the one performed with a high energy sealing device instead of sutures (not used in humans). Animals seem to be more resistant to AL consequences than humans. Incomplete anastomoses models, never constructed in surgery, are more directed to study how to mitigate AL consequences than to analyze AL prevention. Under the extreme conditions of some high-risk models, such as medium-length ischaemic segments, colitis as severe as that induced by TNBS or when cytotoxic medication could not be discontinued; an anastomosis would not be performed in humans. Nevertheless, these models present AL rates that are comparable to humans. Most important, if SCs could be effective in these situations, they would probably be even better in more conventional circumstances.

Perforation models are more open to criticism because the injury is followed immediately by the repair whereas in clinical scenarios, a delay, with peritoneal cavity contamination and wound border inflammation, exists and affects healing. During acute inflammation, some factors that could compromise SC survivorship or effects have been observed (*i.e.* in faecal incontinence[70]), and there are also fundamental cytokines for SC homing and activation[71].

Finally, we discuss the proper SC therapy in this unfavourable environment for SC survival/action (faecal contamination, microbial load, low vascular supply, *etc*).

(1) Regarding SC characterisation, there is a relative heterogeneity in the isolation and characterisation protocols. Hence, there are slight differences in the cellular product composition, a factor that makes it difficult to compare studies. All the studies were published after 2006, when the International Society for Cellular Therapy published their position statement in minimal criteria for defining multipotent MSCs [72]. In addition, most of the studies using ASCs were published after 2013, when similar international standards were published for ASCs[73]. So, isolation protocols must be described more clearly, ideally could be more homogeneous, and publications must specify at least if the minimal international consensus criteria are accomplished.

(2) Another unresolved issue is the best SC delivery system, which influences SC survival, targeting and function in tissues. We analyze systems employed in this field:

The most employed is local injection. SC products can be prepared as simple suspensions (in saline solution, Ringer's solution, *etc*) or combined with biological products (fibrin, thrombin, collagen or gelatine) or biomaterials. SCs could be injected directly into the tissue or sealing a space or fistula within other substances. The delivered doses could be more controlled compared to methods such as biosutures. It is very useful for solid organs or strong structures like skeletal muscle but less useful in thin structures (like some digestive viscera wall) because it is more difficult to apply or exceeds the viscera's capacity. For example, we observed clusters, with SC loss, outside the sphincter in our faecal incontinence experiments[70], and other authors have described insufficient cell retention.

Cell sheet is an advance to improve cell retention and integration. They are prepared on special culture dishes coated with a temperature-responsive polymer that changes from being hydrophobic to hydrophilic when the temperature is lowered. Sheets can be removed as one piece without enzymatic treatment, preventing destruction of cell interactions and with intact extracellular matrix[74,75]. They have been used successfully to improve healing in several fields (*i.e.* heart, trachea, skin, cornea)[76]. In digestive anastomoses/sutures, sheets spontaneously adhere to the serosa rapidly and may help to seal the anastomosis[40]. Future studies will clarify if this approach could be better than injections.

Our group developed biosutures[32] aiming to place SCs directly at the injury and to improve engraftment rates. We applied them in colorectal[32,36] and tracheal[31] anastomoses or anal sphincters[70]. They have been applied mainly in tendon repair [77] but also in organs such as the heart[78,79]. No evidence exists about the best dose or the minimal 'clinically active'. With 1.5×10^6 ASCs, we found that SCs tend to form 'clusters' over the suture, in culture medium and remained adhered after their use [70]. Some modifications have been proposed: to improve cell adherence, Yao *et al*[80] added poly-L-lysine and fibronectin; Horváthy *et al*[81] covered previously sutures with albumin; and Casado *et al*[82] employed pre-treatments with gelatine and NaOH. Muraoka *et al*[83] added growth factors such as myostatin. Other authors have tested sutures solely impregnated by platelet-rich plasma[84,85] or VEGF[86] with interesting results. Therefore, more studies on biosuture preparation and potential adjuvants are needed.

Topical administration has the disadvantages of poor control of the actual administered SC dose and the very high inter-individual variability.

Systemic (IV) administration has the problem of actual homing. Many studies have described high SC homing to injury foci but others have described very low homing [87]. Directing all administered SCs to the injury, avoiding homing to other organs, seems to be very difficult to achieve.

Other potential approaches are to combine SCs with biomaterials or add SCs to mechanical anastomosis devices (i.e. to staple line reinforcements).

(3) Regarding SC doses, more publications are needed to define the best dose or at least a minimal value in which therapeutic effects appear.

(4) Another important issue is SC survival in the anastomotic area. The 8 studies analyzing whether there were cells with different SC markers were able to detect them. Nevertheless, there are contradictory findings in similar fields like faecal incontinence, with some studies not able to find cells with SC markers[88,89].

(5) There are many remaining questions concerning the mechanism of action of SCs. We are going to focus on MSCs. It is possible that other SCs, such as myogenic SCs, have a greater role based on differentiation, but MSCs probably base their function mostly on immunomodulation, anti-inflammatory and angiogeneic capabilities, reducing fibrosis and stimulating resident progenitor cells as all the included studies mention. The immunomodulatory capability of MSCs is based on inhibition of T cell and B cell proliferation and dendritic cell maturation[90] and in the secretion of a large number of cytokines[91]. As some examples, Németh *et al*[92] observed that MSCs attenuated sepsis by macrophage reprogramming to increase IL-10, a cytokine that decreases neutrophil migration and Georgiev-Hristov *et al*[31] found an early change from acute to chronic inflammation with ASCs (neutrophil descent and macrophage increment) in tracheal anastomosis.

To improve SC survival and function in tissues, different strategies have been employed: (1) Combine SCs or their vehicles with cytokines and growth factors, for example, through SCs plasmid transfection or stimulating local production using surgical injury or electricity[93-95]; (2) Induce the expression of paracrine factors (*i.e.* angiogenic or growth factors) by SC genetic modification, which has been successfully used in various animal models of diseases[96]; and (3) Use MSC exosomes, which are nanoscale extracellular vesicles fundamental in intercellular communication and could be responsible for multiple MSCs therapeutic effects. Exosomes can be used to modify MSC functions[97] and open the field of a novel SC-derived, cell-free therapy[98].

To achieve true 'regeneration' of anastomotic tissue with SCs, we need to teach them to differentiate efficiently. Then, we must integrate them in an appropriate delivery system. Finally, a blood supply and innervation need to be generated to allow their integration in the whole organ.

(6) The last critical question is about safety. Preclinical studies and the published clinical experiences have confirmed an adequate safety profile. Our teams have participated in 13 clinicals trials with more than 500 patients receiving autologous or allogeneic local ASCs in digestive fistulising diseases[23-26]; this research has led to the marketing authorization of the first human SC therapy by the European Medicines

Agency, darvadstrocel.

Although there are many potential side effects using SCs, the most worrisome is a possible role in carcinogenesis. We are going to focus on MSCs. Some researchers have observed that MSCs cultured for a long time may develop malignant changes and even tumours in mice[99]. However, subsequent publications attributed those findings to tumour cell cross-contamination[100,101], other studies did not detect it under extreme culture conditions and it has never been observed *in vivo*. The relationship between SCs and tumours is contradictory, as has been reviewed by Ramdasi *et al*[102] and Timaner *et al*[103]. MSCs have enhanced tropism towards tumours and pro-tumour (growing, angiogenesis, immunomodulation, *etc*)[104,105] and anti-tumour (apoptosis, proliferation inhibition, *etc*)[106] properties. This relationship depends on factors like the type of MSCs; the type of cancer cells; *in vivo* or *in vitro* conditions; the MSC secretome; and interactions between MSCs, host immune cells and cancer cells. A possible key factor is related to time: when MSCs are administered with an existing tumour, a suppressive effect has been observed[107], but in some studies with co-administration, tumour growth was higher[108]. Tropism to tumours has been exploited for therapy in experimental models, as reviewed by Chulpanova *et al*[109] and Babajani *et al*[110], and in some preliminary clinical trials[111]. In conclusion, the accumulated preclinical and clinical experience seems to warrant the oncogenic safety of MSCs, but more studies and more long-term follow-up are needed to exclude definitively all the risks.

Regarding other complications, the first clinically severe adverse events potentially relatable to SCs have been reported recently. Three women with macular degeneration developed complications, including vision loss, detached retinas and bleeding, after receiving 'ASCs' (it was really stromal vascular fraction mixed with blood plasma and large numbers of platelets) and remained totally blind[112]. Another case of bilateral retinal detachment was reported[113]. The highly controlled environment of clinical trials is imperative to avoid lamentable events like these.

To finalize, the main limitation of this study is its own nature; we have presented a descriptive review because we consider that there are very few published studies and that they are too heterogeneous to perform a systematic review or meta-analysis.

CONCLUSION

AL is more frequent than desirable despite advances in technology and surgery and may have devastating consequences, so alternative approaches are needed to reduce its incidence. SC therapies have the exciting potential to improve anastomotic healing and different strategies have been explored in preclinical studies.

MSCs from adipose tissue or bone marrow have been the most investigated in different animal models. In general, the 18 published studies have confirmed safety and have shown some encouraging morphological, functional and even clinical results.

More knowledge about SCs and healing biology, and more data on preclinical models (related to SC type, dosage, deliver system and adjuvants, among other topics) are needed to establish definitively efficacy and safety prior to testing in humans in rigorously designed clinical trials. Only research and time will determine SC therapy for preventing AL can become a reality.

ARTICLE HIGHLIGHTS

Research background

Digestive tract anastomoses and sutures are prone to leakages even if all the classical surgical principles for a successful anastomosis are accomplished. Leakage rates have remained almost unchanged for the last decades and usually associate high morbidity and mortality. Leakages are usually due to failed healing. Stem cells (SCs) have emerged as a promising tool to enhance healing in a wide variety of experimental and clinical settings, including particularly unfavorable environments such as anal fistulas and Crohn´s disease. Since 2008, SCs have been proven as an alternative to improve anastomoses outcomes.

Research motivation

To know if SC therapy could improve postoperative healing mechanisms in digestive

anastomosis and sutures in the published literature. If this hypothesis is correct, many patients would benefit from better surgical outcomes reducing morbidity and mortality.

Research objectives

To review the published literature related to SC use for digestive anastomoses and sutures and the registered clinical trials. When this manuscript was confected, there was only one published review including studies published prior to September 2014. This is important for possible future investigations on the field.

Research methods

PubMed, Science Direct, Scopus and Cochrane searches were performed using the key words "anastomosis", "colorectal/colonic anastomoses", "anastomotic leak", "stem cells", "progenitor cells", "cellular therapy" and "cell therapy" in order to identify relevant articles published in English and Spanish during the period 2000-2021. The United States and European Union (EU) official registries of clinical trials, ClinicalTrials.gov and EU Clinical Trials Register, were also searched. Studies employing SCs, performing digestive anastomoses or perforation sutures and monitoring healing were finally included. Reference lists from the selected articles were reviewed to identify additional pertinent articles. Given the great variability in the study designs, animal and anastomotic models, interventions (SCs, doses and vehicles) and outcome measures, performing a reliable meta-analysis was considered impossible, so we present the studies, their results and limitations in a descriptive way.

Research results

Eighteen preclinical studies and three review papers were identified; there are no published clinical studies or registered clinical trials. Colon and colorectal anastomoses are the most frequently examined (ten studies) and rats (12 studies) are the mostly employed animals followed by pigs (4). Three anastomotic models have been described: conventional (4 studies), high risk of AL (8) and insufficient (2); gastric perforation models either included (2 studies) or did not include (1) repair. Most analyzed SCs were Mesenchymal (16 studies); cell transplant was autologous in 8 studies, allogeneic in 7 and xenogeneic in 2 (human); SCs dosage ranged from 5×10^5 to 1×10^7 and delivery routes were mainly local injections (7) and cell sheets (4) followed by biosutures (sutures coated by SCs) or purely topical (2 studies each one). Random assignation of treatments was applied only in 3 publications and blinded evaluations were scarce.

Related to outcome measures, the most frequent evaluation periods were in the first week (9 studies) or during the first month (5). All studies evaluated morphologically the abdominal cavity and/or anastomosis or digestive sutures, and eleven out of 17 analyzed anastomotic or suture strength with bursting pressure evaluation.

All investigations confirmed the safety and absence of relevant adverse events attributable to SCs. It must be highlighted the relatively low rate of severe complications and the extremely low mortality rate reported.

In general, good and encouraging morphological (mainly histological, nearly all the studies), functional (8 studies positive and 3 without effect) and even clinical results have been observed as well as some data suggesting regeneration. Clinically, five studies reported significant lower AL incidence, five fewer adhesions, four fewer abscesses and one less mortality. Eight studies analyzed SC labelling and confirmed SC survival in this potentially septic area.

As potential weaknesses, animal models need to be improved to make them more comparable, and the SC isolation processes need to be standardised.

Research conclusions

There is notable heterogeneity in the studies, making them difficult to compare. Further investigations are needed. The future role of SC therapy in digestive anastomoses/sutures still needs to be determined and seems to be currently far from clinical use.

Research perspectives

In the experimental setting SCs applied to digestive anastomosis or perforation healing have been proven to be safe and may be potentially effective. Areas needing further studying would be: Defining the best model of anastomosis healing; Obtaining deeper knowledge about SCs mechanism of action; Improving SC delivery, survival and function (cytokine or molecule addition, *etc.*); Supplying SCs through minimally

invasive methods; Determining the indications, adjuvants, real efficacy and to confirm safety and definitely discard oncological concerns.

This review suggests that more studies on animal models and with better statistical quality are needed prior to human use. Only in this case SC therapy could be tried on humans in highly controlled settings as clinical trials.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge all their research collaborators and previous publications co-authors for their continuous scientific support and collaboration.

REFERENCES

1 **Peel AL**, Taylor EW. Proposed definitions for the audit of postoperative infection: a discussion paper. Surgical Infection Study Group. *Ann R Coll Surg Engl* 1991; **73**: 385-388 [PMID: 1759770]

2 **Rahbari NN**, Weitz J, Hohenberger W, Heald RJ, Moran B, Ulrich A, Holm T, Wong WD, Tiret E, Moriya Y, Laurberg S, den Dulk M, van de Velde C, Büchler MW. Definition and grading of anastomotic leakage following anterior resection of the rectum: a proposal by the International Study Group of Rectal Cancer. *Surgery* 2010; **147**: 339-351 [PMID: 20004450 DOI: 10.1016/j.surg.2009.10.012]

3 **van Helsdingen CP**, Jongen AC, de Jonge WJ, Bouvy ND, Derikx JP. Consensus on the definition of colorectal anastomotic leakage: A modified Delphi study. *World J Gastroenterol* 2020; **26**: 3293-3303 [PMID: 32684743 DOI: 10.3748/wjg.v26.i23.3293]

4 **Kamarajah SK**, Lin A, Tharmaraja T, Bharwada Y, Bundred JR, Nepogodiev D, Evans RPT, Singh P, Griffiths EA. Risk factors and outcomes associated with anastomotic leaks following esophagectomy: a systematic review and meta-analysis. *Dis Esophagus* 2020; **33** [PMID: 31957798 DOI: 10.1093/dote/doz089]

5 **2015 European Society of Coloproctology collaborating group**. The relationship between method of anastomosis and anastomotic failure after right hemicolectomy and ileo-caecal resection: an international snapshot audit. *Colorectal Dis* 2017 [PMID: 28263043 DOI: 10.1111/codi.13646]

6 **Karim A**, Cubas V, Zaman S, Khan S, Patel H, Waterland P. Anastomotic leak and cancer-specific outcomes after curative rectal cancer surgery: a systematic review and meta-analysis. *Tech Coloproctol* 2020; **24**: 513-525 [PMID: 32206962 DOI: 10.1007/s10151-020-02153-5]

7 **Hammond J**, Lim S, Wan Y, Gao X, Patkar A. The burden of gastrointestinal anastomotic leaks: an evaluation of clinical and economic outcomes. *J Gastrointest Surg* 2014; **18**: 1176-1185 [PMID: 24671472 DOI: 10.1007/s11605-014-2506-4]

8 **Zhang W**, Che X. Effect of indocyanine green fluorescence angiography on preventing anastomotic leakage after colorectal surgery: a meta-analysis. *Surg Today* 2021; **51**: 1415-1428 [PMID: 33428000 DOI: 10.1007/s00595-020-02195-0]

9 **De Nardi P**, Elmore U, Maggi G, Maggiore R, Boni L, Cassinotti E, Fumagalli U, Gardani M, De Pascale S, Parise P, Vignali A, Rosati R. Intraoperative angiography with indocyanine green to assess anastomosis perfusion in patients undergoing laparoscopic colorectal resection: results of a multicenter randomized controlled trial. *Surg Endosc* 2020; **34**: 53-60 [PMID: 30903276 DOI: 10.1007/s00464-019-06730-0]

10 **Akamatsu N**, Sugawara Y, Hashimoto D. Biliary reconstruction, its complications and management of biliary complications after adult liver transplantation: a systematic review of the incidence, risk factors and outcome. *Transpl Int* 2011; **24**: 379-392 [PMID: 21143651 DOI: 10.1111/j.1432-2277.2010.01202.x]

11 **Tsujino T**, Isayama H, Kogure H, Sato T, Nakai Y, Koike K. Endoscopic management of biliary strictures after living donor liver transplantation. *Clin J Gastroenterol* 2017; **10**: 297-311 [PMID: 28600688 DOI: 10.1007/s12328-017-0754-z]

12 **Faiella W**, Atoui R. Therapeutic use of stem cells for cardiovascular disease. *Clin Transl Med* 2016; **5**: 34 [PMID: 27539581 DOI: 10.1186/s40169-016-0116-3]

13 **Trebol Lopez J**, Georgiev Hristov T, García-Arranz M, García-Olmo D. Stem cell therapy for digestive tract diseases: current state and future perspectives. *Stem Cells Dev* 2011; **20**: 1113-1129 [PMID: 21187000 DOI: 10.1089/scd.2010.0277]

14 **Gómez-Barrena E**, Rosset P, Müller I, Giordano R, Bunu C, Layrolle P, Konttinen YT, Luyten FP. Bone regeneration: stem cell therapies and clinical studies in orthopaedics and traumatology. *J Cell Mol Med* 2011; **15**: 1266-1286 [PMID: 21251219 DOI: 10.1111/j.1582-4934.2011.01265.x]

15 **Chivu-Economescu M**, Rubach M. Hematopoietic Stem Cells Therapies. *Curr Stem Cell Res Ther* 2017; **12**: 124-133 [PMID: 26496888 DOI: 10.2174/1574888X10666151026114241]

16 **García-Gómez I**, Elvira G, Zapata AG, Lamana ML, Ramírez M, Castro JG, Arranz MG, Vicente A, Bueren J, García-Olmo D. Mesenchymal stem cells: biological properties and clinical applications. *Expert Opin Biol Ther* 2010; **10**: 1453-1468 [PMID: 20831449 DOI: 10.1517/14712598.2010.519333]

17 **Tsuchiya A**, Kojima Y, Ikarashi S, Seino S, Watanabe Y, Kawata Y, Terai S. Clinical trials using mesenchymal stem cells in liver diseases and inflammatory bowel diseases. *Inflamm Regen* 2017; **37**: 16 [PMID: 29259715 DOI: 10.1186/s41232-017-0045-6]

18 **Mizuno H**, Tobita M, Uysal AC. Concise review: Adipose-derived stem cells as a novel tool for future regenerative medicine. *Stem Cells* 2012; **30**: 804-810 [PMID: 22415904 DOI: 10.1002/stem.1076]

19 **Ma T**, Sun J, Zhao Z, Lei W, Chen Y, Wang X, Yang J, Shen Z. A brief review: adipose-derived stem cells and their therapeutic potential in cardiovascular diseases. *Stem Cell Res Ther* 2017; **8**: 124 [PMID: 28583198 DOI: 10.1186/s13287-017-0585-3]

20 **Maxson S**, Lopez EA, Yoo D, Danilkovitch-Miagkova A, Leroux MA. Concise review: role of mesenchymal stem cells in wound repair. *Stem Cells Transl Med* 2012; **1**: 142-149 [PMID: 23197761 DOI: 10.5966/sctm.2011-0018]

21 **González MA**, Gonzalez-Rey E, Rico L, Büscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009; **136**: 978-989 [PMID: 19135996 DOI: 10.1053/j.gastro.2008.11.041]

22 **Gonzalez-Rey E**, Gonzalez MA, Varela N, O'Valle F, Hernandez-Cortes P, Rico L, Büscher D, Delgado M. Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis. *Ann Rheum Dis* 2010; **69**: 241-248 [PMID: 19124525 DOI: 10.1136/ard.2008.101881]

23 **García-Olmo D**, Herreros D, De-La-Quintana P, Guadalajara H, Trébol J, Georgiev-Hristov T, García-Arranz M. Adipose-derived stem cells in Crohn's rectovaginal fistula. *Case Rep Med* 2010; **2010**: 961758 [PMID: 20224798 DOI: 10.1155/2010/961758]

24 **Guadalajara H**, Herreros D, De-La-Quintana P, Trebol J, Garcia-Arranz M, Garcia-Olmo D. Long-term follow-up of patients undergoing adipose-derived adult stem cell administration to treat complex perianal fistulas. *Int J Colorectal Dis* 2012; **27**: 595-600 [PMID: 22065114 DOI: 10.1007/s00384-011-1350-1]

25 **García-Arranz M**, Herreros MD, González-Gómez C, de la Quintana P, Guadalajara H, Georgiev-Hristov T, Trébol J, Garcia-Olmo D. Treatment of Crohn's-Related Rectovaginal Fistula With Allogeneic Expanded-Adipose Derived Stem Cells: A Phase I-IIa Clinical Trial. *Stem Cells Transl Med* 2016; **5**: 1441-1446 [PMID: 27412883 DOI: 10.5966/sctm.2015-0356]

26 **Panés J**, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A, Nachury M, Ferrante M, Kazemi-Shirazi L, Grimaud JC, de la Portilla F, Goldin E, Richard MP, Leselbaum A, Danese S; ADMIRE CD Study Group Collaborators. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet* 2016; **388**: 1281-1290 [PMID: 27477896 DOI: 10.1016/S0140-6736(16)31203-X]

27 **Cheng F**, Huang Z, Li Z. Efficacy and Safety of Mesenchymal Stem Cells in Treatment of Complex Perianal Fistulas: A Meta-Analysis. *Stem Cells Int* 2020; **2020**: 8816737 [PMID: 33299423 DOI: 10.1155/2020/8816737]

28 **Oyama Y**, Craig RM, Traynor AE, Quigley K, Statkute L, Halverson A, Brush M, Verda L, Kowalska B, Krosnjar N, Kletzel M, Whitington PF, Burt RK. Autologous hematopoietic stem cell transplantation in patients with refractory Crohn's disease. *Gastroenterology* 2005; **128**: 552-563 [PMID: 15765390 DOI: 10.1053/j.gastro.2004.11.051]

29 **Ko JZ**, Johnson S, Dave M. Efficacy and Safety of Mesenchymal Stem/Stromal Cell Therapy for Inflammatory Bowel Diseases: An Up-to-Date Systematic Review. *Biomolecules* 2021; **11** [PMID: 33440772 DOI: 10.3390/biom11010082]

30 **Trébol J**, Carabias-Orgaz A, García-Arranz M, García-Olmo D. Stem cell therapy for faecal incontinence: Current state and future perspectives. *World J Stem Cells* 2018; **10**: 82-105 [PMID: 30079130 DOI: 10.4252/wjsc.v10.i7.82]

31 **Georgiev-Hristov T**, García-Arranz M, García-Gómez I, García-Cabezas MA, Trébol J, Vega-Clemente L, Díaz-Agero P, García-Olmo D. Sutures enriched with adipose-derived stem cells decrease the local acute inflammation after tracheal anastomosis in a murine model. *Eur J Cardiothorac Surg* 2012; **42**: e40-e47 [PMID: 22689184 DOI: 10.1093/ejcts/ezs357]

32 **Pascual I**, de Miguel GF, Gómez-Pinedo UA, de Miguel F, Arranz MG, García-Olmo D. Adipose-derived mesenchymal stem cells in biosutures do not improve healing of experimental colonic anastomoses. *Br J Surg* 2008; **95**: 1180-1184 [PMID: 18690635 DOI: 10.1002/bjs.6242]

33 **Caziuc A**, Calin Dindelegan G, Pall E, Mironiuc A. Stem cells improve the quality of colonic anastomoses - A systematic review. *J BUON* 2015; **20**: 1624-1629 [PMID: 26854462]

34 **Foppa C**, Ng SC, Montorsi M, Spinelli A. Anastomotic leak in colorectal cancer patients: New insights and perspectives. *Eur J Surg Oncol* 2020; **46**: 943-954 [PMID: 32139117 DOI: 10.1016/j.ejso.2020.02.027]

35 **Reischl S**, Wilhelm D, Friess H, Neumann PA. Innovative approaches for induction of gastrointestinal anastomotic healing: an update on experimental and clinical aspects. *Langenbecks Arch Surg* 2021; **406**: 971-980 [PMID: 32803330 DOI: 10.1007/s00423-020-01957-1]

36 **Pascual I**, Fernández de Miguel G, García Arranz M, García-Olmo D. Biosutures improve healing of experimental weak colonic anastomoses. *Int J Colorectal Dis* 2010; **25**: 1447-1451 [PMID: 20544210 DOI: 10.1007/s00384-010-0952-3]

37 **Adas G**, Arikan S, Karatepe O, Kemik O, Ayhan S, Karaoz E, Kamali G, Eryasar B, Ustek D. Mesenchymal stem cells improve the healing of ischemic colonic anastomoses (experimental study).

Langenbecks Arch Surg 2011; **396**: 115-126 [PMID: 20953879 DOI: 10.1007/s00423-010-0717-z]
38 **Yoo JH**, Shin JH, An MS, Ha TK, Kim KH, Bae KB, Kim TH, Choi CS, Hong KH, Kim J, Jung SJ, Kim SH, Rho KH, Kim JT, Yang YI. Adipose-tissue-derived Stem Cells Enhance the Healing of Ischemic Colonic Anastomoses: An Experimental Study in Rats. *J Korean Soc Coloproctol* 2012; **28**: 132-139 [PMID: 22816056 DOI: 10.3393/jksc.2012.28.3.132]
39 **Adas G**, Kemik O, Eryasar B, Okcu A, Adas M, Arikan S, Erman G, Kemik AS, Kamali G, Dogan Y, Karaoz E. Treatment of ischemic colonic anastomoses with systemic transplanted bone marrow derived mesenchymal stem cells. *Eur Rev Med Pharmacol Sci* 2013; **17**: 2275-2285 [PMID: 24065218]
40 **Sukho P**, Boersema GSA, Cohen A, Kops N, Lange JF, Kirpensteijn J, Hesselink JW, Bastiaansen-Jenniskens YM, Verseijden F. Effects of adipose stem cell sheets on colon anastomotic leakage in an experimental model: Proof of principle. *Biomaterials* 2017; **140**: 69-78 [PMID: 28628777 DOI: 10.1016/j.biomaterials.2017.06.011]
41 **Wu Z**, Daams F, Boersema GS, Vakalopoulos KA, Lam KH, van der Horst PH, Kleinrensink GJ, Jeekel J, Lange JF. Colorectal anastomotic leakage caused by insufficient suturing after partial colectomy: a new experimental model. *Surg Infect (Larchmt)* 2014; **15**: 733-738 [PMID: 25314010 DOI: 10.1089/sur.2013.197]
42 **Sukho P**, Boersema GSA, Kops N, Lange JF, Kirpensteijn J, Hesselink JW, Bastiaansen-Jenniskens YM, Verseijden F. Transplantation of Adipose Tissue-Derived Stem Cell Sheet to Reduce Leakage After Partial Colectomy in A Rat Model. *J Vis Exp* 2018 [PMID: 30148499 DOI: 10.3791/57213]
43 **Van de Putte D**, Demarquay C, Van Daele E, Moussa L, Vanhove C, Benderitter M, Ceelen W, Pattyn P, Mathieu N. Adipose-Derived Mesenchymal Stromal Cells Improve the Healing of Colonic Anastomoses Following High Dose of Irradiation Through Anti-Inflammatory and Angiogenic Processes. *Cell Transplant* 2017; **26**: 1919-1930 [PMID: 29390877 DOI: 10.1177/0963689717721515]
44 **Alvarenga V Jr**, Silva PTD, Bonfá ND, Pêgo B, Nanini H, Bernardazzi C, Madi K, Baetas da Cruz W, Castelo-Branco MT, de Souza HSP, Schanaider A. Protective effect of adipose tissue-derived mesenchymal stromal cells in an experimental model of high-risk colonic anastomosis. *Surgery* 2019; **166**: 914-925 [PMID: 31519305 DOI: 10.1016/j.surg.2019.07.023]
45 **Morgan A**, Zheng A, Linden KM, Zhang P, Brown SA, Carpenter JP, Spitz FR, Kwiatt ME. Locally Transplanted Adipose Stem Cells Reduce Anastomotic Leaks in Ischemic Colorectal Anastomoses: A Rat Model. *Dis Colon Rectum* 2020; **63**: 955-964 [PMID: 32168095 DOI: 10.1097/DCR.0000000000001667]
46 **Maruya Y**, Kanai N, Kobayashi S, Koshino K, Okano T, Eguchi S, Yamato M. Autologous adipose-derived stem cell sheets enhance the strength of intestinal anastomosis. *Regen Ther* 2017; **7**: 24-33 [PMID: 30271849 DOI: 10.1016/j.reth.2017.06.004]
47 **Pan H**, Lam PK, Tong SW, Leung KK, Teoh AY, Ng EK. Mesenchymal Stem Cells Combined with Tissue Fusion Technology Promoted Wound Healing in Porcine Bowel Anastomosis. *Stem Cells Int* 2020; **2020**: 5142797 [PMID: 32104185 DOI: 10.1155/2020/5142797]
48 **Komiyama S**, Sakakura C, Murayama Y, Komatsu S, Shiozaki A, Kuriu Y, Ikoma H, Nakanishi M, Ichikawa D, Hujiwara H, Okamoto K, Ochiai T, Nakada A, Nakamura T, Otsuji E. Adipose-derived stem cells enhance tissue regeneration of gastrotomy closure. *J Surg Res* 2013; **185**: 945-952 [PMID: 24095024 DOI: 10.1016/j.jss.2013.05.017]
49 **Liu L**, Chiu PW, Lam PK, Poon CC, Lam CC, Ng EK, Lai PB. Effect of local injection of mesenchymal stem cells on healing of sutured gastric perforation in an experimental model. *Br J Surg* 2015; **102**: e158-e168 [PMID: 25627130 DOI: 10.1002/bjs.9724]
50 **Tanaka S**, Kanetaka K, Fujii M, Ito S, Sakai Y, Kobayashi S, Yamanouchi K, Fujita F, Kuroki T, Eguchi S. Cell sheet technology for the regeneration of gastrointestinal tissue using a novel gastric perforation rat model. *Surg Today* 2017; **47**: 114-121 [PMID: 27262675 DOI: 10.1007/s00595-016-1360-2]
51 **Xue X**, Yan Y, Ma Y, Yuan Y, Li C, Lang X, Xu Z, Chen H, Zhang H. Stem-Cell Therapy for Esophageal Anastomotic Leakage by Autografting Stromal Cells in Fibrin Scaffold. *Stem Cells Transl Med* 2019; **8**: 548-556 [PMID: 30811100 DOI: 10.1002/sctm.18-0137]
52 **Zhang Y**, Sharma A, Joo DJ, Nelson E, AbuRmilah A, Amiot BP, Boyer CJ, Alexander JS, Jalan-Sakrikar N, Martin J, Moreira R, Chowdhury SA, Smart M, Dietz AB, Nyberg SL, Heimbach JK, Huebert RC. Autologous Adipose Tissue-Derived Mesenchymal Stem Cells Introduced by Biliary Stents or Local Immersion in Porcine Bile Duct Anastomoses. *Liver Transpl* 2020; **26**: 100-112 [PMID: 31742878 DOI: 10.1002/lt.25682]
53 **Hara T**, Soyama A, Adachi T, Kobayashi S, Sakai Y, Maruya Y, Kugiyama T, Hidaka M, Okada S, Hamada T, Maekawa K, Ono S, Takatsuki M, Eguchi S. Ameliorated healing of biliary anastomosis by autologous adipose-derived stem cell sheets. *Regen Ther* 2020; **14**: 79-86 [PMID: 31988997 DOI: 10.1016/j.reth.2019.11.001]
54 **Neumann PA**, Twardy V, Becker F, Geyer C, Schwegmann K, Mohr A, Faust A, Lenz P, Rijcken E. Assessment of MMP-2/-9 expression by fluorescence endoscopy for evaluation of anastomotic healing in a murine model of anastomotic leakage. *PLoS One* 2018; **13**: e0194249 [PMID: 29566031 DOI: 10.1371/journal.pone.0194249]
55 **Gershuni VM**, Friedman ES. The Microbiome-Host Interaction as a Potential Driver of Anastomotic Leak. *Curr Gastroenterol Rep* 2019; **21**: 4 [PMID: 30684121 DOI: 10.1007/s11894-019-0668-7]

56 **Oines MN**, Krarup PM, Jorgensen LN, Agren MS. Pharmacological interventions for improved colonic anastomotic healing: a meta-analysis. *World J Gastroenterol* 2014; **20**: 12637-12648 [PMID: 25253969 DOI: 10.3748/wjg.v20.i35.12637]

57 **Brouwer RJ**, Engberts AC, Borger van der Burg BL, van Dongen TT, van Hulst RA, Hoencamp R. Meta-analysis on the effect of hyperbaric oxygen as adjunctive therapy in the outcome of anastomotic healing of experimental colorectal resections in rats. *Diving Hyperb Med* 2018; **48**: 173-185 [PMID: 30199890 DOI: 10.28920/dhm48.3.173-185]

58 **Guyton KL**, Levine ZC, Lowry AC, Lambert L, Gribovskaja-Rupp I, Hyman N, Zaborina O, Alverdy J. Identification of Collagenolytic Bacteria in Human Samples: Screening Methods and Clinical Implications for Resolving and Preventing Anastomotic Leaks and Wound Complications. *Dis Colon Rectum* 2019; **62**: 972-979 [PMID: 31283593 DOI: 10.1097/DCR.0000000000001417]

59 **Vakalopoulos KA**, Daams F, Wu Z, Timmermans L, Jeekel JJ, Kleinrensink GJ, van der Ham A, Lange JF. Tissue adhesives in gastrointestinal anastomosis: a systematic review. *J Surg Res* 2013; **180**: 290-300 [PMID: 23384970 DOI: 10.1016/j.jss.2012.12.043]

60 **Nordentoft T**, Pommergaard HC, Rosenberg J, Achiam MP. Fibrin glue does not improve healing of gastrointestinal anastomoses: a systematic review. *Eur Surg Res* 2015; **54**: 1-13 [PMID: 25247310 DOI: 10.1159/000366418]

61 **Suárez-Grau JM**, Bernardos García C, Cepeda Franco C, Mendez García C, García Ruiz S, Docobo Durantez F, Morales-Conde S, Padillo Ruiz J. Fibrinogen-thrombin collagen patch reinforcement of high-risk colonic anastomoses in rats. *World J Gastrointest Surg* 2016; **8**: 627-633 [PMID: 27721926 DOI: 10.4240/wjgs.v8.i9.627]

62 **Portillo G**, Franklin ME Jr. Clinical results using bioabsorbable staple-line reinforcement for circular stapler in colorectal surgery: a multicenter study. *J Laparoendosc Adv Surg Tech A* 2010; **20**: 323-327 [PMID: 20465429 DOI: 10.1089/lap.2009.0201]

63 **Wang FG**, Yan WM, Yan M, Song MM. Outcomes of transanal tube placement in anterior resection: A meta-analysis and systematic review. *Int J Surg* 2018; **59**: 1-10 [PMID: 30266662 DOI: 10.1016/j.ijsu.2018.09.012]

64 **Slesser AA**, Pellino G, Shariq O, Cocker D, Kontovounisios C, Rasheed S, Tekkis PP. Compression versus hand-sewn and stapled anastomosis in colorectal surgery: a systematic review and meta-analysis of randomized controlled trials. *Tech Coloproctol* 2016; **20**: 667-676 [PMID: 27554096 DOI: 10.1007/s10151-016-1521-8]

65 **Baloyiannis I**, Perivoliotis K, Diamantis A, Tzovaras G. Virtual ileostomy in elective colorectal surgery: a systematic review of the literature. *Tech Coloproctol* 2020; **24**: 23-31 [PMID: 31820192 DOI: 10.1007/s10151-019-02127-2]

66 **Pommergaard HC**, Rosenberg J, Schumacher-Petersen C, Achiam MP. Choosing the best animal species to mimic clinical colon anastomotic leakage in humans: a qualitative systematic review. *Eur Surg Res* 2011; **47**: 173-181 [PMID: 21968050 DOI: 10.1159/000330748]

67 **Yauw ST**, Wever KE, Hoesseini A, Ritskes-Hoitinga M, van Goor H. Systematic review of experimental studies on intestinal anastomosis. *Br J Surg* 2015; **102**: 726-734 [PMID: 25846745 DOI: 10.1002/bjs.9776]

68 **Bosmans JWAM**, Moossdorff M, Al-Taher M, van Beek L, Derikx JPM, Bouvy ND. International consensus statement regarding the use of animal models for research on anastomoses in the lower gastrointestinal tract. *Int J Colorectal Dis* 2016; **31**: 1021-1030 [PMID: 26960997 DOI: 10.1007/s00384-016-2550-5]

69 **Kilkenny C**, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 2010; **8**: e1000412 [PMID: 20613859 DOI: 10.1371/journal.pbio.1000412]

70 **Trébol J**, Georgiev-Hristov T, Vega-Clemente L, García-Gómez I, Carabias-Orgaz A, García-Arranz M, García-Olmo D. Rat model of anal sphincter injury and two approaches for stem cell administration. *World J Stem Cells* 2018; **10**: 1-14 [PMID: 29391927 DOI: 10.4252/wjsc.v10.i1.1]

71 **Ghadge SK**, Mühlstedt S, Ozcelik C, Bader M. SDF-1α as a therapeutic stem cell homing factor in myocardial infarction. *Pharmacol Ther* 2011; **129**: 97-108 [PMID: 20965212 DOI: 10.1016/j.pharmthera.2010.09.011]

72 **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]

73 **Bourin P**, Bunnell BA, Casteilla L, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 2013; **15**: 641-648 [PMID: 23570660 DOI: 10.1016/j.jcyt.2013.02.006]

74 **Okano T**, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide). *J Biomed Mater Res* 1993; **27**: 1243-1251 [PMID: 8245039 DOI: 10.1002/jbm.820271005]

75 **Yamato M**, Utsumi M, Kushida A, Konno C, Kikuchi A, Okano T. Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. *Tissue Eng* 2001; **7**: 473-480 [PMID: 11506735 DOI: 10.1089/107632701524365517]

76 **Yang J**, Yamato M, Shimizu T, Sekine H, Ohashi K, Kanzaki M, Ohki T, Nishida K, Okano T.

Reconstruction of functional tissues with cell sheet engineering. *Biomaterials* 2007; **28**: 5033-5043 [PMID: 17761277 DOI: 10.1016/j.biomaterials.2007.07.052]

77 **Tan EW**, Schon LC. Mesenchymal Stem Cell-Bearing Sutures for Tendon Repair and Healing in the Foot and Ankle. *Foot Ankle Clin* 2016; **21**: 885-890 [PMID: 27871421 DOI: 10.1016/j.fcl.2016.07.015]

78 **Guyette JP**, Fakharzadeh M, Burford EJ, Tao ZW, Pins GD, Rolle MW, Gaudette GR. A novel suture-based method for efficient transplantation of stem cells. *J Biomed Mater Res A* 2013; **101**: 809-818 [PMID: 22961975 DOI: 10.1002/jbm.a.34386]

79 **Hansen KJ**, Favreau JT, Guyette JP, Tao ZW, Coffin ST, Cunha-Gavidia A, D'Amore B, Perreault LR, Fitzpatrick JP, DeMartino A, Gaudette GR. Functional Effects of Delivering Human Mesenchymal Stem Cell-Seeded Biological Sutures to an Infarcted Heart. *Biores Open Access* 2016; **5**: 249-260 [PMID: 27610271 DOI: 10.1089/biores.2016.0026]

80 **Yao J**, Korotkova T, Riboh J, Chong A, Chang J, Smith RL. Bioactive sutures for tendon repair: assessment of a method of delivering pluripotential embryonic cells. *J Hand Surg Am* 2008; **33**: 1558-1564 [PMID: 18984338 DOI: 10.1016/j.jhsa.2008.06.010]

81 **Horváthy DB**, Vácz G, Cselenyák A, Weszl M, Kiss L, Lacza Z. Albumin-coated bioactive suture for cell transplantation. *Surg Innov* 2013; **20**: 249-255 [PMID: 22717700 DOI: 10.1177/1553350612451353]

82 **Casado JG**, Blazquez R, Jorge I, Alvarez V, Gomez-Mauricio G, Ortega-Muñoz M, Vazquez J, Sanchez-Margallo FM. Mesenchymal stem cell-coated sutures enhance collagen depositions in sutured tissues. *Wound Repair Regen* 2014; **22**: 256-264 [PMID: 24635176 DOI: 10.1111/wrr.12153]

83 **Muraoka K**, Le W, Behn AW, Yao J. The Effect of Growth Differentiation Factor 8 (Myostatin) on Bone Marrow-Derived Stem Cell-Coated Bioactive Sutures in a Rabbit Tendon Repair Model. *Hand (N Y)* 2020; **15**: 264-270 [PMID: 30079783 DOI: 10.1177/1558944718792708]

84 **Daradka M**, Alardah MM, Ismail ZB. Effects of autologous platelet-rich plasma coated sutures on intestinal anastomotic healing in rabbits. *Heliyon* 2019; **5**: e02713 [PMID: 31840125 DOI: 10.1016/j.heliyon.2019.e02713]

85 **Aydin MA**, Guler EM, Demiroz AS, Aydin MF, Saglam G. Comparison of Platelet-Rich Plasma-Impregnated Suture Material with Low and High Platelet Concentration to Improve Colonic Anastomotic Wound Healing in Rats. *Gastroenterol Res Pract* 2020; **2020**: 7386285 [PMID: 32565785 DOI: 10.1155/2020/7386285]

86 **Bigalke C**, Luderer F, Wulf K, Storm T, Löbler M, Arbeiter D, Rau BM, Nizze H, Vollmar B, Schmitz KP, Klar E, Sternberg K. VEGF-releasing suture material for enhancement of vascularization: development, in vitro and in vivo study. *Acta Biomater* 2014; **10**: 5081-5089 [PMID: 25204522 DOI: 10.1016/j.actbio.2014.09.002]

87 **Castelo-Branco MT**, Soares ID, Lopes DV, Buongusto F, Martinusso CA, do Rosario A Jr, Souza SA, Gutfilen B, Fonseca LM, Elia C, Madi K, Schanaider A, Rossi MI, Souza HS. Intraperitoneal but not intravenous cryopreserved mesenchymal stromal cells home to the inflamed colon and ameliorate experimental colitis. *PLoS One* 2012; **7**: e33360 [PMID: 22432015 DOI: 10.1371/journal.pone.0033360]

88 **Pathi SD**, Acevedo JF, Keller PW, Kishore AH, Miller RT, Wai CY, Word RA. Recovery of the injured external anal sphincter after injection of local or intravenous mesenchymal stem cells. *Obstet Gynecol* 2012; **119**: 134-144 [PMID: 22183221 DOI: 10.1097/AOG.0b013e3182397009]

89 **Salcedo L**, Mayorga M, Damaser M, Balog B, Butler R, Penn M, Zutshi M. Mesenchymal stem cells can improve anal pressures after anal sphincter injury. *Stem Cell Res* 2013; **10**: 95-102 [PMID: 23147650 DOI: 10.1016/j.scr.2012.10.002]

90 **Nauta AJ**, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* 2007; **110**: 3499-3506 [PMID: 17664353 DOI: 10.1182/blood-2007-02-069716]

91 **Chen L**, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* 2008; **3**: e1886 [PMID: 18382669 DOI: 10.1371/journal.pone.0001886]

92 **Németh K**, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E. Bone marrow stromal cells attenuate sepsis *via* prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009; **15**: 42-49 [PMID: 19098906 DOI: 10.1038/nm.1905]

93 **Frudinger A**, Kölle D, Schwaiger W, Pfeifer J, Paede J, Halligan S. Muscle-derived cell injection to treat anal incontinence due to obstetric trauma: pilot study with 1 year follow-up. *Gut* 2010; **59**: 55-61 [PMID: 19875391 DOI: 10.1136/gut.2009.181347]

94 **Frudinger A**, Pfeifer J, Paede J, Kolovetsiou-Kreiner V, Marksteiner R, Halligan S. Autologous skeletal-muscle-derived cell injection for anal incontinence due to obstetric trauma: a 5-year follow-up of an initial study of 10 patients. *Colorectal Dis* 2015; **17**: 794-801 [PMID: 25773013 DOI: 10.1111/codi.12947]

95 **Sun L**, Xie Z, Kuang M, Penn M, Damaser MS, Zutshi M. Regenerating the Anal Sphincter: Cytokines, Stem Cells, or Both? *Dis Colon Rectum* 2017; **60**: 416-425 [PMID: 28267010 DOI: 10.1097/DCR.0000000000000783]

96 **Pawitan JA**, Bui TA, Mubarok W, Antarianto RD, Nurhayati RW, Dilogo IH, Oceandy D. Enhancement of the Therapeutic Capacity of Mesenchymal Stem Cells by Genetic Modification: A

Systematic Review. *Front Cell Dev Biol* 2020; **8**: 587776 [PMID: 33195245 DOI: 10.3389/fcell.2020.587776]

97 **Miceli V**, Bulati M, Iannolo G, Zito G, Gallo A, Conaldi PG. Therapeutic Properties of Mesenchymal Stromal/Stem Cells: The Need of Cell Priming for Cell-Free Therapies in Regenerative Medicine. *Int J Mol Sci* 2021; **22** [PMID: 33466583 DOI: 10.3390/ijms22020763]

98 **Nikfarjam S**, Rezaie J, Zolbanin NM, Jafari R. Mesenchymal stem cell derived-exosomes: a modern approach in translational medicine. *J Transl Med* 2020; **18**: 449 [PMID: 33246476 DOI: 10.1186/s12967-020-02622-3]

99 **Røsland GV**, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwietz J, Tonn JC, Goldbrunner R, Lønning PE, Bjerkvig R, Schichor C. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. *Cancer Res* 2009; **69**: 5331-5339 [PMID: 19509230 DOI: 10.1158/0008-5472.CAN-08-4630]

100 **Garcia S**, Bernad A, Martín MC, Cigudosa JC, Garcia-Castro J, de la Fuente R. Pitfalls in spontaneous in vitro transformation of human mesenchymal stem cells. *Exp Cell Res* 2010; **316**: 1648-1650 [PMID: 20171963 DOI: 10.1016/j.yexcr.2010.02.016]

101 **Torsvik A**, Røsland GV, Svendsen A, Molven A, Immervoll H, McCormack E, Lønning PE, Primon M, Sobala E, Tonn JC, Goldbrunner R, Schichor C, Mysliwietz J, Lah TT, Motaln H, Knappskog S, Bjerkvig R. Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: putting the research field on track - letter. *Cancer Res* 2010; **70**: 6393-6396 [PMID: 20631079 DOI: 10.1158/0008-5472.CAN-10-1305]

102 **Ramdasi S**, Sarang S, Viswanathan C. Potential of Mesenchymal Stem Cell based application in Cancer. *Int J Hematol Oncol Stem Cell Res* 2015; **9**: 95-103 [PMID: 25922650]

103 **Timaner M**, Tsai KK, Shaked Y. The multifaceted role of mesenchymal stem cells in cancer. *Semin Cancer Biol* 2020; **60**: 225-237 [PMID: 31212021 DOI: 10.1016/j.semcancer.2019.06.003]

104 **Rhodes LV**, Muir SE, Elliott S, Guillot LM, Antoon JW, Penfornis P, Tilghman SL, Salvo VA, Fonseca JP, Lacey MR, Beckman BS, McLachlan JA, Rowan BG, Pochampally R, Burow ME. Adult human mesenchymal stem cells enhance breast tumorigenesis and promote hormone independence. *Breast Cancer Res Treat* 2010; **121**: 293-300 [PMID: 19597705 DOI: 10.1007/s10549-009-0458-2]

105 **Xu WT**, Bian ZY, Fan QM, Li G, Tang TT. Human mesenchymal stem cells (hMSCs) target osteosarcoma and promote its growth and pulmonary metastasis. *Cancer Lett* 2009; **281**: 32-41 [PMID: 19342158 DOI: 10.1016/j.canlet.2009.02.022]

106 **Jafari A**, Rezaei-Tavirani M, Farhadihosseinabadi B, Zali H, Niknejad H. Human amniotic mesenchymal stem cells to promote/suppress cancer: two sides of the same coin. *Stem Cell Res Ther* 2021; **12**: 126 [PMID: 33579346 DOI: 10.1186/s13287-021-02196-x]

107 **Khakoo AY**, Pati S, Anderson SA, Reid W, Elshal MF, Rovira II, Nguyen AT, Malide D, Combs CA, Hall G, Zhang J, Raffeld M, Rogers TB, Stetler-Stevenson W, Frank JA, Reitz M, Finkel T. Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *J Exp Med* 2006; **203**: 1235-1247 [PMID: 16636132 DOI: 10.1084/jem.20051921]

108 **Klopp AH**, Gupta A, Spaeth E, Andreeff M, Marini F 3rd. Concise review: Dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? *Stem Cells* 2011; **29**: 11-19 [PMID: 21280155 DOI: 10.1002/stem.559]

109 **Chulpanova DS**, Kitaeva KV, Tazetdinova LG, James V, Rizvanov AA, Solovyeva VV. Application of Mesenchymal Stem Cells for Therapeutic Agent Delivery in Anti-tumor Treatment. *Front Pharmacol* 2018; **9**: 259 [PMID: 29615915 DOI: 10.3389/fphar.2018.00259]

110 **Babajani A**, Soltani P, Jamshidi E, Farjoo MH, Niknejad H. Recent Advances on Drug-Loaded Mesenchymal Stem Cells With Anti-neoplastic Agents for Targeted Treatment of Cancer. *Front Bioeng Biotechnol* 2020; **8**: 748 [PMID: 32793565 DOI: 10.3389/fbioe.2020.00748]

111 **Lin W**, Huang L, Li Y, Fang B, Li G, Chen L, Xu L. Mesenchymal Stem Cells and Cancer: Clinical Challenges and Opportunities. *Biomed Res Int* 2019; **2019**: 2820853 [PMID: 31205939 DOI: 10.1155/2019/2820853]

112 **Kuriyan AE**, Albini TA, Townsend JH, Rodriguez M, Pandya HK, Leonard RE 2nd, Parrott MB, Rosenfeld PJ, Flynn HW Jr, Goldberg JL. Vision Loss after Intravitreal Injection of Autologous "Stem Cells" for AMD. *N Engl J Med* 2017; **376**: 1047-1053 [PMID: 28296617 DOI: 10.1056/NEJMoa1609583]

113 **Saraf SS**, Cunningham MA, Kuriyan AE, Read SP, Rosenfeld PJ, Flynn HW Jr, Albini TA. Bilateral Retinal Detachments After Intravitreal Injection of Adipose-Derived 'Stem Cells' in a Patient With Exudative Macular Degeneration. *Ophthalmic Surg Lasers Imaging Retina* 2017; **48**: 772-775 [PMID: 28902341 DOI: 10.3928/23258160-20170829-16]

World Journal of Stem Cells

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

World J Stem Cells 2022 January 26; 14(1): 142-145

DOI: 10.4252/wjsc.v14.i1.142

ISSN 1948-0210 (online)

LETTER TO THE EDITOR

Insight into generation of induced mesenchymal stem cells from induced pluripotent cells

Mahmood S Choudhery, Ruhma Mahmood

ORCID number: Mahmood S Choudhery 0000-0003-2038-4817; Ruhma Mahmood 0000-0001-8548-7927.

Author contributions: Choudhery MS and Mahmood R searched the literature and designed and wrote the manuscript.

Conflict-of-interest statement: The authors do not have any financial conflict of interest.

Country/Territory of origin: Pakistan

Specialty type: Cell and tissue engineering

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): D
Grade E (Poor): 0

Mahmood S Choudhery, Department of Biomedical Sciences, King Edward Medical University, Lahore 54000, Punjab, Pakistan

Mahmood S Choudhery, Department of Genetics and Molecular Biology, University of Health Sciences, Lahore 54600, Punjab, Pakistan

Ruhma Mahmood, Stem Cells Laboratory, Allama Iqbal Medical College, Lahore 54000, Punjab, Pakistan

Corresponding author: Mahmood S Choudhery, PhD, Tenured Associate Professor, Department of Genetics and Molecular Biology, University of Health Sciences, Khayaban-e-Jamia Punjab, Lahore 54600, Punjab, Pakistan. ms20031@yahoo.com

Abstract

Mesenchymal stem cells (MSCs) have the potential for use in cell-based regenerative therapies. Currently, hundreds of clinical trials are using MSCs for the treatment of various diseases. However, MSCs are low in number in adult tissues; they show heterogeneity depending upon the cell source and exhibit limited proliferative potential and early senescence in *in vitro* cultures. These factors negatively impact the regenerative potential of MSCs and therefore restrict their use for clinical applications. As a result, novel methods to generate induced MSCs (iMSCs) from induced pluripotent stem cells have been explored. The development and optimization of protocols for generation of iMSCs from induced pluripotent stem cells is necessary to evaluate their regenerative potential *in vivo* and *in vitro*. In addition, it is important to compare iMSCs with primary MSCs (isolated from adult tissues) in terms of their safety and efficacy. Careful investigation of the properties of iMSCs *in vitro* and their long term behavior in animals is important for their translation from bench to bedside.

Key Words: Induced mesenchymal stem cells; Induced pluripotent stem cells; Regenerative potential; Pluripotent; Multipotent; Clinical studies

Core Tip: Regenerative potential of mesenchymal stem cells (MSCs) have been

Received: September 9, 2021
Peer-review started: September 9, 2021
First decision: October 17, 2021
Revised: October 25, 2021
Accepted: December 23, 2021
Article in press: December 23, 2021
Published online: January 26, 2022

P-Reviewer: Jiang W, Wahid M
S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

explored in a number of preclinical and clinical studies for the treatment of various diseases and disorders. However, factors such as low number of MSCs in donor tissues, heterogeneity, limited *in vitro* proliferative potential and early senescence in *in vitro* cultures restrict MSC use clinically. Novel methods to generate induced MSCs from induced pluripotent stem cells have been explored. Development and optimization of such protocols is necessary to evaluate the regenerative potential of induced MSCs *in vivo* and *in vitro*.

Citation: Choudhery MS, Mahmood R. Insight into generation of induced mesenchymal stem cells from induced pluripotent cells. *World J Stem Cells* 2022; 14(1): 142-145
URL: https://www.wjgnet.com/1948-0210/full/v14/i1/142.htm
DOI: https://dx.doi.org/10.4252/wjsc.v14.i1.142

TO THE EDITOR

An interesting article was recently published by Dupuis and Oltra[1] in the "*World Journal of Stem Cells*" about the generation of induced mesenchymal stem cells (iMSCs) from induced pluripotent stem cells (iPSCs). The authors highlighted the importance of production of iMSCs to overcome the problems related to primary MSCs derived from adult tissues. In this regard, they summarized the protocols of iMSC generation from iPSCs. In addition, they discussed the common and method-specific culture components and materials required for iMSC generation. We appreciate the idea of discussion of the current protocols for iMSC generation specifically dividing the available protocols into categories such as MSC switch method, embryoid body formation method, specific differentiation method, pathway inhibitor method and platelet lysate method. However, certain points especially the terminology used, and the information provided is arguable.

MSCs are one the best characterized types of adult stem cells. MSCs can be isolated easily from various adult autologous tissues (such as adipose tissue, bone marrow, *etc.*) as well as neonatal tissues (such as umbilical cord tissue, umbilical cord blood, *etc.*). Due to their regenerative potential, MSCs are currently the focus of cell-based therapy for various diseases and disorders. As a result, a significant number of clinical trials have been registered using MSCs to evaluate their safety and efficacy for future clinical use in myocardial infarction, stroke, meniscus injury, limb ischemia, graft-versus-host disease, skin diseases and autoimmune disorders as well as for wound healing and aesthetic applications (www.clinicaltrials.gov).

The factors such as low number of MSCs in adult tissues, age and donor-related heterogeneity, limited proliferative potential and early senescence in *in vitro* cultures restrict MSC use for research as well for clinical applications[2]. As a result, there has been a great interest in generating MSCs from alternative sources. One such novel alternative method is to generate MSCs (iMSCs) from iPSCs[3,4]. iPSCs are a well-characterized source of pluripotent cells. iPSCs are derived by genetic reprogramming of various types of adult somatic cells to an embryonic stem cell-like state. Forced expression of pluripotent genes (such as *OCT3/4*, *Sox2*, *Klf4*, *c-myc*) convert somatic cells into pluripotent cells. Theoretically, iPSCs have unlimited proliferative potential and ability to differentiate into large numbers of tissues. The generation of iPSCs from mouse fibroblasts was first reported by Takahashi and Yamanaka[5] in 2006. Later, human iPSCs were independently generated by Takahashi *et al*[6] from human fibroblasts[6]. iPSCs are an ideal source of cells for generation of patient specific cells without any ethical and legal concern. Development and optimization of protocols to generate iMSCs from iPSCs is important to overcome the problems associated with primary MSCs isolated from adult tissues. In addition, it is necessary to evaluate regenerative potential of iMSCs *in vivo* and *in vitro* before their clinical applications. Careful investigation of the biological properties of iMSCs *in vitro* and their long term behavior in animals is imperative for their translation from bench to bedside.

The manuscript by Dupuis and Oltra[1] used the term "adult pluripotent stem cells" for MSCs, which is debatable and requires attention. MSCs are multipotent stem cells, although very few studies have identified a subpopulation of very small embryonic-like stem cells (pluripotent) in MSC cultures[7]. In addition, the use of the term "multipotent" for iPSCs may be a typo error. Furthermore, the authors have frequently

used the term "differentiation" for conversion of iPSCs into iMSCs. We are in the view that the use of such terminology may create confusion for readers especially for those who are not the subject specialist. The term differentiation (*i.e.* differentiation of iPSC into iMSCs) is somewhat misleading, as MSCs (iMSCs) are "stem cells," undifferentiated cells with the potential to differentiate into other types of cells. Therefore, appropriate terminology such as conversion, derivation or production seems more suitable for description of iMSCs when obtained from iPSCs.

The authors in the study[1] have only discussed iMSCs based on the cell surface markers. The International Society for Cellular Therapy[8] has recommended the minimum criteria for defining MSCs. According to the International Society for Cellular Therapy, MSCs must exhibit the following attributes: (1) Plastic adherent growth when maintained in standard culture conditions; (2) Positive expression of CD105, CD73 and CD90; (3) Negative expression of hematopoietic lineage surface markers (CD45, CD34, CD14 or CD11b, CD79α or CD19) and HLA-DR; and (4) *In vitro* tri-lineage (osteoblasts, adipocytes, chondroblasts) differentiation capability. To make their review article more comprehensive and for better understanding of iMSCs, more relevant information about other MSC attributes (especially tri-lineage differentiation) suggested by the International Society for Cellular Therapy is desirable.

In addition, the study by Dupuis and Oltra[1] discussed the factors associated with restricted use of MSCs for clinical applications. However, there is no discussion of how the use of iMSCs will overcome these problems. From the rationale of the study, it was expected that there will be proper information showing suitability of iMSC use for patients.

iPSCs are derived from various tissues as described in the study. Do iMSCs derived from different iPSCs lines show the same characteristics? What is the behavior of iMSCs in long-term cultures? Furthermore, the protocols described in the review manipulate the cell culture environment, and therefore their safety for clinical use is questionable. The discussion of relevant challenges in the use of iMSCs and future perspectives could possibly highlight the importance of careful research and use in patients.

iMSCs are a next generation alternative source of stem cells with potential therapeutic applications in regenerative medicine[3,4,9]. iPSC-derived iMSCs could significantly elevate the therapeutic values for clinical applications. However, there are a number of challenges ahead that need to be addressed before their clinical use. One such challenge is their proper characterization and development and optimization of reliable and efficient protocols for consistent production of a homogenous population of iMSCs. The behavior of iMSCs in *in vitro* cultures especially in long-term cultures and *in vivo* in animal studies needs to be addressed for better understanding of their safety. In addition, it is important to compare iMSCs with MSCs (isolated from adult tissues) in terms of their safety and efficacy in preclinical and clinical studies. Overall, the search for suitable alternative sources of MSCs are the focus of current research to minimize associated problems. However, careful investigation of new methods for development of MSCs (iMSCs) is the key for successful translation of clinical research from bench to bedside.

REFERENCES

1 **Dupuis V**, Oltra E. Methods to produce induced pluripotent stem cell-derived mesenchymal stem cells: Mesenchymal stem cells from induced pluripotent stem cells. *World J Stem Cells* 2021; **13**: 1094-1111 [PMID: 34567428 DOI: 10.4252/wjsc.v13.i8.1094]

2 **Wang YH**, Tao YC, Wu DB, Wang ML, Tang H, Chen EQ. Cell heterogeneity, rather than the cell storage solution, affects the behavior of mesenchymal stem cells *in vitro* and in vivo. *Stem Cell Res Ther* 2021; **12**: 391 [PMID: 34256842 DOI: 10.1186/s13287-021-02450-2]

3 **Rogers RE**, Haskell A, White BP, Dalal S, Lopez M, Tahan D, Pan S, Kaur G, Kim H, Barreda H, Woodard SL, Benavides OR, Dai J, Zhao Q, Maitland KC, Han A, Nikolov ZL, Liu F, Lee RH, Gregory CA, Kaunas R. A scalable system for generation of mesenchymal stem cells derived from induced pluripotent cells employing bioreactors and degradable microcarriers. *Stem Cells Transl Med* 2021; **10**: 1650-1665 [PMID: 34505405 DOI: 10.1002/sctm.21-0151]

4 **Zhao Q**, Gregory CA, Lee RH, Reger RL, Qin L, Hai B, Park MS, Yoon N, Clough B, McNeill E, Prockop DJ, Liu F. MSCs derived from iPSCs with a modified protocol are tumor-tropic but have much less potential to promote tumors than bone marrow MSCs. *Proc Natl Acad Sci U S A* 2015; **112**: 530-535 [PMID: 25548183 DOI: 10.1073/pnas.1423008112]

5 **Takahashi K**, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]

6 **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]

7 **Bhartiya D**. Are Mesenchymal Cells Indeed Pluripotent Stem Cells or Just Stromal Cells? *Stem Cells Int* 2013; **2013**: 547501 [PMID: 24187558 DOI: 10.1155/2013/547501]

8 **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]

9 **Guo NN**, Liu LP, Zheng YW, Li YM. Inducing human induced pluripotent stem cell differentiation through embryoid bodies: A practical and stable approach. *World J Stem Cells* 2020; **12**: 25-34 [PMID: 32110273 DOI: 10.4252/wjsc.v12.i1.25]

Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
Telephone: +1-925-3991568
E-mail: bpgoffice@wjgnet.com
Help Desk: https://www.f6publishing.com/helpdesk
https://www.wjgnet.com