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ABOUT COVER

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REVIEW

How the interplay among the tumor microenvironment and the gut microbiota influences the stemness of colorectal cancer cells

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Abstract

Colorectal cancer (CRC) remains the third most prevalent cancer disease and involves a multi-step process in which intestinal cells acquire malignant characteristics. It is well established that the appearance of distal metastasis in CRC patients is the cause of a poor prognosis and treatment failure. Nevertheless, in the last decades, CRC aggressiveness and progression have been attributed to a specific cell population called CRC stem cells (CCSC) with features like tumor initiation capacity, self-renewal capacity, and acquired multidrug resistance. Emerging data highlight the concept of this cell subtype as a plastic entity that has a dynamic status and can be originated from different types of cells through genetic and epigenetic changes. These alterations are modulated by complex and dynamic crosstalk with environmental factors by paracrine signaling. It is known that in the tumor niche, different cell types, structures, and biomolecules coexist and interact with cancer cells favoring cancer growth and development. Together, these components constitute the tumor microenvironment (TME). Most recently, researchers have also deepened the influence of the complex variety of microorganisms that inhabit the intestinal mucosa, collectively known as gut microbiota, on CRC. Both TME and microorganisms participate in inflammatory processes that can drive the initiation and evolution of CRC. Since in the last decade, crucial advances have been made concerning to the synergistic interaction among the TME and gut microorganisms that condition the identity of CCSC, the data exposed in this review could provide valuable insights into the biology of CRC and the development of new targeted therapies.

Key Words: Colorectal cancer; Colorectal cancer stem cells; Tumor microenvironment factors; Tumor stroma; Gut microbiota; Cancer progression

Core Tip: Colorectal cancer (CRC) represents one of the most prevalent tumors worldwide. The tumor microenvironment (TME) through its proinflammatory role, among others, actively participates in CRC progression and the disturbance of gut microbiota (dysbiosis) can influence this inflammatory process. CRC stem cells (CCSC) are a tumor cell subpopulation that drives CRC initiation, progression and treatment failure. The features and behavior of CCSC are modulated by several factors including TME and gut microbiota. Here, we will give an overview of the synergistic interaction among TME and intestinal microorganisms that condition the CRC environment and shape CCSC characteristics allowing CRC evolution.

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INTRODUCTION

Colorectal cancer (CRC) is a multi-step process where intestinal cells acquire malignant phenotypic characteristics that allow them to proliferate, migrate, invade and establish in new tissues[1]. In the last decades, screening strategies and treatments have been improved, decreasing the proportion of CRC patients by as much as 65%–88%[2]. However, this disease remains the third most prevalent type of cancer, having an incidence of 10% and ranking second in mortality (9.4% among all cancer deaths) according to global cancer statistics[3]. The leading cause of patient deaths and relapses is the appearance of new CRC subtypes and the acquired resistance to currently used therapies[4]. Moreover, a great number of CRC are diagnosed with distal metastases and these patients have a poor survival rate due to a lack of response to therapy[2]. One of the causes that affect the treatment of this type of tumor by inducing resistance and the appearance of recurrences, is the presence of a small subpopulation of cells called CRC stem cells (CCSC). This small number of cells have mutations in specific oncogenes that allow them to develop the ability to induce tumor initiation, self-renew, differentiate, dedifferentiate, and acquire multidrug resistance[1,5]. The origin of this cell subpopulation is still controversial. They may originate from colorectal normal cells, colorectal normal stem cells, or CRC cells by genetic alterations or by the influence of environmental factors that induce epigenetic changes[5].

It is known that in the tumor niche, different cell types, structures, and biomolecules coexist and interact with cancer cells favoring the growth and development of the tumor. Together these components constitute the tumor microenvironment (TME). In the last decades, several investigations have demonstrated that tumor surrounding ambiance through its proinflammatory role, among others, actively participates in the development, progression and chemoresistance of CRC[1,4].

Researchers have deepened the study of the influence of the complex variety of microorganisms that inhabit the intestinal mucosa, collectively known as the gut microbiota, on this inflammatory microenvironment. Besides contributing to innate and adaptive immune function, it has been observed that the imbalance in the species present in the intestinal microbiota and the consequent variation in microbial products can promote the development of CRC and compromise the efficacy of its therapy[6].

Since all the factors mentioned are involved in the CRC progression and therapy resistance and considering the great influence of CCSC in several events of this disease, this review aims to analyze the available literature that is focused on the interaction of TME and the intestinal microbiota that favors the development and maintenance of CCSC properties.

COLON CANCER STEM CELLS: FEATURES AND BEHAVIOR

CRC is a heterogeneous pathology that has a variable clinical course and prognosis[7]. The etiology of this disease combines genetic alterations in colorectal epithelial cells with unhealthy lifestyles, such as smoking, alcohol consumption and poor nutritional habits[8,9]. In addition, it has been seen that sex, age, family history of CRC and the persistence of inflammatory processes or infectious agents in the intestinal tract, can be also considered risk factors[5,9-12]. In all these cases, the synergy among genetic mutations, epigenetic alterations and the influence of the TME and gut microorganisms promotes the acquisition of molecular and phenotypic features that allow tumor progression[5,6,11,13,14]. Therefore, within the tumor niche, cells present great heterogeneity but are still strictly organized. In the last 20 years, the focus has been on the study of cancer stem cells (CSC) derived from colorectal tissue (CCSC),

a subpopulation of cells that have a substantial tumorigenic capacity and maintain intestinal tumor growth[15]. CSC are responsible for resistance to multiple drugs maintaining a state of undifferentiation and slow cell division and also favoring the efficiency of desoxyribonucleic acid (DNA) damage repair mechanism[16]. Besides, they have similar features to normal stem cells, such as self-renewal, multipotency, cell cycle arrest, quiescence, and reversibility from their resting state[17,18]. As shown in Figure 1, the ability of CSC to maintain their population response to symmetric/asymmetric division, resulted in the first situation in two identical daughter stem cells and, in the second situation in two distinct cells with or without CSC properties[19,20]. In addition to the division theory, CSC undergo a bidirectional conversion process between stem and non-stem phenotype[20]. Although initially a hierarchical model has been established, in which CSC are the initiators of a monoclonal developmental hierarchy, emerging data highlight the concept of phenotypic plasticity of CSC. This new theory is supported by a dynamic state of interconversion between CSC and non-CSC that can be driven by the TME[21-23]. As the reader can see in Figure 1, during this phenomenon, cells can easily exchange their status within the tumor transforming from CSC to intermediate phenotypes to stemless states and vice versa[15,18,22,24]. Therefore, based on the data provided by the literature and shown in Figure 1, it can be concluded that any cell type is capable of initiating and promoting cancer development[24]. This model contributes with new concepts to the classical theory of the origin/behavior of CSC that highlight the importance of taking into account the study of phenotypic plasticity and the reversible state of this type of cells and that support the criterion that cancer cells with or without stem characteristics must be eradicated for successful therapy.

CCSC constitute about 2% of the cell population in the tumor nest and this percentage can be higher with tumor progression, particularly after chemotherapy or radiotherapy treatments[17,18,25]. Since an increase in the proportion of this cell subtype is an indicator of poor prognosis, in the last decades the identification and targeting of CCSC have become one of the key topics of study[26]. The recognition of CCSC is possible by the detection of typical phenotypic characteristics such as the expression of surface markers, membrane transporters and enzymes. Some of them are Prominin-1/cluster of differentiation 133 (CD133), a transmembrane glycoprotein that is associated with metastasis, invasiveness and chemoresistance in CRC[18]; cluster of differentiation 44 (CD44) a receptor of hyaluronic acid in extracellular matrix related to the epithelial to mesenchymal transition (EMT) program and poor survival in CRC patients[5,27]; cluster of differentiation 166 (CD166) and cluster of differentiation 24, both adhesion molecules whose expressions are associated with the aforementioned markers, and that contribute to stratify low, intermediate, and high-risk CRC cases[5,28]; leucine rich repeat containing G-protein coupled receptor 5 (LGR5) a key CCSC biomarker that decreases in advanced stages of CRC[20,29] and aldehyde dehydrogenase (ALDH), an intracellular enzyme found in high concentrations in most of CSC participating in self-renewal, differentiation and self-protection[20,30,31]. In addition, the study of the ATP-binding cassette transporter superfamily through Hoechst 33352 dye efflux is also employed to detect CCSC[15,32]. In experimental models, the identification and characterization of CCSC can also be performed by fluorescence-activated cell sorting, selection by cell culture properties, *in vivo* transplantation of cells derived from spheroids or organoids, and lineage tracing techniques with labeled CCSC[22]. The above mentioned markers are hallmarks of CCSC and are involved in CRC pharmacotherapy and pathophysiology[33,34], but can also be present in enterocytes and cells of other tissues[20]. Hence, to increase the detection sensitivity and specificity, it is essential to combine the analysis of different biomarkers with CCSC isolation techniques.

Another substantial aspect to consider in the study of CSC is their association with other cellular processes such as EMT, autophagy and the response to cellular stress[15]. In particular, EMT is a physiological process that is also involved in tumor progression. The activation of this program reduces intercellular adhesion and causes epithelial cells to acquire mesenchymal properties that increase the invasiveness and migration of tumor cells[35]. Several studies have reported a link between EMT and the acquisition of CCSC characteristics in both, *in vitro* and *in vivo* assays[35-38]. These investigations show that transcription factors and signaling pathways that are altered in the EMT program are also deregulated in CSC, generating this subpopulation to exhibit phenotypes like EMT[39]. However, recent evidence indicates that EMT may not be necessary to acquire CSC properties. Then, although these processes can go along with each other, they can also happen through independent paths[15]. One of the tumor events that is known to be related to EMT and CSC is the high metabolic demand of TME and the existence of a tortuous vasculature that promotes a hypoxic environment. This phenomenon induces the release of factors such as hypoxia-inducible factor 1α (HIF-1α) that promotes not only EMT but also autophagy associated with CSC. In CRC it was demonstrated that blocking this factor with the consequent inhibition of autophagy reduces cell proliferation and the acquisition of stem-like characteristics[40].

Another cause that has been reported that promotes a stem-like phenotype on several types of tumor cells is the cellular imbalance derived from oxidative stress[15]. In breast and lung cancer cell lines, studies demonstrate that oxidative stress upregulates the CSC marker SRY-box transcription factor 2 (Sox2) activity, and stem-like properties[41,42]. However, in CRC cells it was shown that the reduction of intracellular reactive oxygen species inhibits the formation of CRC stem-like cells[43]. Since this type of cellular stress is considered potentially cytotoxic, more studies are necessary to know the mechanisms by which it has a positive effect on the development of CCSC[15].

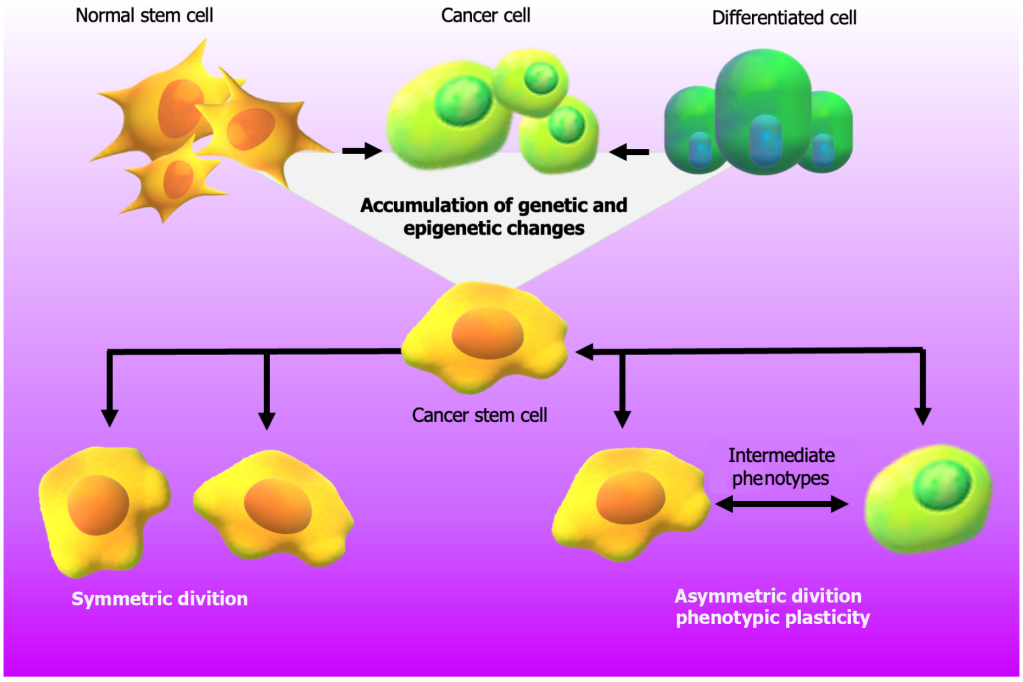

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Figure 1 Theory-based models of cancer stem cell. The ability of cancer stem cells (CSC) to maintain their population response to symmetric/asymmetric division, resulting in the first situation in two identical stem cells daughters and in the second situation in two distinct cells with or without CSC properties. In addition to the division theory, CSC undergo a bidirectional conversion process between stem and non-stem phenotype. During this phenomenon, cells can exchange their status within the tumor transforming from CSC to intermediate phenotypes to stemless states and vice versa. Also differentiated cells, normal stem cells or cancer cells through the accumulation of genetic and epigenetic changes are capable of initiating and promoting cancer development. These general theories are applicable to colon CSC.

Furthermore, it is important to note that like all processes and phenomena related to tumorigenesis and malignant progression, CCSC and their features are modulated by the aberrant activation of various signaling pathways. Wnt, NOTCH, hedgehog (HH), and transforming growth factor-β (TGF-β) are important cascades that are usually misregulated in CCSC and play a central role in the therapy resistance of these cells[5,44].

Thus, understanding CCSC features and all the events and factors associated with cell plasticity constitute a fundamental tool for the development of new target therapeutic strategies.

INFLUENCE OF THE TME ON CCSC FEATURES

It has been reported that multiple links exist between inflammatory processes and stemness in CRC[2]. In this context, the role of the tumor stroma is crucial. The TME in CRC is a physical shelter for CSC[5] composed of biomolecules from the extracellular matrix, an aberrant vasculature and multiple stromal and immune cell types. These cells include mesenchymal stem cells, cancer-associated fibroblasts (CAFs), endothelial cells (ECs), pericytes, and tumor infiltrating immune cells which comprehend: Macrophages, neutrophils, natural killer cells, Treg cells and cytotoxic T lymphocytes[2,4]. The interaction between CRC cells and the different types of cellular and non-cellular elements of TME involves complex and dynamic crosstalk by paracrine signaling[22]. Therefore, self-renewal, differentiation and properties of CRC cells and CCSC are modified by factors released by the surrounding stroma[1]. These factors are cytokines, growth factors and small nucleic acids, which have different mechanisms of action. Next, we will discuss those derived from TME that modulate CCSC properties and that are summarized in Table 1.

Cytokines have been shown to play a key role in CRC stemness. It was reported that TME-derived factors with a pro-inflammatory action such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β foster EMT phenotype and stem cell proliferation in human colon cancer cells[45,46]. Besides, it is known that CAFs, one of the most studied cells in the TME, produce IL-6, which promotes the expression of CCSC markers such as ALDH1 and LGR5[1,47].

The acquisition of a stem-like phenotype is also influenced by the expression and secretion of growth factors[48,49]. It was demonstrated that the epidermal growth factor and the insulin-like growth factor regulate and promote CCSC growth[50]. Moreover, Muñoz Galván *et al*[49] have proved that the treatment of CRC derived cells with hepatocyte growth factor (HGF) and/or macrophage migration

Table 1 Tumor microenvironment factors associated with stemness in colorectal cancer

TME factor	Action	Ref.
Growth/inducible factors		
Epidermal growth factor	Regulates and promotes CCSC growth	[50]
Insulin-like growth factor	Regulates and promotes CCSC growth	[50]
TGF-β	Participates in the initiation of the EMT, invasion, metastasis and initiation of angiogenesis associated to CCSC	[13,29,50]
Bone mophogenetic protein 4	Induces differentiation and decreases the tumorigenic potential of CCSC	[16,60,63]
Bone mophogenic protein 2	Stimulates the differentiation of CCSC inducting autophagic degradation of β-catenin	[44,63]
Hepatocyte growth factor	Activates Wnt signaling and the clonogenicity from CCSC	[53,54]
Macrophage migration inhibitory factor	Increases CCSC properties	[49]
Vascular endothelial growth factor	Promotes growth, epithelial to mesenchymal transition and stemness	[50,51]
Platelet derived growth factor	Promotes growth, epithelial to mesenchymal transition and stemness	[50]
Osteopontin	Regulates EMT and participates in the activation of the Wnt/β-catenin signaling pathway, promoting stemness	[4,156]
HIF-1A	Activates Wnt/β-catenin pathway inducing self-renewal of CCSC. Promotes survival and maintenance of CCSC	[40,157]
Citokines/immune associated proteins		
IL-1β	Modulates the expression of CCSC markers	[158]
IL-4	Facilitates the communication of CCSC with stromal cell, maintains their properties and evades the immune system	[5,44]
IL-6	Promotes the expression of the CCSC markers, ALDH1 and LGR5	[1,47]
IL-8	Induces stemness and EMT	[50,159]
IL-17A	Promotes invasiveness and self-renewal and increases CCSC properties	[12]
IL-22	Promotes invasiveness and self-renewal and increases CCSC properties	[12]
IL-33	Induces the expression of core stem cell genes in CRC-derived cells	[160]
Chemokine (C-C motif) ligand 2	Promotes CCSC properties	[4,49]
Tumor necrosis factor- α	Modulates CCSC features and induces cell death	[158,161]
Parathyroid hormone related-protein	Activates Wnt/β-catenin pathway and promotes events related to stemness	[162-164]
Non-coding RNA		
miR-135 a/b and miR-17	Promote stemness through the activation of Wnt/β-catenin signaling	[157]
miR-34 and miR-93	Inhibit stemness	[157]
miR-92a-3p	Promotes Wnt signaling activation and consequently the expression of β-catenin target genes related to stemness, the EMT program, and chemoresistance	[165]
miR-20a and miR-106 a/b	Repress TGF-β activity and stemness	[157]
miR-146 and Let-7	Affect stem cell fate or proliferation, activation of several stemness markers in a colon cancer cell line	[157]
miR-221/222 and miR-21	Induce the development and maintenance of CCSC	[157]
miR-21	Promotes the activation of the Wnt/β-catenin signaling pathway and increases the population of CCSC	[157]
miR-145	Represses miR-21 and its expression inversely correlates with that of CCSC markers	[157,166]
miR-137	Suppresses CCSC tumorigenicity	[167]
miR-147	Decreases the expression of CCSC markers	
miR-200, miR-203, miR-141 and miR-429	Regulate CCSC through negative modulation of EMT and self-renewal	[157]

lncRNA H19	Promotes CCSC phenotype and drug resistance	[168]
Signaling pathway ligands		
Wnt ligands	Increase CCSC characteristics and enhances tumor-initiating potential	[5,157]
Delta like canonical Notch ligand 4	Participates on CSC maintenance	[44]
Jagged1	Participates on CSC maintenance	[66]
SHH	Promotes CCSC survival, self-renewal and drug resistance	[67,68]
Enzymes		
Phospholipase D2	Promotes CRC stemness	[4,49]
Extra-cellular matrix components		
Tenascin, fibronectin, collagen type I, secreted protein acidic and rich in cysteine, galectin	Contribute to stemness and CCSC activities	[1]

EMT: Epithelial to mesenchymal transition; CCSC: Colorectal cancer stem cells; CRC: Colorectal cancer; lncRNA: Long non-coding ribonucleic acid; miR: Micro ribonucleic acid; SHH: Sonic Hedgehog protein; TGF-β: Transforming growth factor beta; IL: Interleukin.

inhibitory factor increases the number and size of colonospheras and significantly enhances the expression of putative markers like CD133[49].

Proangiogenic factors like vascular endothelial growth factor (VEGF) and platelet derived growth factor are also implicated in promoting growth and metastasis, both processes directly related to stemness[50]. Furthermore, it was demonstrated that clusters of ECs improve the survival of CCSC and promote their spread[51].

As it is known, all these TME factors modulate the activation of different signaling pathways, altering gene expression and thus modifying the molecular and phenotypic profile of tumor cells[5,44,50]. Wnt signaling is a key stem cell pathway involved in the maintenance of the CCSC and the TME[13,52]. One decade ago, Vermeulen *et al*[53] observed that high activity of the Wnt signaling was associated with CCSC features. Furthermore, this activity was mainly observed near fibroblasts in the tumor niche. Vermeulen *et al*[53] then demonstrated that HGF derived from CAFs activates Wnt signaling and the clonogenicity from CCSC[53]. This research had a great impact on the study of CSC and recently, Essex and collaborators replicated these studies and obtained similar results. They found that TME regulates the activation of the Wnt signaling pathway, increases CCSC characteristics and enhances tumor-initiating potential[54]. Regarding this, it is known that several Wnt ligands are secreted mostly by CAFs[53-56]. Moreover, other TME factors participate in the activation of the Wnt/β-catenin pathway (Table 1).

Some ligands from other signaling pathways are also related to stem cell phenotype. TGF-β is a growth factor that belongs to a superfamily of molecules including inhibins and bone morphogenetic proteins (BMP)[13]. It has the ability to promote or suppress tumor development depending on the interactions that take place in the TME[57]. As a pro-tumor factor, TGF-β regulates immune responses and participates in many neoplastic events such as proliferation, EMT and stemness[13]. TGF-β signaling pathway mutations and CCSC are linked[58] and in accordance with this, Zhou *et al*[29] found an association between TGF-β signaling and the expression of LGR5 biomarker in CRC[29]. Even more, Gu *et al*[59] have recently demonstrated that the expression of genes related to CCSC features like the carcinoembryonic antigen-related cell adhesion molecule alters TGF-β signaling and promotes CRC[59]. Some other members from the TGF-β family, like bone morphogenetic protein 4 and bone morphogenetic protein 2 (BMP4 and BMP2, respectively), have the capacity to induce CCSC differentiation and increase the response to standard chemotherapy[16,60-62]. Besides, the modulation of the BMP4 pathway by hormones like triiodothyronine was reported in CCSC, decreasing its tumorigenic potential [44,63,64]. This result suggests that CCSC features are modulated not only by local molecules from the TME but also by endocrine factors[44].

Notch signaling is also associated with the expression of CSC features in CRC cells[16,65]. In fact, it was reported that delta like canonical notch ligand 4 and jagged 1, both notch ligands, are overexpressed in this type of tumor providing essential signals for CCSC maintenance[44,66]. Moreover, since HH signaling is implicated in CRC development[20], in the last years several investigations were conducted on the association between this pathway and CCSC properties. Regan *et al*[67], have shown that the activation of the non-canonical HH pathway is required for CCSC survival and depends on sonic hedgehog protein (SHH) ligand[67]. Recently, it has been also observed that the modulation of HH-related proteins expressions by non-coding ribonucleic acids (ncRNAs) impacts on CCSC self-renewal capacity and drug resistance[68]. In line with this, Skoda and collaborators showed that treatment with HH pathway inhibitors such as vismodegib and sonidegib weakens the ability of CCSC [69]. Since no significant differences have been found in clinical trials[70], more studies are needed to determine the effects of the inhibition of this pathway in CRC patients.

Besides the aberrant activation of several signaling pathways, hypoxia is known as a hallmark of CCSC and TME interaction[5]. This is a condition in the tumor niche whose main cause is the poor vasculature associated with the tumor and the upregulation of HIF-1α, a factor released mainly by ECs [40,71,72]. This condition activates Wnt/β-catenin pathway inducing self-renewal and maintenance of CCSC[50,73]. Also, HIF-1α promotes cancer cell proliferation and CCSC survival[40].

Furthermore, short ncRNAs like microRNAs (miRs) and long ncRNAs are secreted not only by tumor cells but also by stromal cells in the TME[4]. In the last decades, the study of ncRNAs has gained importance in CRC. In the framework of factors and signaling pathways related to CCSC biology, these small nucleic acids have a key role[74]. miRs related to stemness in CRC are exposed in Table 1.

As previously mentioned, the interaction between CRC cells and their TME also involves non-cellular elements. Colonic stromal cells mediate the remodeling of the extra-cellular matrix favoring the healing or progress of the disease[75]. Recently, it has been demonstrated, by lineage tracing, that components of the extra-cellular matrix regulate dormancy in CCSC[76]. Tenascin, fibronectin, collagen type I, secreted protein acidic and rich in cysteine (SPARC), galectin and some other components of the tumor matrix are associated with stemness and CCSC activities[1].

Finally, another important concept to consider in the tumor nest is that CCSC also release various factors and cytokines that enable them to communicate with stromal cells, maintain their properties and evade the immune system, such as IL-4 and the cluster of differentiation 200[5,44].

The aforementioned data (and shown in Table 1) suggest that TME instructs the development, properties, plasticity, maintenance and dissemination of CCSC. In the last decade, the remarkable influence of the stroma on CRC development prompted the postulation of a novel classification of this disease based on its impact on tumor gene expression[5]. This CRC staging contains four consensus molecular subtypes (CMS) plus a group called "unclassified" since their features do not fit into the other CMS. All these subtypes are summarized in Table 2[1,5,77-79]. As the reader can see in this table, the influence of TME determines a low or high degree of immune and inflammatory response depending on the CMS, highlighting the importance of factors from TME in the distinctive characteristics of each CRC subtype. Taking into account that the mentioned inflammatory/immune process (that is relevant for CRC classification) can be influenced by the intestinal microorganisms, next we will discuss the interactions of this microbiota with tumor cells and their microenvironment that modulate the behavior and characteristics of CCSC since it is the focus of this review.

ESTABLISHED DYNAMICS BETWEEN THE GUT MICROBIOTA, THE TME AND CCSC

As we mentioned in this work, the inflammatory microenvironment contributes to promoting CRC initiation and progression. However, the role of the cell types involved in this process, including intestinal microorganisms, has not been completely understood yet.

The human microbiome, a concept that is mentioned throughout this section, represents microorganisms with their genetic elements and the interactions arising with the environment in which they are found[80]. Advances in the characterization of this human microbiome have led to the consideration that the role of the microbiota in metabolic functions and maintenance of homeostasis is more important than previously believed. Currently, the human is considered as a holobiont organism inhabited by millions of microorganisms including bacteria, archaea and fungi[81]. The gut microbiota is a complex ecosystem that contains more than 500 bacteria species involved in physiological processes like immune regulation and maintenance of human health[6] and its composition relies fundamentally on diet and lifestyle[74].

In physiological conditions, stromal and immune cells from the gut mucosa interact with this ecosystem to maintain intestinal equilibrium[82]. Cells from the immune system recognize antigens from foreign cells and generate memory and effector cells, which control or avoid the generation of diseases[82].

It has been observed that sustained shifts in this ecosystem, known as intestinal dysbiosis, have unfavorable repercussions on health[74,83]. In this sense, the presence of harmful microorganisms (“drivers”) could induce changes in the intestinal mucosa and favor the colonization by opportunistic bacteria (“passengers”)[84]. This model is known as driver-passenger[84] and could involve changes in the immune system allowing the advance of the damage in the intestinal epithelium tissue[85,86]. This imbalance of the local microbiota promotes the restructuring of the intestinal environment and alters the immune status of the host contributing to the appearance of malignant cells and a favorable niche for tumor development, invasion and metastasis[85,87,88]. The mechanisms of these microorganisms that influence directly the immune system are different and involve the synthesis of immunomodulatory compounds and metabolites, like short-chain fatty acids (SCFAs), polyamines and other fermentation products[89,90]. Moreover, it is known that the intratumoral composition of microorganisms affects T-cell-mediated cytotoxicity and anti-tumor immune surveillance[91]. The unfavorable changes in the intestinal microbiota can promote a pro-inflammatory environment and impair anti-cancer immunity [91]. In this context, cells from TME secrete factors like interferon-γ, TGF-β, IL-6, IL-8, CXCL1 and TNF-α,and favoring the differentiation of T helper 17 Lymphocytes to develop an adaptive immune

Table 2 Consensus molecular subtypes of colorectal cancer

	CMS1-immune (14%)	CMS2-canonical (37%)	CMS3-metabolic (13%)	CMS4-mesenchymal (23%)	Unclassified (13%)
General features	Hypermutated	Epithelial	Epithelial	TGF-β activation. Angiogenesis	Mixed phenotype of multiple CMS
	Microsatellite unstable	WNT and MYC signaling activation	Metabolic dysregulation	Upregulation of EMT	
Mutations	*BRAF, MSH6, RNF43, ATM, TGFBr2, PTEN*	*APC, KRAS, TP53, PIK3CA*	*APC, KRAS, TP53, PIK3CA*	*APC, KRAS, TP53, PIK3CA*	
TME	Decrease of CAFs	Decrease of CAFs	Decrease of CAFs	Increase of CAFs; Immunosuppressive signature	
	High immune and inflammatory signature	Low immune and inflammatory signature	Low immune and inflammatory signature		

This Table is based on Islas *et al*[1], 2022; Fidelle *et al*[79], 2020; Trinh *et al*[169], 2018; Becht *et al*[78], 2016; Guinney *et al*[77], 2015. *APC: Adenomatous polyposis coli gene; ATM: Ataxia telangiectasia mutated gene; BRAF: Serine/threonine-protein kinase B-raf gene*; CAFs: Cancer associated fibroblasts; CMS: Consensus molecular subtype; EMT, Epithelial to mesenchymal transition; *KRAS: Ki-ras2 kirsten rat sarcoma viral oncogene homolog gene; MSH6: MutS homolog 6 gene; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene; PTEN: Phosphatase and tensin homolog gene; RNF43: Ring finger protein 43 gene*; TGF-β: Transforming growth factor beta; *TGFBr2: Transforming growth factor beta receptor 2 gene*; TME: Tumor microenvironment; *TP53: Transformation-related protein 53 gene.*

response that contributes to immune-prone carcinogenesis and CRC development[79,87,92]. In this regard, increasing evidence suggests that gut microorganisms condition CRC patients response to immunotherapy, because they alter the expression of elements such as anti-programmed cell death protein 1 (PD-1) and its ligand (PD-L1) and anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4)[91,93]. PD-1/PD-L1 has been highly studied in the last years on the tumor-microbiome-immune axis[91]; in fact, several investigations provide evidence that PD-L1 is overexpressed on different tumor cells and stromal immune cells, allowing tumors to evade attacks *via* T-cell infiltration[91,94,95].

The increased expression of PD-L1 in CRC cells both *in vitro* and *in vivo* is a mechanism involved in the influence of certain pathogenic bacteria associated with an immunosuppressive TME[96]. In contrast, bacteria associated with healthy microbiota improve the efficacy of anti-PD-L1 therapy by enhancing the accumulation of cytotoxic T cells in the TME[97]. This suggests that TME reprogramming through manipulation of the microbiota can modulate the response to immunotherapies in CRC[98]. Concerning all this information, CRC could be considered as a bacterial-induced disease and disturbance in microbiota could be potentially useful as diagnostic biomarker, indicator of risk and predictor of response to therapies for this type of cancer[74,88].

On the other hand, CRC modifies the local metabolic environment[99]. In this context, it is important to mention that metabolites and factors derived from CRC cells and TME cells such as spermidine, L-valine, L-lysine or stearic acid confer an advantage for the growth and development of certain bacterial species, conditioning changes in the intestinal microbiota[99]. Although different factors produce changes in gut microbiota, recently it has been seen that the shift in the metabolome of tumor cells and TME cells is a key aspect in this event[86,99,100]. Thus, TME can be the consequence or the cause of intestinal dysbiosis.

The gut microorganisms cited below in this section are described in the available literature due to their role in CCSC development and maintenance. They are also summarized in Table 3.

Regarding CSC properties, some pathogenic bacteria such as *Helicobacter pylori* and *Porphyromonas gingivalis* can promote the expression of markers associated with stemness such as CD44 and CD133 in gastrointestinal tumors[101,102]. This association between the presence of certain bacteria genera in the gut and the expression of CSC markers has led to the study of the effects of microorganisms shifts and bacterial metabolites on CRC. Several models of tumorigenesis induced by bacteria have been proposed, suggesting how the interactions of host-microorganism promote the development and progression of this type of cancer[101]. In fact, it is known that the metabolites from the intestinal microbiota have the potential to act as tumorigenic factors. However, others can act as anti-tumorigenic factors since many of these microbiota-derived products are capable of inhibiting CRC progression[103]. Kim *et al*[43] have demonstrated that ursodeoxycholic acid, a secondary bile acid produced by Clostridium species, including *Clostridium absonum* and *Clostridium baratii*, regulates the oxidative stress suppressing CCSC growth and CRC cells proliferation[43]. Moreover, it has been observed that niacin, a product of the metabolism of some intestinal bacteria, such as *Lactobacillus acidophilus*, has different effects on CCSC. Depending on the dose, this vitamin can promote proliferation or death in this cell subtype[104]. Additionally, bowel microorganisms produce SCFAs such as butyrate, propionate and acetate[92]. It has been reported that these SCFAs favor beneficial bacteria proliferation and stimulate regulatory T cells to reduce inflammatory mediators, regulating immune response[105]. Butyrate participates in epithelial integrity maintenance and has antitumor effects. Several investigations show that in CRC, this product

Table 3 Microorganisms present in the intestinal mucosa associated with stemness in colorectal cancer

Microorganism	Action	Ref.
Bacterioides dorei, Bacterioides vulgatum, Parabacterioides distasonis, Lachnoclostridium sp., and Mordavella sp	Inhibit the action of factors related to CCSC phenotype. Inhibit CRC development and progression	[59, 114]
Bacterioides fragilis	Releases an enterotoxin that promotes immune TME cells activation with secretion of factors related to CCSC	[118]
Citrobacter rodentium	Protects the inflammatory CCSC niche	[121]
Clostridium septicum	Contributes to CRC development and to the activation of signaling pathways associated with CCSC	[59]
Enterococcus faecalis	Induces the expression of TGF-β, thereby activating signaling pathways associated with CCSC. Activates Wnt/β-catenin signaling and pluripotent transcription factors associated with CCSC	[113, 115]
Escherichia coli	Upregulates the expression of CCSC-associated genes. Releases genotoxin colibactin which induces the production of growth factors related to CCSC	[112, 117,79]
Fusobacterium nucleatum	Stimulates the secretion of immune factors related to CCSC	[79]
Helicobacter pylori	Promotes the expression of markers associated with stemness	[101, 102]
Lactobacillus acidophilus	Promotes proliferation or death in CCSC depending on dose	[104]
Porphyromonas gingivalis	Promotes the expression of markers associated with stemness	[101, 102]
Shigella, and Citrobacter	Upregulate the expression of CCSC-associated genes	[112]

CCSC: Colorectal cancer stem cells; CRC: Colorectal cancer; TGF-β: Transforming growth factor beta; TME: Tumor microenvironment.

inhibits events associated with CSC such as invasion and proliferation[6]. Interestingly, butyrate inhibits cell proliferation to a greater extent in CRC derived cells than in non-cancerous cells[92]. Although butyrate was reported as an anti-tumor and chemopreventive agent[92,106], other studies have shown that it has variable outcomes on CCSC[92]. So, more investigations are necessary to determine the mechanistic action of this type of fatty acid. Experiments with other SCFAs like acetate and propionate with similar results demonstrated that these acids have opposing effects[6,107]. Besides that, a large number of microbial products such as deoxycholic acid, lithocholic acid chenodeoxycholic acid, taurochenodeoxycholic acid and others, are associated with the promotion of gastrointestinal tumors including CRC[6,108]. Recent studies have found that in CRC patients the microbial composition of the colonic crypt is different from that of the intestinal lumen. In the environment of the crypt of the colorectal tumor, groups such as *Proteobacteria* and anaerobes, such as *Acinetobacter, Stenotrophomonas* and *Delftia* were found[109]. Therefore, specific microorganisms could have a role in the maintenance of CCSC, located in the crypt, through the production of specific metabolites[110]. However, more studies are needed in this field since the molecular mechanisms underlying the effects of intestinal microbial products on CCSC have not yet been fully elucidated.

Currently, the study of mechanisms involved in the communication between the microbiota, the tumor cells and their microenvironment has gained impact on CRC. One reported mechanism for this interaction is through pattern recognition receptors located on intestinal epithelial cells that have the ability to detect distinctive microbial macromolecular ligands called pathogen-associated molecular patterns such as lipopolysaccharides and peptidoglycans[111]. Congruently, a recent work documented an altered function of CSC in a CRC murine model due to intruding bacteria like *Escherichia, Shigella*, and *Citrobacter*. This effect results in the activation of a toll-like receptor (TLR), a class of pattern recognition receptors, and the consequent upregulation of stem cell-associated genes such as *Cd44v6* and *Lgr5*[110,112]. In line with this, the microorganisms are capable of activating several signaling pathways in tumor cells and/or TME cells inducing the secretion of factors associated with CCSC features. In this context, it has been observed in a murine model that microorganisms such as *Enterococcus faecalis* cause colitis after infection and induce expression of TGF-β, thereby activating the Smad signaling pathway[113]. A recent study has demonstrated an inverse correlation between the expression of molecules associated with TGF-β signaling pathway and stem cells- related genes in CRC. Moreover, the authors of this work have compared feces from mice with defects in TGF-β signaling with feces from wild-type (WT) mice, and have shown that the first ones had increased bacterial species associated with the development and progression of CRC, such as *Clostridium septicum*, and diminished amounts of favorable microorganisms including *Bacteroides vulgatus* and *Parabacteroides distasonis*[59]. Similar results were obtained by Wang and collaborators who showed that the amounts of beneficial species (*Bacterioides dorei*, *Lachnoclostridium sp*., and *Mordavella sp*.) are recovered in WT mice but not in those

with mutated TGF-β signaling after chemotherapy treatment[114]. These investigations demonstrate the close relationship between the microbiota, the production and release of TGF-β and CCSC in the tumor.

Concerning other signaling pathways, Wang *et al*[115] have shown that *Enterococcus faecalis* are capable of polarizing macrophages by activating Wnt/β-catenin signaling and pluripotent transcription factors associated with the dedifferentiation, reprogramming and development of CCSC such as cellular myelocytomatosis oncogene, Kruppel-like factor 4, octamer-binding transcription factor 4 (Oct4), and Sox2[115]. These events respond to the microbiota-induced bystander effect theory based on the fact that macrophages induce genetic mutations and chromosomal instability in intestinal cells[116].

Other signaling pathways associated with pro-inflammatory and growth factors can be activated in response to bacterial products. For instance, the unbalance in the amount of the gut bacteria *Escherichia coli*, correlates with CRC progression by producing the genotoxin colibactin[79]. This toxin accelerates tumor progression and involves the production of growth factors related to CCSC, such as the HGF and the consequent activation of its signaling pathway[79,117]. Also, the enterotoxin produced by *Bacteroides fragilis* promotes immune TME cells activation with the secretion of IL-17 which favors CCSC properties [118]. Furthermore, as we have previously mentioned, gut microorganisms shape the immune environment promoting tumor evolution and CCSC features. For example, *Fusobacterium nucleatum* stimulates IL-8 secretion by TME cells and the inhibition of T and NK cell functions[79]. This bacteria has been deeply studied, since clinical analysis of specimens from CRC patients showed that the levels of *F. nucleatum* are significantly higher in neoplastic tissues than in adjacent normal tissues, and correlate with tumor invasion and metastasis[119]. These results support the role of *F. nucleatum* in the regulation of CCSC plasticity and EMT[101]. Also, it is known that *F. nucleatum* and other microorganisms like Epstein–Barr virus are capable of incorporating human ncRNAs favoring microbial growth [74]. In this regard, Tarallo *et al*[120] found a human and microbial ncRNA signature in CRC in which many miRs associated with CSC features, are overexpressed including miR-21 and miR-200[74,120]. A recent study conducted by Wang showed that *Citrobacter rodentium* infection induces the inhibition of miR-34a, which protects the inflammatory CCSC niche[121]. These investigations suggest a close relationship between the intestinal microbiota and the regulation of ncRNAs involved in CCSC properties.

Finally, not only the shift in the number of microorganisms is responsible for stemness and CRC progression, but the interaction and collaboration between several types of bacteria in biofilm communities also participate in bowel inflammation and CRC. It was demonstrated that biofilms correlate with an increase in IL-6 secretion by TME cells playing a key role in proliferation, cell transformation and stemness[79].

The data in this section demonstrate a close interrelationship between the gut microbiota, the TME, and CCSC. This information highlights the relevance of further investigating the intestinal microbiota switch in patients with CRC and the associated mechanisms that lead to TME changes and promote stemness.

THERAPEUTIC TARGETING OF TME AND THE GUT MICROBIOTA: A KEY TOOL TO MODULATE STEMNESS IN CRC

Standard chemotherapeutic approaches for CRC are based on attacking the replicative mechanisms of tumor cells to induce tumor regression. However, considering CSC properties, this subpopulation usually results unharmed by the treatment because they present a low division rate as well as a great capacity to correct DNA defects[122]. This entails therapy resistance of CSC and the subsequent treatment failure and disease progression. It is interesting to note that in CRC, CSC represent around 2.5% of neoplastic cells but due to their phenotypic plasticity, they constitute a dynamic population[123, 124]. This fact, together with the lack of response to therapies, highlights the need of new clinical strategies targeting CCSC[125].

As we explain throughout this review, the influence of the TME and the intestinal microorganisms on CSC properties makes these factors a promising tool in therapy. Many therapeutic agents are capable of inhibiting those events associated with the maintenance of CCSC. For instance, Apatinib napabucasin, Bigelovin, Wogonin and Metformin are drugs whose mechanisms are associated with the inhibition of EMT or angiogenesis in CRC[1]. Moreover, it has been demonstrated that therapeutic agents such as Genistein cause the inhibition of CSC characteristics by glioma-associated oncogene1 signaling pathway [126]. Targeting the activation of those signaling pathways associated with CCSC can also be considered as a mechanism to reduce stemness in CRC tumors and thus improve the response to the therapy. LGK974, Foxy-5, PRI-724[127] and DKN-01[126] are agents that act targeting the Wnt/β-catenin pathway. However, the clinical application of most of these drugs is still under study.

The tumor protective niche also could be modified to eradicate CCSC and overcome chemoresistance. As we have mentioned in previous sections, in the TME, immune cells modulate cancer development and progression. For that reason, in the last decade the treatment of patients with immune checkpoint inhibitors such as CTLA-4 and PD-1/PD-L1 has been studied. Even though employing these drugs leads to various systemic and organic complications, immunotherapy may be promising in sorting these

obstacles and could ameliorate the response of CRC patients to the treatment[1,128]. In fact, coadjuvant therapy with FOLFOX (a combination of leucovorin, fluorouracil and oxaliplatin which are first-line chemotherapeutic drugs)[129], and PD-1/PD-L1 inhibitors had an objective response rate of 50% in clinical trials[130]. In addition, in a phase II trial in CRC metastatic patients, immune checkpoints inhibitors like nivolumab and nivolumab-ipilimumab show improvement in patients survival rate[130]. Moreover, monoclonal antibodies against CAFs and antifibrotic drugs were also tested in clinical studies[5]. Another type of antitumor therapy was accomplished through the production of a cell-based vaccine with specific antigens of CCSC[5].

In addition, plenty of compounds were designed in the last decade to target CCSC signaling pathways[5]. These strategies include the inhibition of HH signaling components, NOTCH pathway inhibitors, anti-angiogenic agents and Wnt ligand blockers. All these drugs are undergoing clinical trials [129]. Despite being an encouraging strategy, it still has limitations like the inhibition of signaling pathways involved in physiologic processes.

In the last years, the particularities exhibited by extracellular vesicles (EVs) have led researchers to consider them as a therapeutic delivery strategy of great value in CRC and other types of tumors. Within the different types of EVs are the exosomes, which are secreted by a variety of cells. These vesicles carry out the molecular content of donor cells and enable cellular communication over short and long distances. These EVs are loaded with coding nucleic acids, ncARNs and bioactive proteins which determine their functions. Exosomes can target a specific tissue and internalize in a cell type by the recognition of surface ligands/receptors[131]. In this regard, Han *et al*[132] investigated the delivery of human cord blood-derived MSC exosomes loaded with miRs as CRC targeted therapy. The results showed an inhibition of tumor growth *in vitro* and *in vivo*, as well as a selective increase of these ncRNAs in CRC cells[132]. The relation between miRs and CCSC was mentioned in previous sections so their delivery may be strong weapons to confront drug resistance and CCSC maintenance[5]. Circular RNAs are ncRNAs that exhibit cell-type and tissue-specific signatures. There has recently been considerable attention on these ncRNAs as they modulate miRs expression[129]. In CRC, recent studies have focused on their study as biomarkers. However, they have not been applied in patients' therapy yet[133, 134]. Moreover, the importance that these small molecules could have in CRC is unknown[129].

Foods containing biologically active ingredients are termed functional foods or nutraceuticals[135, 136]. In the past years, the influence of diet on CRC development and evolution was demonstrated. A diet with natural products like phytochemicals and nutritional herbs has shown protective effects in overcoming CRC associated dysbiosis[137,138]. Diets enriched in dairy are a major source of products that are known to have a protective effect on CRC development such as, calcium, vitamin D and folate [138]. Sulforaphane, a sulfur-rich compound found in cruciferous vegetables like broccoli, has been documented to diminish CSC markers and improve the chemotherapeutic efficacy of drugs commonly used in CRC treatment such as cisplatin, doxorubicin and fluorouracil[137]. It has been observed that dietary polyphenols like quercetin have similar effects[137,139]. Other polyphenols or flavonoids are known to target ABCG-2 transporters and miRs strictly associated with CCSC[139]. Curcumin is one of several substances present in turmeric plants. It has been demonstrated that this bioactive agent inhibits the activation of several signaling pathways related to CSC characteristics. The treatment with this natural product on a CSC model diminished the expression of CD44 and CD133 markers[137]. Moreover, some other natural products have been observed that interfere with intrinsic CSC pathways, like epigallocatechin-3-gallate (EGCG), resveratrol and genistein[140].

Diet can also manipulate the gut microbiota. Indeed, this is achieved by the administration of probiotics in the diet. As probiotics and their active metabolites can exert immunomodulatory and anti-tumorigenic effects[135], the study of them and their metabolites has gained ground in recent decades. Probiotics are live microorganisms, normally lactic acid bacteria, recognized as safe by the United States Food and Drug Administration[135]. Defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[141], they can improve health by administration along vegetable fibers and other prebiotics stimulating beneficial bacterial growth in the intestine[142].

Probiotics administration can be done by different routes, commonly through functional foods, but also by commercial supplements or vaccines[135,138]. It is known that probiotic oral vaccines promote mucosal immunity that prevents enteric infections and could complement the standard therapy in the patient[143]. Microorganisms administration including probiotics and synbiotics (pharmaceutical preparation that contains probiotics and prebiotics that implies a synergy between both) are a potential resource for prophylaxis and therapy in CRC[138,144]. In addition, the luminal cocktail of microorganisms in the bowel can be modified not only by dietary approaches but also with the use of antibiotics or fecal microbiota transplantation (FMT)[145,146]. In particular, FMT has gained considerable interest in recent years as a strategy to treat different gastrointestinal disorders[147-149]. It consists of introducing a healthy microbial population from a disease-free host into a diseased host that has a dysbiotic community to restore microbial homeostasis[150]. Although there are limited data on the use of FMT in the treatment of CRC, several studies are under development to answer relevant questions such as if CRC can be detected, treated or prevented with this method. Rosshart and collaborators observed that mice treated with this method improved their resistance against colorectal tumorigenesis induced by azoximetane[151]. Besides, it has been seen that FMT in Balb-c mice prevents intestinal damage, and chemotherapy-induced toxicity[152]. Interestingly, the fecal microbiota from CRC patients

has been shown to cause tumors in healthy and germen-free $Apc^{min/+}$ mice through the activation of the Wnt signaling pathway. In these mice, the intestinal barrier is also altered and the presence of pro-inflammatory cytokines is increased[153]. These data reveal that the composition of the microbiota may play a determinant role in TME conditions during tumorigenesis. Nevertheless, the subjacent mechanisms of all these treatments or how they ameliorate the side effects of chemotherapy is not clear yet.

In summary, we need a favorable and efficient clearance of tumor cells, all tumorigenic cells including CCSC and a restructuring of the TME for the complete eradication of CRC. Based on everything described in this review, a specific combination of techniques and therapies for each tumor and patient would be necessary to achieve this goal.

FUTURE PERSPECTIVE

According to the information stated in the previous sections, in CRC occurs an alliance between the TME, intestinal microorganisms and CCSC that favors tumor progression. In this scenario, it is emerging a new query regarding the direct effects of CCSC on gut microbiota. Perhaps the appearance of CCSC by spontaneous mutations favors (through paracrine signals and the release of specific factors) a dysbiotic and pro-inflammatory environment but in this regard, new investigations are necessary to evaluate the regulation of CCSC on CRC microbiota. So, there is great potential in the study of the interrelationship between these three components in the tumor niche, mostly for the development of new therapies aimed at the eradication of CCSC and non-stem cells, the restructuring of the TME and the growth induction of microorganisms that are beneficial to the intestinal mucosa.

Many of the therapies currently in use or under clinical evaluation are associated with systemic toxicity since they do not act on a well-defined target[137]. Therefore, the combination of radiotherapy and chemotherapy has still remained the strategy of choice in CRC[145] and not much attention is paid to nutritional accompaniment. Since the gut microbiota seems to be a pivotal factor in inflammatory disease and CRC development, overcoming therapy resistance could also improve with changes in diet. For this purpose, is crucial the development of foods containing compounds with anti-CCSC activity such as flavonoids but with better bioaccessibility and bioavailability[154]. Moreover, bacteriotherapy is a great opportunity to customize CRC treatment and the following tools that we will mention could be useful in this type of therapy. The modification of patient microbiome tending to resolve dysbiosis through the administration of beneficial bacteria could significantly improve conventional treatment [93]. Even more, considering that some microbial species exhibit tumor targeting specificity, this strategy could ameliorate cytotoxicity in non-tumor cells. Regarding bacterial products, given their low molecular weight and hydrophobicity, they can easily enter tumor tissues and exert their action[155]. These features result in the use of microorganisms with potential preventive or palliative action in CRC currently receiving special attention. In fact, microbe-based therapies, and bacteria-mediated modulatory strategies are studied to be used for the delivery of drugs to the tumor site and to produce anti-cancer vaccines[145,155]. However, the information about the toxins, metabolism of microbial-derived agents and complications from bacteriotherapy is still limited[155]. Thus, placing emphasis on clinical research that allows the use of these new therapies, overcoming the obstacles related to it, will be essential in the coming years.

In addition, as we discussed in the previous section, it is also necessary to focus on the restructuring of the TME in favor of improving conventional CRC treatment. Restructuring the extracellular matrix, modulating the immune response with vaccines, antibodies, or inhibitory drugs, employing drugs that induce changes in the secretion profile of TME cells, switching macrophages polarization and inhibiting CAFs and processes like fibrosis and inflammation are some of the potential effective techniques under investigation[1,5,116,128].

The development of vaccines containing CSC-specific antigens is also under investigation[5]. However, since many of the antigens present in this cell subtype are also found in differentiated cells or normal stem cells, this is a challenge to overcome for successful therapy.

So, the combination of conventional therapies with new targeted inhibitors (*e.g.* inhibitors of signaling pathways or molecules derived from TME) plus an appropriate diet that favors beneficial colonic microbiota, as well as the use of targeting methods such as charged nanoparticles or specific bacterial species, could constitute a reliable alternative to fight with CRC chemoresistance and relapses. The use of different *in vitro* and *in vivo* preclinical models of CCSC such as colonospheras, organoids and xenografts, is essential to achieve this goal and bring it to clinical research.

In the near future, the challenge will be the development of selective and combined therapies to promote: (1) CSC eradication; (2) Eradication of cancer cells, owing to their phenotypic plasticity, even in the absence of CSC features; and (3) Reduction of the damage to cells outside the tumor bulk.

In any case, it is clear that the standardization of treatment protocols is not always effective for this disease. It is advisable to resort to a combined and personalized therapy that considers the needs and responses of each patient.

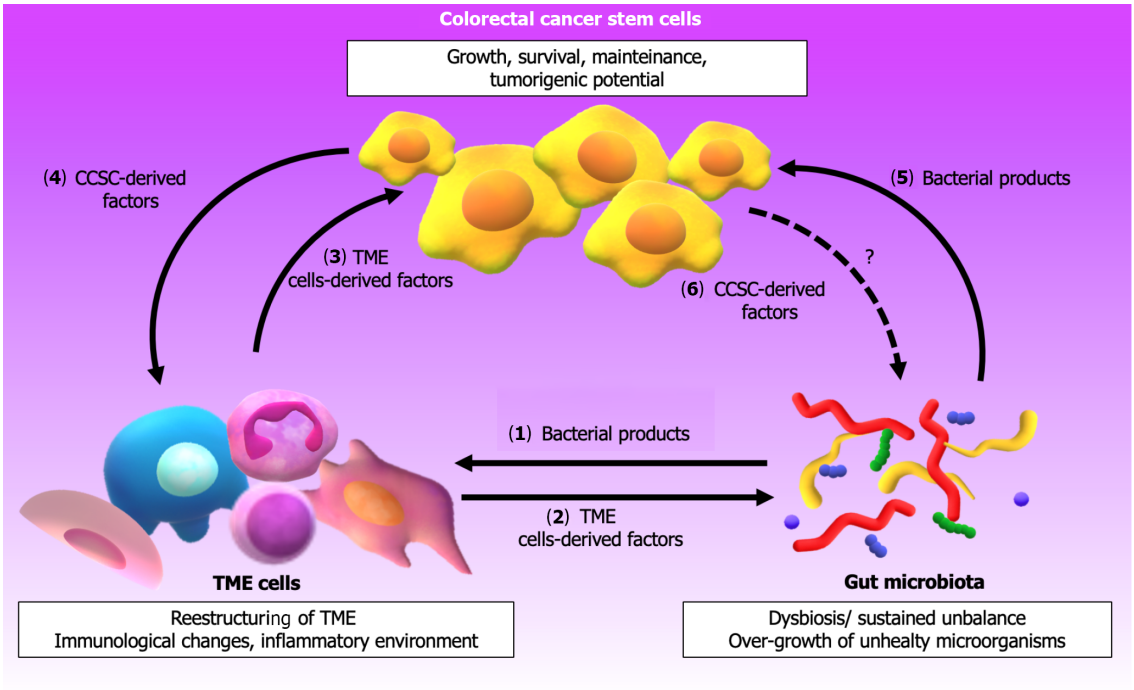

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Figure 2 The interplay among the tumor microenvironment and the gut microbiota influences the stemness of colorectal cancer cells. (1) Gut microorganisms and/or their derived products in a dysbiosis context influence the restructuration of tumor microenvironment (TME), favoring the release of several factors (growth factors, cytokines, non-coding ribonucleic acids and enzymes), immunological changes and an inflammatory environment; (2) The factors released by TME cells impact on intestinal microbiota promoting the growth of unhealthy microorganisms and their sustained unbalance; (3) Moreover, these TME factors can modulate the properties and behavior of colorectal cancer stem cells (CCSC) promoting effects such as their growth, survival, maintenance and tumorigenic potential; (4) In this context, CCSC response expressing factors that enable them to communicate with stromal cells and also influence a TME restructuration; (5) Microorganisms and/or their derived products can directly modulate the features and properties of CCSC, which in response; and (6) Probably affect the intestinal microbiota. All these associated events contribute to colorectal cancer progression. CCSC: Colorectal cancer stem cells; TME: Tumor microenvironment.

CONCLUSION

Figure 2 shows the interplay between the TME and the gut microbiota that influences the properties/behavior of CCSC. Besides, the reader can appreciate that CCSC influence on cells from TME favoring CRC progression but probably also on gut microbiota. The knowledge described in the present review provides data that may promote future research aimed at addressing the complexity of the components in the CRC-associated microenvironment and microbiota. Compounding such complexity, CRC is not an isolated neoplasm, but it's rather emerging as a dynamic pathology whose actors are capable, regrettably, of contributing to evasion mechanisms of the current therapeutic strategies. Although the incidence and mortality from CRC have decreased in recent years, a large number of patients still suffer from relapses due to resistance to treatment. The development of metastases and chemoresistance is undoubtedly one of the greatest challenges in CRC therapy. As we have seen in this work, the properties of the CCSC make this cell subtype have the main responsibility for the recurrences. The shift in the tumor niche and the intestinal microbiota favors the acquisition of CSC characteristics, promoting a worse prognosis of CRC. Although much is currently known about the interrelationship between components of the TME, the microorganisms present in the intestinal mucosa and CCSC, there is still much to be discovered in this field.

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FOOTNOTES

Author contributions: Novoa Díaz MB and Carriere P contributed to conceptualization, methodology, investigation, formal analysis, visualization, writing-original draft, and writing-review and editing; Gentili C contributed to conceptualization, methodology, resources, investigation, formal analysis, visualization, writing-original draft, supervision, writing-review and editing, project administration, and funding acquisition.

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REVIEW

Delineating the glioblastoma stemness by genes involved in cytoskeletal rearrangements and metabolic alterations

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Abstract

Literature data on glioblastoma ongoingly underline the link between metabolism and cancer stemness, the latter is one responsible for potentiating the resistance to treatment, *inter alia* due to increased invasiveness. In recent years, glioblastoma stemness research has bashfully introduced a key aspect of cytoskeletal rearrangements, whereas the impact of the cytoskeleton on invasiveness is well known. Although non-stem glioblastoma cells are less invasive than glioblastoma stem cells (GSCs), these cells also acquire stemness with greater ease if characterized as invasive cells and not tumor core cells. This suggests that glioblastoma stemness should be further investigated for any phenomena related to the cytoskeleton and metabolism, as they may provide new invasion-related insights. Previously, we proved that interplay between metabolism and cytoskeleton existed in glioblastoma. Despite searching for cytoskeleton-related processes in which the investigated genes might have been involved, not only did we stumble across the relation to metabolism but also reported genes that were found to be implicated in stemness. Thus, dedicated research on these genes in GSCs seems justifiable and might reveal novel directions and/or biomarkers that could be utilized in the future. Herein, we review the previously identified cytoskeleton/metabolism-related genes through the prism of glioblastoma stemness.

Key Words: Glioblastoma; Stemness; Cytoskeleton; Metabolism; Biomarkers; Therapy

Core Tip: Glioblastoma stemness intensifies the resistance to treatment *via* increased invasiveness. Among the processes crucial for glioblastoma stem cells, metabolism is known to influence invasion. However, the cytoskeleton is currently negligent in glioblastoma stemness research, while it also regulates invasion. Herein, we review the link between stemness and cytoskeleton/metabolism-related genes that we previously identified in glioblastoma. These genes influence stemness *via* numerous biological processes; for some genes, clinical trials are currently ongoing. Others were connected to glioblastoma stemness for the first time. Future glioblastoma-related research should delve into the cytoskeleton since the concept is already encouraging.

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INTRODUCTION

Glioblastoma (GBM) has remained an incurable condition with increasing incidence in many countries [1,2]. Although GBM is less prevalent than breast, colon, or lung cancer, it outperforms other tumors by affecting patients in the prime of their lives and causing them to lose many years of life[3]. The initial intervention in newly diagnosed GBM includes a surgical approach, with post-surgery temozolomide (TMZ) and radiation therapy[4]. Adding tumor-treating electric fields (TTFields) to maintenance TMZ chemotherapy was found to prolong progression-free and overall survival, but is currently limited due to the lack of methods to predict or quantify the efficacy of TTFields (the imaging features associated with treatment response are unclear and there are no predictive neuroimaging markers). Moreover, the treatment device is required to be worn for a predetermined period (typically approximately 75% of the time) or until there is a clinical progression of the disease, which introduces a delay in getting used to the device and makes patients anxious with regard to the intended therapy effect[5]. Strong motivation to predict TTField efficacy in a patient-specific manner was provided[6]. Nevertheless, glioblastoma recurrence is practically inevitable which, combined with a grim prognosis and ineffective treatment, underlines the importance of further research into this deadliest tumor[3,7].

One of the GBM traits that implicate the lack of effective treatment is the heterogeneity that can be explained by both clonal evolution and the presence of stem cells[8]. Stemness refers to the molecular events that underlie the essential characteristics of self-renewal and differentiation into daughter cells [9]. On the cellular level, some processes were indicated as crucial for GBM stemness, namely epigenomic regulation, posttranscriptional regulation, and metabolism[10]. Glioblastoma stemness research in recent years has also bashfully introduced a key aspect of cytoskeletal rearrangements [11, 12] while it has been long time since this machinery is well-known for controlling two processes that influence cancer malignant behavior, *i.e.*, cellular division and invasion[13]. The stemness itself is also responsible for potentiating the resistance to treatment[14,15], *inter alia* due to increased invasiveness [16]. In addition, more recent studies have identified the role of metabolism in GBM invasion[17]. Although non-stem glioblastoma cells are less invasive than GBM stem cells (confirmed by sevenfold reduced cell migration through the Matrigel, or 3.8-times and 6.8-times lower expression of matrix metalloproteinase-14 and -16)[18], the same cells also acquire stemness with greater ease if they are characterized as invasive cells and not tumor core cells[19,20].

The above-mentioned data imply that GBM stemness should be further explored for any phenomena related to the cytoskeleton and metabolism, as they may provide the missing puzzle from the point-of-view of invasion. Moreover, the cytoskeleton and metabolism are related; for instance, the cytoskeleton is involved in carbohydrate metabolism[21] and at the same time the actin and tubulin require energy from nucleotide hydrolysis to maintain structural dynamics[22]. Cytoskeletal rearrangements and metabolic alterations are important not only for GBM cells but also for neuronal and glial progenitors. For example, cytoskeleton dynamics underlie the cellular asymmetry while metabolic reprogramming ensures a transition in energy production from glycolytic to oxidative[23,24]. Nevertheless, it is possible to discriminate normal glial cells from glioblastoma; the cancerous cells present decreased cortical but increased intracellular stiffness, and preferentially metabolized glucose into lactate despite the abundance of oxygen[17,25]. Stiffness and metabolic adaptations can also influence stem cell differentiation[26,27]. Moreover, the cellular cross-talk that utilizes cytoskeleton or metabolites affects physical dynamics and signaling of various cell types including astrocytes, neurons, and oligodendrocytes[28, 29]. In cancers, such cross-talk renders abnormal protrusions or extensions termed as tumor microtubes that contribute to glioma resistance[30]. These structures are rich in cytoskeletal proteins, such as actin and tubulin, and are known to modify energetic metabolism of the receiving cells *via* transport of

mitochondria[31].

Our previous research has proved that interplay between metabolic alterations and cytoskeletal rearrangements exists in GBM[32]. Of genes described below in the present review (some previously identified genes were not included if their implication in stemness was not found in the literature) (Supplementary Table 1)[33-37], the example of a relationship between metabolism and cytoskeleton can be visualized (Figure 1) based on the literature on methylenetetrahydrofolate dehydrogenase 2 (MTHFD2)[38-41] and ribonucleotide reductase subunit M2 (RRM2)[42-45]. In our previous research, despite searching for cytoskeleton-related processes in which the investigated genes might have been involved, not only did we stumble across the relation to metabolism, but we also reported some genes which were found to be implicated in glioblastoma stemness. Thus, the dedicated work on these genes in the GBM stem cells (GSCs) seems justifiable and might reveal novel therapeutic directions and/or biomarkers that could be utilized in the future. Herein, we review the previously identified cytoskeleton/metabolism-related genes through the prism of GBM stemness. Literature screening allowed the decision to split these genes based on whether their role in stemness is known from GBM or another tumor, the latter suggesting an urgent need to experimentally verify the observations in the glioblastoma context.

GENES WITH CONFIRMED ROLE IN GLIOBLASTOMA STEMNESS

Bone morphogenetic protein 4

Based on the literature abundance, the best-known from its implication in glioblastoma stemness is bone morphogenetic protein 4 (*BMP4*). The bone morphogenetic proteins are growth factors from the TGF-β superfamily that undergo expression during embryogenesis and control development. Initially denoted as crucial for osteogenesis, they are now described as regulators of gastrulation, neurulation, mesoderm patterning, proliferation, and differentiation in many tissues[46]. About 15 years ago, it was found that the signaling *via* BMPs and their cognate receptors (BMPRs) influenced the activity of normal brain stem cells but could also inhibit the cancer-initiating GBM stem-like cells[47]. Later the same year, these authors confirmed that *in vivo* delivery of *BMP4* blocked the tumor growth and associated mortality, which occurred in all mice following intracerebral grafting of human glioblastoma[48]. This protein was suggested as a non-cytotoxic therapeutic agent that can be utilized in combination with stem cell-based therapy[49]; this complements its usage as an agent used to differentiate GSCs into normal glial cells [50]. *BMP4* has been found promising to the extent that it entailed the development of novel therapies. For example, one that utilizes the oncolytic vaccinia virus was developed to alleviate glioblastoma and prevent its recurrence[51]. Later on, the cell-based treatment option of BMP4-secreting human adipose-derived mesenchymal stem cells was found to reduce proliferation and migration both *in vitro* and *in vivo*, as well as prolong survival in a murine model[52]. Still, Videla Richardson *et al*[53] admitted that little is known about this morphogen regarding triggered cellular events, which prompted the authors to establish several GSC-enriched cell lines growing as adherent monolayers and not floating neurospheres. Distinct lineage preferences were noticed depending on the expression pattern of BMP signaling-astrocyte fate or neuronal commitment was noticed and, under certain conditions, even a smooth muscle-like phenotype[53]. Providing new findings to the available data, *BMP4*-overexpressing neural stem cells were found to promote GSCs apoptosis *via* Smad1/5/8 signaling[54]. Moreover, recent studies indicate a formerly underestimated link between *BMP4* and metabolism or mechanotransduction which affects oxygen consumption or matrix stiffness[55]. The latter is known to be associated with cytoskeletal remodeling[56,57]. With regard to the cytoskeleton, *BMP4* was found to re-organize actin dynamics *via* activation of Rac1, Rho, and Cdc42[58]. The impact of *BMP4* in inducing asymmetric cell division was also noted, limiting the GSCs expansion[59]. The newest literature data on *BMP4* consider it on a broader scale, either evaluating other GBM aspects and referring to *BMP4*, or investigating upstream/downstream molecules. Ciechomska *et al*[60] explored *EGFR* alterations in glioblastoma since GSCs with various *EGFR* levels respond differently to therapy; the authors found that EGFR/FOXO3a/BIM signaling pathway determined chemosensitivity of BMP4-differentiated GSCs to TMZ. On the other hand, Wu *et al*[61] identified BIRC3 as an inducer of glioblastoma stemness *via* downstream *BMP4* inactivation. At last, the most recent paper by Verploegh *et al*[62] summarized the cellular viability variance in response to *BMP4* and proposed early-response markers for sensitivity to *BMP4*. Three cultures with the highest sensitivity for *BMP4* revealed a new cell subpopulation that presented a reduced cell proliferation but an elevation of apoptosis. These changes in composition correlated with treatment efficacy; the latter was predicted using *OLIG1/2* expression. Furthermore, upregulated *RPL27A* and *RPS27* were considered early-response markers. Interestingly, *RPS27* is one of the genes identified in our previous study that prompted us to investigate the aspects presented in this review. This gene will be described below in a separate subsection.

Glutamate ionotropic receptor NMDA type subunit 2B

Glutamate ionotropic receptor NMDA type subunit 2B (GRIN2B) encodes one subtype of glutamate-binding GluN2 subunit, which is a part of the N-methyl-D-aspartate receptor (NMDAR). Ionotropic

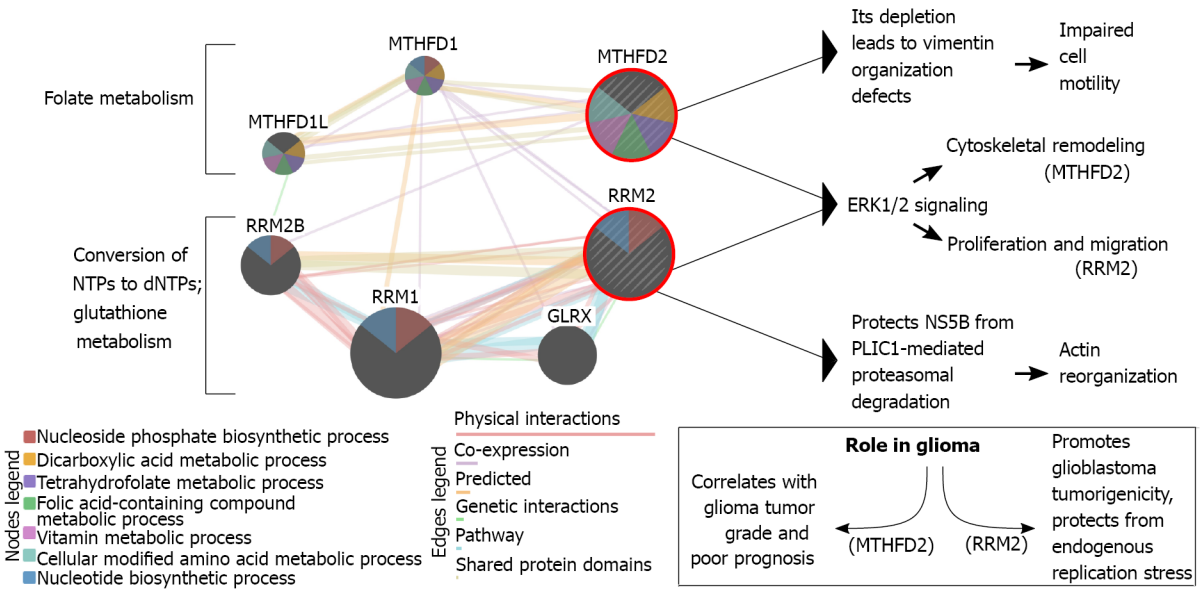

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Figure 1 Example of the interplay between cytoskeleton and metabolism using the biological function of methylenetetrahydrofolate dehydrogenase 2 and ribonucleotide reductase subunit M2 enzymes. Typically, methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) dehydrogenase is known for its activity in folate metabolism, whereas ribonucleotide reductase subunit M2 (RRM2) reductase is known for the conversion of ribonucleotide triphosphates to deoxyribonucleotide triphosphates which requires metabolic resources supplied by reduced glutathione. However, these two enzymes (encircled in red) are also involved in cytoskeletal rearrangements that are summarized on the right side of the figure. Literature data indicate that they also affect the same pathway (*i.e.*, ERK1/2 signaling) but render various outcomes. Moreover, their role in glioma has already been proposed (bottom-right panel). Figure created using Inkscape and GeneMania (*MTHFD2* and *RRM2* as query genes; five "resultant" genes included to highlight interconnectivity; exemplary metabolism-related processes included from the built-in functional analysis). NTP: Ribonucleotide triphosphates; dNTPs: Deoxyribonucleotide triphosphates; MTHFD: Methylenetetrahydrofolate dehydrogenase; RRM2: Reductase subunit M2.

glutamate receptors from this family mediate Ca^{2+}, *i.e.*, the permeable component of excitatory synaptic transmission in the central nervous system (CNS)[63]. NMDARs assemble from four subunits: two GluN1 and two GluN2. The former subunits are widely expressed in the nervous system, while four subtypes of GluN2 subunits (from "A" to "D") are characterized by various expression patterns[64]. *GRIN2B* encodes the GluN2B subunit, which is abundantly expressed in the prenatal period, then declines in most brain parts[65]. The presence of GluN2B in such an early stage implies that it contributes to brain development, circuit formation, synaptic plasticity, as well as migration and differentiation[66]. Glutamate-dependent synaptic transmission is frequently dysfunctional in gliomas[67], and regarding this specific subunit, an enrichment of expression was noticed in GSCs[68]. In our previous research, with the use of literature data, we related this gene with the cytoskeleton since GluN2B interacts with cytoskeletal protein α-actinin-2 *via* the carboxyl-terminal domain[63]. It might be of importance as α-actinin-2 is closely associated with multimerins which are possible markers and therapeutic targets in low-grade glioma[69]. Moreover, one of the multimerins encoded by the *MMRN1* gene was found to be correlated to stemness and chemoresistance, although these observations were based on the leukemia model[70]. Nevertheless, *GRIN2B* is confirmed to influence stemness not only in glioblastoma but also in lung cancer. She *et al*[71] identified *GRIN2B* expression to be higher in primary tumors than in normal tissues, and at the same time higher in metastatic lesions than in primary tumors which contributed to poorer prognosis. Moreover, the same authors observed inhibition of tumorsphere formation during *GRIN2B* silencing.

Homeobox protein A10 and A1

The homeotic genes, in vertebrates denoted as homeobox, are highly conserved and regulate the proper development of various body segments during ontogeny[72]. Homeobox protein A10 (*HOXA10*) is implicated in the embryogenesis of the uterine epithelium, stroma, and muscle[73]. In response to hormones, it undergoes periodical expression in the mature endometrium, controlling receptivity during the implantation window[74]. Concerning GBM stemness, the functionality of *HOXA10* was presented as a direct result of the activation of protein from the *Trithorax* family, which serves as a histone methyltransferase, *i.e.*, MLL. Afterward, *HOXA10* activated other *HOXA* genes, such as *HOXA7* and *HOXC10*[75]. In another study, *HOXA10* was marked as one of the strongest candidates (alongside the *HOX -A9*, *-C4*, and *-D9* genes), having value as a therapeutic target and biomarker for both GBM and GSCs[76]. Our previous research echoed the data that *HOXA10* facilitated cytoskeleton remodeling (*via CK15*)[77], promoted tumorigenesis in glioma[78], and regulated homologous recombinant DNA

repair and subsequently TMZ resistance in GBM[79]. Since stemness also contributes to treatment resistance[14], the last two events complement each other mutually. Another homeotic gene that we identified in our previous study was *HOXA1*, a homeobox that is abundantly expressed in the mesoderm and neuroectoderm at the level of the brainstem precursor[80]. Upregulation of *HOXA1* was noted in GBM, which inversely correlated with the survival of patients[81]. This homeotic member was also implicated in regulating the cytoskeleton *via* E-cadherin. Namely, *CDH1*-dependent signaling was found to increase *HOXA1* expression through Rac1, *i.e.*, the same pathway that regulates actin cytoskeleton at cadherin adhesive contacts[79]. With regard to GBM stemness, Schmid *et al*[82] observed upregulated HoxA locus (encompassing, *e.g.*, *HOXA1*) after they dedifferentiated murine astrocytes into GSCs *via* Rb knockout, Kras activation, and Pten deletion. These cells were sufficient to form GBMs in their transplant mouse model. Although the insights did not provide further mechanistic details, the regulation loop of *HOXA1* and HOXA transcript antisense RNA (HOTAIRM1) was found to be involved in stemness maintenance[81,83]. This was presented in colorectal carcinoma and uveal melanoma. Still, taking into account the study by Schmid *et al*[82], the profound investigation of *HOXA1* in GSCs in this aspect should be considered.

Matrix metalloproteinase 13

Matrix metalloproteinases are constituents of extracellular matrix (ECM) belonging to the zinc-containing endopeptidases family that encompasses 23 members[84]. Functionally, these calcium-dependent molecules are responsible for the degradation and remodeling of other proteins that constitute ECM. Moreover, their roles in various biological and physiological processes dependent on hormones, growth factors, and cytokines were described[85]. It is known that different ECM components modulate cancer stem cells' properties; regarding glioblastoma, the confirmed ones were type I collagen, laminin α2, fibronectin, periostin, decorin, and lumican[86]. Matrix metalloproteinase 13 (*MMP13*) is a collagenase almost universally upregulated in the pan-cancer view[87]; in GBM, its overexpression increases migration and invasion[88], as well as confers poor prognosis[89]. The relationships between *MMP13* and the cytoskeleton[33] or metabolism[90] are known. In terms of stemness, Inoue *et al*[91] suggested that highly invasive potential GSCs depended on MMP13 enzymatic activity; the authors also proposed MMP13 as a potential therapeutic target.

MTHFD2

The folate cycle is responsible for appropriate cellular metabolism by regulating ATP production, methylation reactions for DNA/protein synthesis, or developing immunomodulatory molecules that orchestrate signaling and cytotoxicity[92]. The differences between *MTHFD1* and *MTHFD2*, two enzymes implicated in the folate pathway, include the use of different co-enzyme (NADP *vs* NAD), functionality (*MTHFD1* has three distinct enzymatic activities while *MTHFD2* is bifunctional), and location (cytoplasm *vs* mitochondria). Compared to *MTHFD1*, which generates NADPH and formate for purine biosynthesis, *MTHFD2* is overexpressed in rapidly proliferating malignant tumors. It is considered the "main switch" that enables mitochondria to produce additional growth-facilitating one-carbon units and generates NADH necessary for protection from reactive oxygen species[93]. *MTHFD2* is also an excellent example to present the link between metabolism and cytoskeleton. Lehtinen *et al*[39] have found that *MTHFD2* depletion leads to vimentin organization defects, and identified this gene as a regulator of cell migration and invasion. Regarding glioma, *MTHFD2* was found to be associated with tumor grade and prognosis[38]. Inhibition of this enzyme in GSCs induced apoptosis and affected not only central carbon metabolic pathways (*e.g.*, glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle) but also unfolded protein response, highlighting a novel connection between one-carbon metabolism and reaction to cellular stress[94]. Nishimura *et al*[95] suggested that the purine synthesis pathway, as well as folate-mediated one-carbon metabolism, seem to be crucial for the maintenance of tumor-initiating cells. The same authors also concluded that EGF-induced expression of *MTHFD2* may be mediated by Myc, with the latter regulating the expression of metabolic enzymes for the maintenance of brain tumor-initiating cells.

Plant homeodomain finger-like domain-containing protein 5A

Alternative splicing maintains post-transcriptional gene regulation, which enables a single gene to be transcribed into various RNAs, diversifying the proteome. Abnormal splicing function can lead to tumor-related processes, *e.g.*, proliferation, angiogenesis, and metastasis[96]. Spliceosome, a dynamic machinery responsible for splicing, is made of small nuclear ribonucleoproteins (snRNPs; five molecules are known: U1, U2, U4, U5, and U6) and numerous non-snRNP proteins[97,98]. U2 snRNP comprises U2 snRNA, SF3a complex, and SF3b complex, which are responsible for recognizing branchpoint sequences during initial spliceosome assembly stages[99]. Splicing factors comprising the SF3b complex include plant homeodomain (PHD) finger-like domain-containing protein 5A (*PHF5A*), which facilitates interactions between the U2 snRNP and RNA helicases[100] but can also bind chromatin *via* its PHD that is composed of a small zinc finger structural fold[101,102]. The knockdown of *PHF5A* results in reduced GBM viability and cell cycle arrest[103]. Trappe *et al*[104] revealed that systematic deletion of its yeast homolog is lethal, showing that *PHF5A* is crucial for cell viability. The flagship paper on *PHF5A*

in brain tumor[105] indicates that the gene is required to expand GSCs and that in these tumor-initiating cells, but not untransformed neural stem cells, *PHF5A* contribute to the identification of exons having unusual C-rich 3′ splice sites in thousands of essential genes. The same authors inhibited *PHF5A*, which reduced GSCs-driven tumor formation *in vivo* and inhibited the growth of established GBM patient-derived xenograft tumors.

Ribosomal protein S27

One of the most dynamic and largest molecular motors (driven by a complex thermal ratchet translocation mechanism) are ribosomes[106]. Metallopanstimulin-1, also known as ribosomal protein S27 (*RPS27*), is a constituent of the human 40S ribosome that is mainly found in the cytoplasm while it can also relocate to the nucleus[107] or even extracellular space[108]. Regarding the nuclear location, it is able to interact with DNA *via* its C4-type zinc finger[109]. In glioblastoma, *RPS27* was found to be correlated with age in IDH-mutated glioma patients and with Ki67 in GBM patients. Interestingly, it is detected in astrocytic tumors but not in normal astrocytes unless the tissue was inflamed[109]. This allowed the same authors to emphasize that in comparison to inflammatory tissue (in which only a small number of macrophages were positive for *RPS27*), almost all macrophages in tumor tissue were distinctly enriched in *RPS27* expression. As for GSCs, the ribosomes and related proteins were generally found to reprogram glioma cells to induce plasticity and stemness[110]. Among these molecules, *RPS27* was considered oncogenic with higher expression at the GSC-dominant area[111]. Inquisitive findings revealed that *RPS27* is also detected in the microvascular proliferation area and pseudopalisading cells around necrosis[110]. It is worth underlining that aberrant vessels are crucial for the formation of pseudopalisading necrotic regions that provide shelter for residing cancer stem cells from anti-tumor agents, which enable these cells to expand and promote proliferation and growth[112]. As mentioned above, upregulated *RPL27A* and *RPS27* were considered to be early-response markers related to the presence of *BMP4*. This suggests a link that should be further investigated since the signaling of ribosome translation was found to be overexpressed during the response to stress in glioblastoma.

RRM2

A balanced supply of deoxyribonucleotide triphosphates (dNTPs) is a prerequisite of DNA synthesis. Still, *de novo* synthesis of dNTP is also possible *via* the reaction catalyzed by the ribonucleotide reductase (RR) that reduces the C2′-OH bond of the four ribonucleotides triphosphates to form corresponding dNTPs[113]. *RRM2* encodes the β subunit of RR; each RRM2 monomer contains the tyrosyl radical and non-heme iron[114]. Since a sufficient supply of dNTPs drives an uncontrolled DNA replication in cancer[115], it is not surprising that *RRM2* was frequently subjected to molecular therapy[116,117]. Currently, several RRM2 inhibitors have been developed, *e.g.*, radical scavengers, iron chelators, subunit polymerization inhibitors, or expression silencers[118-120]; this is to inhibit proliferation, division, but also invasion[32]. In glioblastoma, *RRM2* is responsible for the advancement of GBM tumorigenicity and protection from endogenous replication stress *via* the BRCA1-RRM2 axis[45]. For glioma in general, regulation of proliferation and migration *via* ERK1/2 and AKT signaling was noted[44]. Available literature also links the *RRM2* to the cytoskeleton *via* hPLIC1; the latter decreases during *RRM2* downregulation, which entails actin cytoskeleton re-organization[42]. Perrault *et al*[121] have suggested that *RRM2* can be a chemoresistance driver that dictates how GBM cells respond to TMZ. The same authors further verified that *RRM2*-overexpressing cells had enhanced DNA repair efficiency. Moreover, the use of a selective FDA-approved RRM2 inhibitor, 3-AP Triapine, enabled Perrault *et al* [121] to observe that in comparison to both TMZ and control, glioblastoma treated with the 3AP + TMZ formed fewer neurospheres that were also significantly smaller. Another group found that *RRM2* expression dramatically declined after 12 d of dasatinib treatment compared to naïve GSCs of the GSC8 cell line[122].

Serum amyloid A protein 2

In order to re-establish homeostasis, both adaptable and primordial mechanisms exist; the latter comprises the acute-phase response (APR) that is a set of changes that occur after inflammation, infection, or trauma[123]. During APR, the changes include the altered levels of serum proteins, with the most notable being C-reactive protein and serum amyloid A (SAA)[124]. Being an apolipoprotein, SAA is related to plasma high-density lipoprotein and is implicated in the cholesterol transport to the liver for excretion as bile[125]. Its other functions include regulation of amyloidogenesis, tumor pathogenesis, anti-bacterial events, and inflammatory response[126]. The role of SAA in tumor progression was suggested owing to its cytokine-like properties that influence the course of inflammation[127]. *SAA2* is one of the paralogs of the family and was investigated as a lung cancer biomarker a few years ago[128]. The description of its role in glioblastoma is limited, yet it is already known that *SAA2* increases GBM proliferation and invasion[129]. Knebel *et al*[130] have confirmed that SAA production occurs not only in the liver but also in tumor cells; the authors emphasized that exploring the SAA influence on the cytoskeleton and invasiveness using more complex assays is needed. In terms of GBM stemness, Adamski *et al*[131] recently have compiled the literature data and stated that *SAA2* is implicated in a drug-promoted cellular dormancy, with the latter being closely connected to stem cell characteristics.

The group also indicated the ability of *SAA2* to sustain inflammatory conditions in the brain, which consequently supports TMZ resistance and induces the expression of stemness markers in glioblastoma.

Wilms' tumor protein 1

The 5-methylcytosine (5mC) and its derivatives have altered patterns in a range of tumors. 5mC can be recognized and oxidized to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine by Ten-Eleven translocation (TET) enzymes[132,133]. One of the transcription factors that directly interacts with TET proteins is Wilms' tumor protein 1 (WT1): A master regulator essential for urogenital, epicardium, and kidney development that can act as a tumor suppressor or oncoprotein in multiple tumors[134, 135]. Initially cloned as a suppressor of Wilms' tumor, WT1 is now considered to be an oncoprotein in hematologic malignancies and a variety of solid tumors, as well as the protein with the highest potential for cancer immunotherapy[136-138]. According to the phase I/II clinical trial, WT1 peptide-based vaccine for glioblastoma patients was considered safe and induced cellular and humoral immune response[139]. This is important due to the fact that *WT1* is involved in GBM tumorigenicity *via* increasing proliferation and decreasing apoptosis[140]. As for the impact on the cytoskeleton, this protein was found to interact with actin both in the cytoplasm and nucleus, as well as supposedly binds to RNA in a cytoskeleton-dependent regulation manner[141]. Focusing on GBM stemness, Mao *et al*[142] found that *WT1* was expressed predominantly in mesenchymal GSCs which, compared to proneural stem cells subtype, are characterized by higher proliferation, greater radioresistance, and implication in worse patients' prognosis. Uribe *et al*[143] reviewed that mesenchymal GSCs develop tumors having more blood vessels, hemorrhagic lesions, and necrotic areas; the expression pattern in these stem cells generally facilitates inflammation, angiogenesis, migration, invasion, and glycolysis-mediated metabolism. Undoubtedly, more insights are needed concerning GBM molecular pathways in which *WT1* is implicated.

GENES WITH STILL UNCONFIRMED ROLE IN GLIOBLASTOMA STEMNESS

Chemokine-like factor superfamily 6

Cytokines are soluble proteins that are secreted by immune and non-immune cells in response to stimulants such as immunogens or mitogens; this allows them to maintain the immune response and homeostasis[144]. Chemokines constitute a specific type of small (8-13 kDa) cytokines that promote the directed chemotaxis of nearby cells[145]. Consisting of nine members, the chemokine-like factor superfamily (CMTM) is expressed throughout the human tissues and regulates immune, circulatory and muscular systems, as well as the hematopoiesis[146-149]. The aberrant *CMTM* expression is implicated in various diseases, *e.g.*, rheumatoid arthritis, atopic dermatitis, focal cerebral ischemia, male infertility, as well as tumorigenesis and metastasis[150-153]. The influence of *CMTM6* on glioblastoma is known, but the research in this entity seems to be in the initial state. Guan *et al*[154] revealed that the highest *CMTM6* expression was noted in the glioblastoma (WHO grade IV) compared with WHO grade II and III gliomas. Enrichment was also observed in both microvascular proliferation and hyperplastic blood vessels, which are both essential for tumor progression. In GBM, *CMTM6* was also associated with one of the genes of immune checkpoints, *i.e.*, *TIM-3*. From a broader glioma scale, the same authors summarized it as a molecule diminishing T-lymphocyte-dependent anti-tumor immunity, reducing patient survival and indicating poor prognosis. However, it is still yet to be elucidated what role *CMTM6* may play in the GBM stemness. Currently, its contribution to such characteristics is confirmed on the basis of data from head-and-neck squamous cell carcinoma. Chen *et al*[155] observed poorer patient prognosis during *CMTM6* overexpression that correlated with overactive Wnt/β-catenin signaling, *i.e.*, the pathway crucial for tumorigenesis, epithelial-to-mesenchymal transition (EMT) and cancer stem cells maintenance. Silencing of *CMTM6* led to PD-L1 downregulation, decreased tumor growth, and increased $CD8^+$ and $CD4^+$ T-cell infiltration. Eventually, the authors not only suggested the therapeutic suitability of *CMTM6* but also concluded that this protein is implicated in EMT, stemness, and T-cell dysfunction. Similar research in the glioblastoma context is advisable, especially since *CMTM6* can stabilize PD-L1 protein to impair T-cell function[156,157], as well as their combined expression had prognostic significance in pancreatic ductal adenocarcinoma and triple-negative breast cancer[158]. Nowadays, the role of PD-L1 in cancer and immunotherapy is unquestionable[159]; focusing on another protein related to this well-established molecule might bring novel strategies.

Dual specificity phosphatase 7

Signal transduction is based on phosphorylation and dephosphorylation events performed by kinases and phosphatases, leading to a cellular program relevant to the encountered stimulus[160]. Dual specificity phosphatases (*DUSP*) are responsible for the dephosphorylation of threonine and tyrosine residues on mitogen-activated protein kinases, rendering them inactive[161]. Even if *DUSP7* was only noted as downregulated in glioblastoma, whereas *DUSP1*, *DUSP5*, and *DUSP6* were induced within pseudopalisading and perinecrotic GBM regions[162], the role of *DUSP7* in preserving the pluripotency of non-cancerous stem cells was certified in a murine model[163]. However, its contribution could be

distinct from *DUSP1*, *DUSP5*, and *DUSP6* but similar to *DUSP2*, *DUSP8*, and *DUSP9* which were clustered together with *DUSP7* in the study of Mills *et al*[162]. At last, it is worth noting that *DUSP7* guides chromosome dynamics which is known for being regulated by cytoskeletal proteins[164,165]. The study linking this phosphatase to metabolism revealed that *DUSP7* knockout accelerates metabolic disorder and insulin resistance in mice with a high-fat diet[166].

Kinesin family member 20A

Cytoskeletal elements that act as scaffolds for intracellular cargo transport are microtubules. Motor proteins known as kinesins and dyneins orchestrate microtubule-related transport that is essential for cell differentiation or survival[167]. Kinesins constitute a large superfamily responsible for cargo trafficking, as well as controlling microtubule growth and stability[168]. Increased expression of kinesin superfamily representatives KIF4A, -9, -18A, and -23 was associated with poor prognosis in low-grade glioma and glioblastoma[169]. The pro-cancerous characteristics of *Kinesin family member 20A* (*KIF20A*) were noted more than 15 years ago in pancreatic cancer, which presented a reduction of proliferation once *KIF20A* was downregulated[170]. Currently, accumulating evidence shows that this kinesin is overexpressed in multiple tumors[171]. In glioblastoma, *KIF20A* downregulation induces cell cycle arrest and apoptosis *via* suppressing PI3K/AKT pathway[172]. Regarding cytoskeleton-related events, it is not only essential for cytokinesis but also interacts with Rab6 to regulate Golgi-related vesicle trafficking[173]. Although the role of *KIF20A* in GBM stemness has not yet been confirmed, it was suggested outside of the glioblastoma context in a study by Qiu *et al*[174]. The authors conceived the importance of *KIF20A* in controlling proliferation *vs* differentiation of tumor-initiating cells, based on both the fact that cancer stem cells share many mechanisms with neural progenitors, as well as their observations where *KIF20A* was implicated in balancing symmetric and asymmetric divisions during cerebral cortical development[175]. The *KIF20A* inactivation affected cortical neural progenitor cells that switched from proliferative to differentiative mode. During divisions, daughter cell-fate specification was controlled by *KIF20A* in coordination with *RGS39* and *SEPT710*[174,176].

Neurofibromatosis type 2 protein

Neurofibromatoses (type 1, type 2, schwannomatosis) are distinct, dominantly inherited disorders that have in common the occurrence of nerve sheath tumors[177]. Type 1 neurofibromatosis presents with neurofibromas, cafe-au-lait spots/macules, freckling, and optic gliomas, whereas type 2 neurofibromatosis is characterized by bilateral vestibular schwannomas, ependymomas, and meningiomas [178]. Each disease has a different underlying genetic alteration: Type 1 neurofibromatosis is related to the *neurofibromatosis type 1 protein* (*NF1*) gene, type 2 is linked to *NF2*, while schwannomatosis to integrase interactor 1 (*INI1*, also known as *SMARCB1*). The protein product of *NF2* has the same name as its gene but can also be referred to as Merlin. Although this tumor suppressor is not mutated in GBMs, it exhibited oncogenic properties in glioblastoma when phosphorylated at serine 518; this post-translational modification inactivates Merlin's anti-cancer capabilities, which affects the expression of EGFR or Notch1 and its downstream targets, *i.e.*, *HES1* or *CCND1*[179]. Other authors demonstrated that upon *NF2* re-expression, a regulation of YAP, cIAP1/2, and the Hippo signaling pathway led to the inhibition of glioma growth and progression[180]. Merlin is also known for regulating cell morphology or motility, and its loss renders dramatic changes in cellular adhesion and cytoskeleton organization [181,182]. Specifically, this protein is closely related to ezrin, radixin, and moesin (collectively denoted as "ERM"), *i.e.*, critical proteins that enable the anchorage between membrane proteins and cortical cytoskeleton[183]. Ultimately, the link between *NF2* and stemness might be related to CD44, the receptor of which cytoplasmic tail can interact with both Merlin and "ERM" proteins[184,185]. Literature data state that *NF2* exhibits tumor suppressor function *via* negative regulation of CD44[186], whereas this receptor has been repeatedly indicated as a marker of cancer stem cells in various tumors, such as leukemia and carcinoma of breast, colon, ovarian, prostate, or pancreas[187-191]. Knowing that CD44 is also an upstream regulator of the aforementioned Hippo signaling pathway[192], of which components regulate the stem cell niche, self-renewal, maintenance, and differentiation[193-196], one could investigate Merlin in the GBM stemness context taking into the account the NF2-ERM-CD44-Hippo regulation network.

Retinoid X receptor gamma

The signal transduction molecules being vitamin A derivatives are retinoids, they regulate cellular differentiation and proliferation *via* members of the nuclear receptors superfamily, including retinoic acid receptors (RARs) and retinoid X receptors (RXRs)[197]. The RXR family members (RXRA, RXRB, and RXRG) form heterodimers within the superfamily, *e.g.*, with vitamin D, retinoic acid, or peroxisome proliferator-activated types of receptors[198,199]. RXRs have tumor suppressor properties and, as partners of *RARA* and *RARB*, they are implicated in the anti-proliferative effects of retinoic acid[197]. *RXRG* was found to modulate differentiation and apoptosis in various tumors, indicating its function in cancer pathogenesis[200]. Glioblastoma-related research certifies the general view that *RXRG* contributes to anti-neoplastic effect *via* its ligands; in study by Papi *et al*[201], the treatment of GBM with 6-OH-11-O-hydroxyfenantrene had anti-proliferative and anti-invasive effects. However, the literature

data on glioblastoma stemness seem to focus on RARs rather than RXRs. Ying *et al*[202] evaluated the cellular and molecular responses of GSCs to all-*trans* retinoic acid; this treatment changed cells morphology (*e.g.*, decreased neurosphere-forming capacity), caused growth arrest at G_1/G_0 to S transition, reduced cyclin D1 expression, and elevated p27 expression. Moreover, differentiation markers such as Tuj1 and GFAP were induced, while stem cell markers, such as CD133, Msi-1, Nestin, and Sox-2, had decreased expression. Friedman *et al*[203] provided similar observations with regard to Nestin level or neurosphere formation but also indicated that GBM differentiation induced by all-*trans* retinoic acid is executed *via* the ERK1/2 pathway. Evidently, retinoid-related research in the GBM context frequently focuses on all-*trans* retinoic acid while this isomer is bound only by RARs and not by both RARs and RXRs, as is the case with another retinoic lipid: *9-cis*[204]. Even if two of the best-known retinoid receptors (*RARA* and *RXRA*) are described in detail by Rodriguez *et al*[205] in the GBM stemness context, the data on *RXRG* is still lacking and should begin with evaluation of whether *9-cis* retinoid acid is able to manifest the anti-glioblastoma effects *via RXRG* and subsequently ERK1/2 pathway.

SPARC/Osteonectin, CWCV, Kazal-like domains 1

ECM is a component containing elastin, collagen, laminins, glycoproteins, fibronectin, and proteoglycans. Together, these elements bind *via* cell adhesion receptors and form a complex macromolecular network[206]. Matricellular proteins are made of matrix-binding proteins and cytokines that can be located within the cell or secreted outside[207]. SPARC/Osteonectin, CWCV, Kazal-like domains 1 (SPOCK1), also referred to as testican-1, is an ECM proteoglycan from a matricellular family of proteins that regulate matrix remodeling and affects tumor progression[208-210]. As the interplay between ECM and cytoskeleton is known[211], it is not surprising that changes in *SPOCK1* lead to alterations in cytoskeletal components. For example, Schulz *et al*[212] noticed that *SPOCK1* upregulation paralleled that of *EPB41L4B*, the latter being a cortical cytoskeleton protein underlying cellular membrane. With regard to brain tumors, testican-1 contributes to GBM metastasis and resistance to TMZ, as well as promotes glioma invasion, migration, and proliferation *via* Wnt/β-catenin and PI3K/AKT pathways[213,214]. Mediating TMZ chemoresistance *via SPOCK1* in GBM was independently confirmed by Sun *et al*[215]. Although not yet directly concluded by any scientific group, it is conceivable that the impact of *SPOCK1* on TMZ resistance renders a similar GSCs-related effect as *SAA2* which was described in one of the previous sections.

Ubiquitin-like with PHD and ring finger domains 1

The proteins' turnover and degradation depend on ubiquitination that is orchestrated by the ubiquitin-proteasome system (UPS)[216], of which alterations can lead to several tumor types[217,218]. One of the ubiquitin-protein ligases responsible for the UPS specificity is ubiquitin-like with PHD and ring finger domains 1 (*UHRF1*)[219], a molecule also interacting with DNA methyltransferase 1, which together constitute the main regulatory axis of cellular senescence[220]. *UHRF1* was already identified as a novel oncogene and/or druggable epigenetic target for various tumors[221-223], and Jung *et al*[220] suggested its role as a switch molecule between senescence and cancer. In GBM, *UHRF1* is overexpressed by upstream CD47 and regulates downstream silencing of tumor suppressor gene $p16^{INK4A}$, leading to increased proliferation[224]. Regarding cytoskeleton, *UHRF1* contributes to microtubule organization through its downstream targets: *BRCA2*, *HOOK1*, *KIF11*, and *KIF18A*[225]. The role of *UHRF1* in different types of stem cells is documented but overlooks GSCs. Namely, it was found to be required for the proliferative potential of basal stem cells in response to airway injury[226], as well as regulate the transcriptional marks at bivalent domains in pluripotent stem cells[227]. On the other hand, *UHRF1* decrease was found to be a major cause of DNA demethylation in embryonic stem cells[228] and led to the activation of retroviral elements and delayed neurodegeneration[229]. It is evident that research in the glioblastoma context should be pursued in the future, especially since some epigenetic features, next to transcriptional ones, are unique in GSCs compared to neural stem cells and may include druggable targets for new therapeutic approaches[230].

DISCUSSION

Despite molecular advancements, there is still a considerable need for glioblastoma biomarkers[231], especially since the relatively ineffective treatment leaves the patients with a very dismal chance of survival[232]. One of the glioblastoma traits involved in the absence of effective treatment is tumor heterogeneity which can be explained by clonal evolution and the presence of stem cells[8].

Many independent studies on various tumor types have reported common genes as potential therapeutic or diagnostic biomarkers[233]. Al-Fatlawi *et al*[234] contemplated that biomarker signatures for different cancer types should be similar, due to the fundamental mechanisms shared between tumors, *e.g.*, survival, tumor growth, or invasion. Thus, we presume that our description of stemness-related genes, especially those still unconfirmed in GBM, adds significant value to the current knowledge and provide insights into novel therapeutic or diagnostic directions.

For clarity, a graphical presentation was prepared to emphasize the role of described genes specifically in stem cells, setting aside the rest of the information provided for each gene (Figure 2). At first glance, the most frequently regulated processes are proliferation and chemoresistance, followed by differentiation, tumor growth, invasion, and apoptosis. Except for *BMP4* (increase in asymmetric cell division and apoptosis), *NF2* (reduced self-renewal, tumor growth, stemness maintenance), *RXRG* (decrease in invasion and proliferation), and *DUSP7* (insufficient data for a definite conclusion), the remaining genes exhibit pro-cancerous properties. This corresponds to what was described in subsections, separately for each gene. Interestingly, two genes that promote invasiveness of stem cells (*SPOCK1*, *MMP13*) are known to affect the cytoskeleton[33,212] and, in terms of *MMP13*, also the metabolism[90]. Two genes that were also found to regulate both the cytoskeleton and metabolism were *MTHFD2* and *RRM2*. On the one hand, they control the organization of vimentin and actin; these proteins are known for influencing glioblastoma migratory potential[235,236]. On the other hand, the contribution of *MTHFD2* and *RRM2* to metabolism is related to folate and glutathione cycles that are implicated in the resistance of GBM to therapy[237,238].

In order to gravitate towards the link between metabolism, cytoskeleton, and GBM stemness, the appropriate representatives of each process (including the most frequently regulated processes that were mentioned above), were compiled into a cross-talk network. This allowed us to integrate the aim of our review with the main processes that are regulated by genes described in this work, additionally with the inclusion of GBM biomarkers (acquired from review by Sasmita *et al*[231]). Prevalent interaction types include co-expression and physical interaction between these representatives, there is also a high interconnectivity of the entire network, confirming that these molecular events are related. The cross-talk is visualized in Supplementary Figure 1, whereas the datasets used in the workflow are summarized in Supplementary Table 2.

The narrative of this review was intended to elaborate on the background of the biological machinery in which each successive gene is involved, then proceed with details regarding the regulation of glioblastoma, cytoskeleton/metabolism, and stemness (GBM-related or, if not present in the literature, any available). It is worth emphasizing that the herein described genes constitute more than half of the "top genes" that we established in our previous *in silico* study *via* a multi-stage methodology that included, *e.g.*, enrichment analysis, machine learning algorithm, and differential expression analysis [32]. The remainder was not presented due to a lack of stemness-related literature data (Supplementary Table 1). For the part available in this paper, the majority of genes (*BMP4*, *GRIN2B*, *HOXA10*, *HOXA1*, *MMP13*, *MTHFD2*, *PHF5A*, *RPS27*, *RRM2*, *SAA2*, *WT1*) were confirmed to influence GSCs. The attempt to associate *CMTM6*, *DUSP7*, *KIF20A*, *NF2*, *RXRG*, *SPOCK1*, and *UHRF1* with glioblastoma stemness revealed the promising implication in crucial biological processes that should be validated in future experiments. For *BMP4*, *WT1*, and *RXRG*, their contribution to novel therapeutic strategies was above-mentioned on the basis of literature data, prompting us to investigate whether any clinical trials utilize the products of described genes as drug components or targets. According to the ClinicalTrials website (https://clinicaltrials.gov/), cancer-related data can be found for six genes (Table 1); however, the seventh trial on *GRIN2B* was also included because it focused on brain research and highlights that selective *GRIN2B* antagonist is already developed. Moreover, the details on NF2-related intervention are not yet disclosed[239]. Collectively, these studies are in the early phases, certifying that there is still a room for further research.

CONCLUSION

Taken together, a promising set of genes involved in cytoskeletal rearrangements and metabolic alterations were found to influence glioblastoma stemness *via* a plethora of biological processes. Most of the described genes exhibit pro-cancerous properties; among them, clinical trials on *GRIN2B*, *RRM2*, *WT1*, and *KIF20A* are ongoing and focus on selective inhibitors or peptide-based vaccines. Concerning tumor suppressors, the anti-cancer effect can also be achieved *via* delivery of recombinant proteins (*BMP4*), ligands for tumor suppressors (*RXRG*), or counteracting the pathways that become hyperactive following an anti-oncogene loss (*NF2*). The cytoskeletal phenomena currently linked to the described genes require experimental verification of their contribution to GSCs expansion. Future GBM stemness-related research should generally delve into cytoskeleton and related molecular events, since the concept is already encouraging.

Table 1 Clinical trials that utilize the products of described genes as drug components or targets

Gene	Compound	Condition	Trial number and phase	Intervention details
BMP4	hrBMP4	Glioblastoma	NCT02869243 (phase I)	Delivery of human recombinant BMP4
GRIN2B	EVT 101	Healthy volunteers (brain function assessment)	NCT00526968 (phase I)	Delivery of selective GRIN2B antagonist
RRM2	COH29	Solid tumors	NCT02112565 (phase I)	Delivery of ribonucleotide reductase inhibitor
WT1	DSP-7888	Gliomas (incl. GBM)	NCT02750891 (phase I/II)	Delivery of WT1 peptide-based cancer vaccine
KIF20A	KIF20A peptide	Small cell lung cancer	NCT01069653 (phase I)	Delivery of KIF20A peptide-based vaccination
NF2	IAG933	Solid tumors	NCT04857372 (phase I)	Not yet disclosed (the drug presumably counteracts the YAP/TAZ hyperactivity that occur following NF2 loss)
RXRG	*9-cis* retinoic acid	Breast cancer	NCT00001504 (phase I)	Delivery of RXRG ligand

NF2: Neurofibromatosis type 2 protein; BMP4: Bone morphogenetic protein 4; RXRG: Retinoid X receptor gamma; MMP13: Metalloproteinase 13; RRM2: Reductase subunit M2; SPOCK1: SPARC/Osteonectin; CWCV: Kazal-like domains 1; ECM: Extracellular matrix; WT1: Wilms' tumor protein 1; KIF20A: Kinesin family member 20A; GRIN2B: Glutamate ionotropic receptor NMDA type subunit 2B.

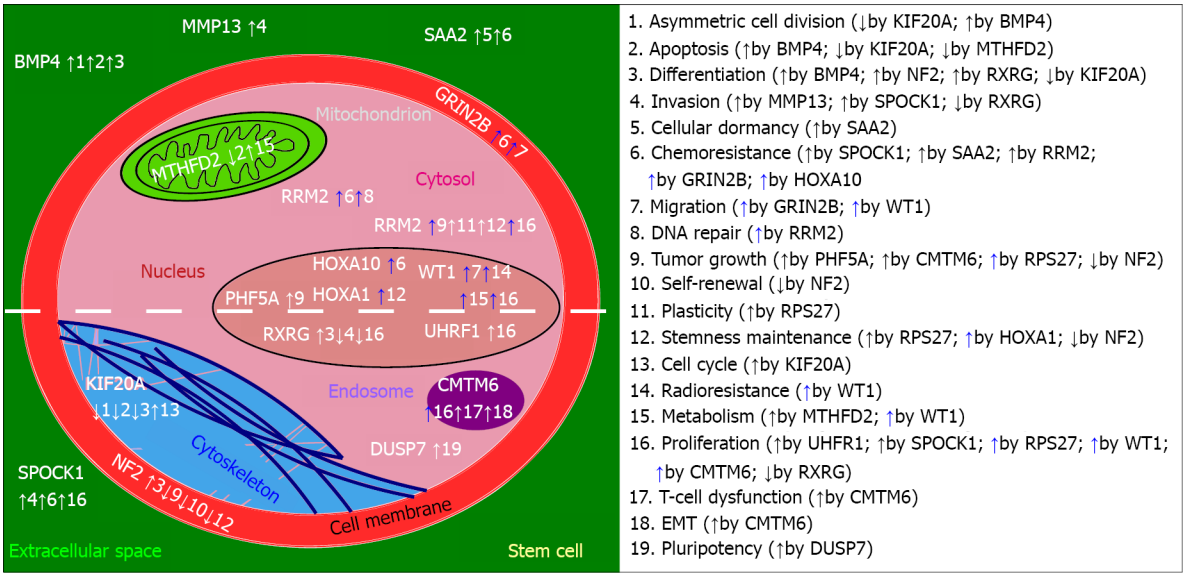

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Figure 2 Impact of described genes on biological processes related to stem cells. The "↑" or "↑" (blue) symbol indicates activation of the process while "↓"denotes inhibition. The impact of genes on processes (numbered from 1 to 19) is either directly confirmed (solid arrow next to the number) or recapitulated based on available data from various literature sources (dashed arrow next to the number). The "↓" (blue) symbol was not required as any gene inhibited the given process in an indirect manner. The white dashed line dividing the stem cell into two halves separates the genes with a confirmed role in glioblastoma stem cells (above the line) from those involved in cancer stemness outside the glioblastoma context (below the line). Figure created using Inkscape. NF2: Neurofibromatosis type 2 protein; BMP4: Bone morphogenetic protein 4; RXRG: Retinoid X receptor gamma; MMP13: Metalloproteinase 13; RRM2: Reductase subunit M2; SPOCK1: SPARC/Osteonectin; CWCV: Kazal-like domains 1; ECM: Extracellular matrix; CMTM: Chemokine-like factor superfamily.

FOOTNOTES

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REVIEW

Tissue-specific cancer stem/progenitor cells: Therapeutic implications

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Abstract

Surgical resection, chemotherapy, and radiation are the standard therapeutic modalities for treating cancer. These approaches are intended to target the more mature and rapidly dividing cancer cells. However, they spare the relatively quiescent and intrinsically resistant cancer stem cells (CSCs) subpopulation residing within the tumor tissue. Thus, a temporary eradication is achieved and the tumor bulk tends to revert supported by CSCs' resistant features. Based on their unique expression profile, the identification, isolation, and selective targeting of CSCs hold great promise for challenging treatment failure and reducing the risk of cancer recurrence. Yet, targeting CSCs is limited mainly by the irrelevance of the utilized cancer models. A new era of targeted and personalized anti-cancer therapies has been developed with cancer patient-derived organoids (PDOs) as a tool for establishing pre-clinical tumor models. Herein, we discuss the updated and presently available tissue-specific CSC markers in five highly occurring solid tumors. Additionally, we highlight the advantage and relevance of the three-dimensional PDOs culture model as a platform for modeling cancer, evaluating the efficacy of CSC-based therapeutics, and predicting drug response in cancer patients.

Key Words: Cancer stem cells; Therapy resistance; Tissue-specific cancer stem cell markers; Patient-derived organoids; Pre-clinical cancer models

Core Tip: Therapeutic approaches targeting cancer stem cell (CSC) markers hold great promise toward developing effective anti-cancer treatment. Tissue-specific CSCs (TSCSCs) possess unique expression profile that allows for their identification, isolation, and targeting. TSCSCs, isolated from patient tumor tissues, were shown to form organ analogs or patient-derived organoids (PDOs) under specific culturing conditions *in vitro.* These models simulate the original tumor characteristics in a three-dimensional culture dish. As such, PDOs have the potential to be used in patient-specific *in vitro* drug clinical trials and proof-of-concept studies on CSC-targeted therapies.

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INTRODUCTION

Cancer disease remains a leading cause of death worldwide. Despite significant progress directed toward developing anti-cancer therapies, the successful management of cancer remains impeded by multiple challenges, including metastatic dissemination, conventional-therapy resistance, and disease relapse[1,2]. Accumulating evidence suggests that the cancer stem cells (CSCs) subpopulation plays a vigorous role in sustaining the tumorigenic properties, thus contributing to tumor re-growth and progression[3] (Figure 1). This subpopulation of multipotent cells possesses unique properties of self-renewal and differentiation and is capable of extensively proliferating and generating different lineages of cancerous cells, which constitute the tumor bulk and contribute to the heterogeneous phenotype found in tumors[2,4].

CSCs may arise from the transformation of normal stem cells (SCs) found within tissues or from the de-differentiation of differentiated cells[5]. They were first identified in acute myeloid leukemia[6], and compelling evidence later showed that they exist in a variety of solid tumors where they act as key drivers of tumor progression and metastasis[7,8]

CSCs harbor multiple resistance mechanisms that enrich cancer hallmarks and result in the failure of conventional anti-cancer therapies. One underlying mechanism is the disrupted intracellular pathways that profoundly control CSCs behavior. For instance, overexpression of the Notch pathway plays a dual role that is context and cell-type-dependent, acting either as an oncogene or tumor suppressor[9-11]. In the context of CSCs, the Notch pathway has been implicated in proliferation, angiogenesis, metastasis, stemness maintenance, tumor immune evasion, and resistance to radiation[9,11-13]. Moreover, the Wnt pathway has been linked to the activation of dormant CSCs, their proliferation, maintenance, and inhibition of apoptosis. This pathway also plays a role in the metastasis and de-differentiation of CSCs [14,15]. Besides, the Hedgehog pathway is associated with increased proliferation, maintenance, and self-renewal of CSCs, as well as their migration, invasiveness, and resistance to chemotherapy[14,16,17]. Additionally, the NF-κB pathway is implicated in self-renewal, maintenance, and inhibition of apoptosis of CSCs, as well as regulation of epithelial to mesenchymal transition (EMT), angiogenesis, and metastasis[18]. Finally, the aberrant expression of the JAK/STAT3 pathway promotes cell survival and stemness properties, as well as metastasis and resistance to chemotherapy[14,19]. The intrinsic regulation of CSCs also occurs at the level of stemness-related transcription factors (TFs) such as OCT-4, SOX2, KLF4, c-MYC, STAT3, and NANOG, as well as epigenetics and epi-transcriptomics, which contribute to stemness maintenance and plasticity of CSCs[11]. Additionally, CSCs are regulated at an extrinsic level by their microenvironment, specifically by cancer-associated fibroblasts and tumor-associated macrophages. The tumor microenvironment is a major player in modulating CSCs resistance, metastasis, and heterogeneity[11,20].

The resistance mechanisms of CSCs further include their overexpression of DNA repair genes, resulting in resistance to radiotherapy and other DNA-damaging agents[21]. Also, they express upregulated multidrug efflux pumps such as ATP-binding cassette (ABC) transporters that mediate the active transport of chemotherapeutic drugs out of the cell[22]. CSCs were shown as well to overexpress aldehyde dehydrogenases (ALDHs) which are enzymes involved in the detoxification of aldehydes, chemotherapeutic agents, and reactive oxygen species[23]. Another mechanism that promotes the survival of CSCs is their ability to exist at a reversible quiescent state in the G_0 phase, which contributes to their drug resistance since most chemotherapeutic agents target highly proliferative tumor cells[24]. Thus, standard therapies succeed at reducing tumor size but tend to spare the highly resistant CSCs subpopulation. The successful elimination of tumors, therefore, necessitates targeting the residual dormant CSCs to yield long-lasting eradication of cancer and prevent relapse.

In this review, we provide a recapitulation of the main tissue-specific CSC (TSCSC) biomarkers in five of the most diagnosed solid tumors. Importantly, we highlight the beneficial role of these CSCs in

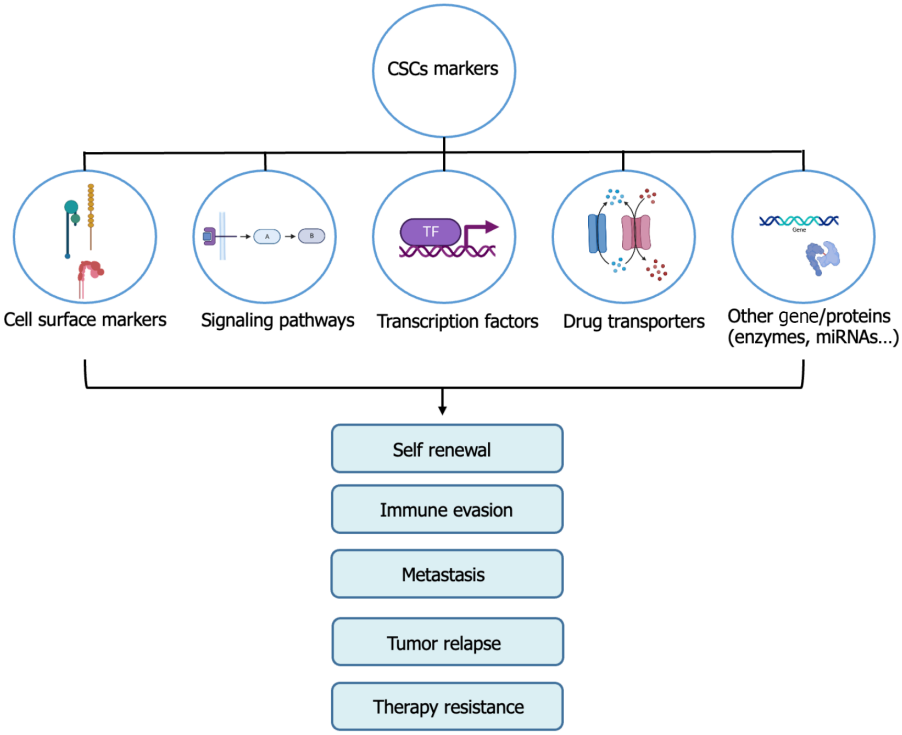

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Figure 1 Schematic presentation of cancer stem cell markers and their contribution to cancer development, progression, and resistance to therapy. Several cancer stem cell (CSC) markers and regulatory signaling pathways are involved in the sustenance and activation of self-renewal, immune evasion, and tumor metastasis, and contribution to tumor re-growth and therapy resistance. CSC markers serve as potential therapeutic targets for cancer treatment. CSCs: Cancer stem cells.

providing relevant preclinical cancer models and thus improving CSC-targeted therapies.

TISSUE-SPECIFIC CANCER STEM CELLS

Given the importance of CSCs in tumor progression and prognosis, several attempts were made to identify and isolate CSCs from the tumor mass based on the markers they express. CSCs express a wide spectrum of markers, some of them being more universal than others. Several markers, mostly located on the cell surface, are often used in combination to ensure a more tissue-specific isolation of targeted CSCs. Here we provide an updated overview of the most prominent TSCSC surface markers, focusing on five solid cancers (prostate, colon, bladder, breast, and lung). Refer to Table 1 for the full list of markers.

Prostate-specific cancer stem cells

The presence of prostate CSCs (PCSCs) was identified by Collins *et al*[25] using SCs markers (integrin $\alpha_2\beta_1$ and CD133) that were previously identified in the normal prostate epithelium[25,26]. This subpopulation of PCSCs isolated from human prostate cancer (PC) biopsies showed a high expression of CD44, CD133, and integrin $\alpha_2\beta_1$. The isolated cells exhibited high proliferative ability and were highly invasive on Matrigel™. Moreover, they possessed a high self-renewal ability and could also differentiate into cells expressing the same phenotype as PC cells, thus re-establishing the original heterogeneous tumor from which they were isolated[27].

CD133 (Prominin-1), a cell surface glycoprotein, remains one of the most used biomarkers to identify and isolate PCSCs either alone or in combination with other markers. In fact, $CD133^+$ PC cells that were isolated from human PC cell line exhibited self-renewal ability, which was correlated with their expression of stemness genes[28]. These cells could also generate a heterogeneous tumor mass when transplanted into immunocompromised mice. Moreover, they displayed high clonogenic abilities and led to the formation of tumor spheres (prostaspheres) that were more malignant than the ones formed by $CD133^-$ PC cells. Furthermore, the $CD133^+$ cells were chemo-resistant and demonstrated high proliferation[28]. Interestingly, a well-established combination of $CD133^+$ and $CD44^+$ PC cells allowed the isolation of PCSCs and the formation of spheroids characterized by heterogeneous PC cells[29].

Table 1 Summary of the most prominent biomarkers required to identify and isolate the tissue-specific cancer stem cells in prostate, colon, bladder, breast, and lung tumors

TSCSCs markers	PCSCs	CCSCs	BCSCs	BrCSCs	LCSCs	Ref.
CD24	-	+	+	-		[64,90,159,160]
CD26		+				[161]
CD29	+	+		+	+	[99,162-164]
CD44	+	+	+	+	+	[31,63,74,108,165]
CD47			+	+	+	[78,166,167]
CD49b (integrin α_2 or ITGA2)	+	+	-	+	+	[168-171]
CD49f (integrin α_6 or ITGA6)	+	+	+	+	+	[99,169,172,173]
CD51	+	+				[69,174]
CD61				+		[99]
CD66c		+	-			[84,175]
CD67LR			+			[84]
CD87					+	[116]
CD90		+	+	+	+	[99,110,176,177]
CD117	+			+	+	[38,116,178]
CD126		+		+	+	[179-181]
CD133	+	+	+	+	+	[28,51,87,99,107]
CD151	+					[35]
CD166	+	+		+	+	[46,104,182,183]
CD326 (EpCAM or ESA)	+	+	+	+	+	[48,56,116,184,185]
Integrin $\alpha_2\beta_1$	+	+				[27,186]
TRA-1-60	+	+				[35,187]
Trop2	+					[45]
CXCR4	+	+		+	+	[102,162,188,189]
ABCB5		+				[73]
ABCG2	+	+	+	+	+	[49,102,177,190,191]
MAGE-A3			+			[177]
GLDC					+	[102]
ALDH	+	+	+	+	+	[44,68,96,102,177]
BCMab1			+			[79]
Lgr5		+		+	+	[53,99,192]
Prox1	+	+				[70,193]
EMA (MUC1)	+	+	-			[77,194,195]
E-cadherin	+	+				[196,197]
ZEB-1	+	+		+	+	[198-200]
PSA	-					[201]
CK5	+		+	+	+	[117,202-204]
CK17			+			[89]
CK18	-		-	-		[89,205,206]

CK20		-	[89]
Ar-v7	+		[207]

+: Over-expressed; -: Under-expressed; Blank: Not found in the literature/controversial. TSCSCs: Tissue-specific cancer stem cells; PCSCs: Prostate cancer stem cells; CCSCs: Colon cancer stem cells; BCSCs: Bladder cancer stem cells; BrCSCs: Breast cancer stem cells; LCSCs: Lung cancer stem cells; EpCAM: Epithelial cell adhesion molecule; TRA-1-60: T cell receptor alpha locus; ALDH: Aldehyde dehydrogenase; EMA: Epithelial membrane antigen.

CD44 (also referred to as P-Glycoprotein 1) is a transmembrane glycoprotein that interacts with several extracellular matrix components, such as collagen, hyaluronic acid, osteopontin, and matrix metalloproteinases. It is one of the most conventional markers used to identify and isolate PCSCs. The expression of CD44 allowed the isolation of cells that were able to differentiate into all types of PC epithelium leading to complete reconstitution of the original tumor bulk when injected into immunocompromised mice[30]. Notably, $CD44^+$ PC-derived cells expressed elevated levels of several mRNAs associated with stemness[31]. This marker was also associated with several aspects of PC tumorigenesis including proliferation, invasion, adhesion, EMT initiation, metastasis, and therapy resistance[32].

T cell receptor alpha locus (TRA-1-60) is a carbohydrate addition to podocalyxinis, which is a cell surface antigen that belongs to the CD36 family. TRA-1-60 is expressed on pluripotent SCs conferring them the ability to induce differentiation. TRA-1-60 was shown to be overexpressed in PC cells as compared to the adjacent normal prostate tissue, which qualifies it as a favorable marker to specifically target PCSCs while sparing normal cells[33]. Moreover, it was detected in the peripheral blood of patients with metastatic PC[34]. The isolation of TRA-1-60^+ cells led to the generation of spheres and initiation of PC in a more efficient manner as compared to other known PCSCs markers. TRA-1-60 was then combined with two other markers of PCSCs (CD166 and CD151) leading to a more enhanced sphere-forming ability. Furthermore, the injection of the triple-marker-positive cells was able to form tumors with at least 5-fold more efficiency as compared to TRA-1-60^+ cells alone[35].

CD117 (also termed c-Kit) is a member of the Type-III tyrosine kinase receptors known to be involved in several cancer mechanisms by binding to its stem cell factor (SCF) ligand[36]. CD117 overexpression was detected in PC[37]. A recent study suggested that CD117 may be considered a potential marker for PCSCs because it was shown to display a broad spectrum of tumorigenic abilities[38]. In fact, CD117 stimulated PC cell proliferation and migration. Moreover, $CD117^+$ cells were able to form 1.35-fold larger prostaspheres as compared to $CD117^-$ cells. Most importantly, $CD117^+$ cells expressed stemness genes and their implantation into immunocompromised mice led to PC initiation[38].

CD49f (integrin α6 or ITGA6) is a transmembrane glycoprotein that was demonstrated to be a putative marker of PCSCs. $CD49f^{high}$ cells were shown to be tumor-initiating cells in the Pten-null PC model[39]. Moreover, CD49f was shown to be the most selective marker for targeting colony-forming cells[40]. Additionally, it was expressed on the surface as well as in the middle of prostatospheres[41]. Importantly, the expression of CD49f allowed the isolation of sphere-forming SCs[42].

In addition to the ones discussed above, there are several markers that can be used to target PCSCs including ALDH1A1 (ALDH 1 family member A1)[43,44], trop-2 (Tumor-associated calcium signal transducer 2)[45], CD166 (activated leukocyte cell adhesion molecule)[46,47], EpCAM (Epithelial cell adhesion molecule)[48], and ABCG2 (ATP binding cassette super-family G member 2)[49].

Colon-specific cancer stem cells

Colon CSCs (CCSCs) were first identified and isolated by Ricci-Vitiani *et al*[50] after the injection of colon cancer (CC) $CD133^+$ cells into immunocompromised mice, which led to the generation of the original tumor mass contrary to their $CD133^-$ counterparts. The $CD133^+$ cells were able to exponentially grow *in vitro* as undifferentiated spheres while preserving the same phenotypic properties of the initial colon tumor[50]. O'Brien *et al*[51] in 2007 also showed that all CC-initiating cells were $CD133^+$ cells that were able to either maintain themselves as undifferentiated CCSCs or to differentiate and therefore sustain the tumor heterogeneity[51].

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) (also recognized as FEX; HG38; GPR49; GPR67) is a seven-transmembrane G-protein coupled receptor. LGR5 is an "orphan" receptor abundantly expressed in active SCs of the intestinal crypts[52]. LGR5 was shown to be overexpressed in CC[53]. A growing body of evidence supports the idea that LGR5 is a main marker of CCSCs. For instance, human $LGR5^+$ CC cells were visualized as the CSC pool in proliferating CC tissue[54]. Furthermore, LGR5 was demonstrated to be a marker of tumor-initiating cells, where implantation of $LGR5^+$ cells was able to form colon tumors, indicating that LGR5 provides a dynamic stemness characteristic in CC[55]. Additionally, LGR5 was correlated with tumor proliferation due to the ability of $LGR5^+$ cells to form more multipotent spheres as compared to $LGR5^-$ cells[56]. Notably, LGR5 was shown to be involved in the colony formation capacity of CCSCs[56,57]. Importantly, LGR5 was found to have an essential role in CC metastasis where organoids derived from $LGR5^+$ cells led to liver cancer formation in the absence of a primary tumor[55]. In addition, LRG5 was selected to be the most suitable CSC marker that identifies immature cancer cells in regional lymph nodes of CC patients[58].

EpCAM (also known as CD326) is a Type-I transmembrane glycoprotein that serves as an epithelial cell adhesion molecule. Interestingly, EpCAM along with its reprogramming TFs were shown to be overexpressed in CC-initiating cells leading to a high self-renewal ability and increased invasiveness [59]. In fact, EpCAM was considered to be a robust CCSCs marker[60]. Indeed, it was used along with CD133 and CD44 to initiate CC in mice[61]. Furthermore, EpCAM provided more enhanced CSC-like properties when combined with LRG5 and CD44[56]. Moreover, EpCAM was proven to promote CC invasion and metastasis, as $EpCAM^{high}/CD44^{+}$ cells were visible in corresponding liver metastasis regions of CC patients[62].

CD44 was also shown to be a robust marker for CCSCs. In fact, a single $CD44^{+}$ cell was able not only to generate a sphere, but also to form a tumor with similar characteristics as the primary one from which it was isolated[63]. Moreover, the expression of CD44 was correlated with CC proliferation[4]. Furthermore, CD44 was reported as a stemness marker in spherical clusters[64]. In addition, CD44 was considered a reliable marker for the prediction of hepatic cancer metastasis in CC patients[65].

ALDH1 is also selected as a potential marker for CCSCs. ALDH1 expression increased during CC tumorigenesis and the implantation of only 25 $ALDH1^{+}$ cells into immunocompromised mice led to the generation of xenograft tumors even in the absence of other CCSCs markers such as CD133 and CD44 [66]. Furthermore, ALDH1 expression conferred high tumorigenic abilities and chemo-resistance to CC cell lines[67]. Interestingly, ALDH1 was linked to lymph node and vascular invasion in CC patients[68].

Among the most specific CSCs related to CC are LGR5, CD44 and EpCAM. However, the combination of multiple markers allows more accurate detection of CSCs which was proven when LGR5, CD44 and EpCAM resulted in more potent CSCs properties as compared to each marker alone [56]. Other markers are also attributed to CCSCs such as CD59[69], Prox1 a regulator of Notch-independent $LGR5^{+}$ SCs[70,71], CD24[4,64], CD166[72], and ABCB5 (ATP binding cassette super-family B member 5)[73].

Bladder-specific cancer stem cells

Bladder CSCs (BCSCs) were first isolated in 2009 by using markers for normal basal bladder SCs ($CD44^{+}$). It was found that the $CD44^{+}$ subpopulation of bladder cancer (BC) cells was 10 to 200 more likely to form tumors in immunocompromised mice in comparison with their CD44 counterparts[74]. Additionally, $CD44^{+}$ BCSCs efficiently maintained the heterogeneity of the initial tumor mass after serial transplantation[74].

Epithelial membrane antigen (EMA, also known as MUC1) is a membrane-bound glycoprotein that belongs to the family of mucins[75]. EMA^{+} bladder cells are usually located in the mature differentiated layer of the urothelium, whereas EMA^{-} cells are found in the basal layers, where SCs reside. It was demonstrated that EMA^{-} BC cells had a greater colony-forming ability when compared with the unsorted BC population[75,76]. BCSCs can thus be identified through the combination of EMA^{-} and $CD44^{+}$ BC cells[77].

CD47 (also known as integrin associated protein) is a transmembrane protein overexpressed on the surface of $CD44^{+}$ BCSCs compared to the $CD44^{-}$ subpopulation and was thus hypothesized to be a BCSCs marker[78,79]. CD47 acts like a "don't eat me" signal by interacting with the signal regulatory protein-1 receptor on the surface of macrophages and neutrophils. Thus, CD47 has an immunosuppressive role, protecting the BSCSC from phagocytosis[78,79], that makes it a promising target for cancer therapy[80,81].

ALDH1A1 has also been used to isolate BCSCs. In fact, $ALDH1A1^{+}$ cells retained the stem-cell ability to divide asymmetrically, yielding both $ALDH1A1^{+}$ and $ALDH1A1^{-}$ cells[82]. Additionally, $ALDH1A1^{+}$ BCSCs exhibited a greater tumorigenic potential both *in vitro* (sphere formation ability) and *in vivo* (xenografts in immunocompromised mice) compared to $ALDH1A1^{-}$ BC cells[82]. Knocking down the ALDH1A1 gene in BCSCs reduced their proliferation, confirming the key role played by the ALDH enzyme in BCSCs division and renewal[83]. Furthermore, ALDH1A1 BCSCs maintained the original tumor heterogeneity after sequential transplantations into immunocompromised mice[83]. Finally, $ALDH^{+}$ BCSCs demonstrated an enhanced ability to migrate and invade tissues contrary to $ALDH^{-}$ BC cells[82].

$67LR^{+}$ (67KDa Laminin Receptor)/ $CD66c^{-}$ (also known as CEACAM6) BC cells were demonstrated to have stemness properties. These markers, similar to CD44, are also present in normal bladder SCs[84]. He *et al*[85] showed that $67LR^{+}$ BCSCs were 5 to 10 times more potent in initiating tumors *in vivo* compared to $67LR^{-}$ ones[85]. In addition, $67LR^{+}$ BCSCs expressed a panel of genes involved in stemness and resistance to chemotherapy and radiation[85,86]. Similarly, $CD66c^{-}$ cells were demonstrated to be more tumorigenic than the $CD66c^{+}$ counterparts[85].

$CD133^{+}$ BC cells were shown to upregulate the expression of genes involved in pluripotency. This subpopulation of BC cells was also more resistant to the chemotherapeutic agent cisplatin and to radiation. Additionally, $CD133^{+}$ BCSCs exhibited a greater tumorigenicity both *in vitro* and *in vivo*, as well as a more aggressive proliferation in immunocompromised mice in comparison to $CD133^{-}$ BC cells [87].

Additional markers are also used for the identification of BSCSC namely MAGE-A3 (Melanoma antigen family A, 3)[88], BCMab1[79], and several members of the cytokeratin family of proteins ($CK5^{+}$, $CK17^{+}$, $CK18^{-}$, $CK20^{-}$)[89].

Breast specific cancer stem cells

The importance of breast CSCs (BrCSCs) markers was first demonstrated by Al-Hajj *et al*[90] only a subpopulation of human breast cancer (BrC) cells appeared to lead to the formation of tumors in immunocompromised mice. Al-Hajj *et al*[90] isolated $ESA^+CD44^+CD24^{-/Low}$ cells from human BrC tissue, and showed that as low as 200 of these cells were enough to initiate cancer in immunocompromised mice, whereas more than 50000 BrC cells with a different phenotype were unable to form tumors[90].

CD44 and CD24 are often used in combination to detect and isolate BrCSCs[91]. In addition to its key role in adhesion, cell survival, metastasis and angiogenesis, CD44 act as a TF to regulate metastasis and stemness of BrCSCs[92,93]. On the other hand, CD24 is a cell surface adhesion glycoprotein which plays a key role in cell-cell and cell-extracellular matrix (ECM) interactions[94,95]. Even though CD24 is overexpressed in a number of cancers (including BrC), only $CD44^+CD24^{-/Low}$ BrCSCs were able to form tumors in immunocompromised mice[90]. $CD44^+CD24^{-/Low}$ BrCSCs were also shown to be more resistant to chemotherapy[91].

ALDH1 has also been used to target BrCSCs, as it was shown that $ALDH1^+$ BrC cells were more resistant to chemotherapy and were able to form tumors in immunocompromised mice in comparison to $ALDH1^-$ cells[96]. ALDH1 is essential for the early development of the stemness properties of BrCSCs [97]. Interestingly, the subpopulation of BrC cells expressing ALDH1 is distinct from the $CD44^+CD24^{-/Low}$ BrCSCs, with minimal overlap between the two (approximately 1%)[91]. Moreover, $ALDH1^+/CD44^+$ BrCSCs were highly tumorigenic, with a higher metastatic potential, and greater resistance to cancer therapies[91].

To date, CD44, CD24 and ALDH1 remain the most used biomarkers to isolate BrCSCs. Although there is little overlap between $CD44^+CD24^{-/Low}$ and $ALDH1^+$ BrCSCs, cells that share all three markers were more tumorigenic[98]. Moreover, the CD44/CD24 markers were more associated with cell proliferation and tumorigenesis while the ALDH1 marker was positively correlated with tumor metastasis [98]. Nonetheless, other markers have been studied and found suitable for the identification of BrCSCs, such as CD133 (in triple negative BrC; TNBC), GD2 (ganglioside in TNBC), CD49f, $CD61^+$ (β3 integrin in Her2 BrC), CD29 (β1 integrin), CD90, and EpCAM[99-101].

Lung cancer stem cell markers

Lung cancer is histologically divided into non-small cell lung carcinoma cells (NSCLC) and small cell lung carcinoma (SCLC)[102]. Due to a higher incidence and the greater ease to obtain NSCLC tissue, NSCLC CSCs (referred to afterward as lung CSCs; LCSCs) markers have been better characterized.

CD166 (also known as ALCAM) has also been associated with stemness properties of NSCLC. CD166 is a member of the immunoglobulin superfamily of cell adhesion molecules and participates in both homophilic and heterophilic interactions. Additionally, CD166 plays an important role in migration and invasion of LCSCs[103]. CD166 was characterized by Zhang *et al*[104] as the most robust cell marker for isolating LCSCs among other candidates (CD44, EpCAM and CD133)[104]. In contrast to $CD166^-$ NSCLC cells which failed to form tumors *in vivo*, $CD166^+$ LCSCs were able to initiate tumors in immunocompromised mice. Furthermore, $CD166^+$ NSCLC cells had enhanced self-renewal properties and were able to consistently form spheres *in vitro*.

The $CD133^+$ subpopulation of NSCLC cells were able to indefinitely divide and form spheres in an *in vitro* setting, whereas $CD133^-$ NSCLC cells were characterized by a slow growth and an inability to form spheres[105]. These results also parallel the *in vivo* ability of $CD133^+$ LCSCs to form tumors in immunocompromised mice compared to $CD133^-$ cells; the $CD133^+$ xenografts were histologically similar to the initial cancer mass[105,106]. Moreover, the expression of CD133 in LCSCs was associated with increased resistance to chemotherapy and radiation[105,107]. Finally, $CD133^+$ LCSCs are more prone to metastasize than their $CD133^-$ counterparts, especially to lymphoid organs. In fact, detection of $CD133^+$ metastatic NSCLC in lymph nodes is indicative of a poor prognosis[107].

CD44 has also been studied as a marker to isolate LCSCs. Accordingly, $CD44^+$ NSCLC cells demonstrated a greater ability to form spheres *in vitro* and to initiate tumors in immunocompromised mice in comparison to $CD44^-$ cells. Additionally, $CD44^+$ LCSCs upregulated several stemness TFs to maintain their pluripotent properties. $CD44^+$ LCSCs were also more resistant to the chemotherapeutic agent cisplatin compared to $CD44^-$ cells[108]. Moreover, the expression of CD44 in LCSCs was associated with an enhanced ability to metastasize and invade tissues[20].

CD90 (also known as Thy-1) is a glycosylphosphatidylinositol-anchored surface protein that is involved in cell-cell as well as cell-ECM interactions[109]. Initial studies have shown that $CD90^+$ NSCLC cells demonstrated greater self-renewal and proliferative properties and expressed a higher level of stemness genes. Additionally, when compared to a control, as few as 5000 $CD90^+$ LCSCs were able to initiate tumors in immunocompromised mice, indicating the stronger tumorigenicity associated with CD90[110].

ALDH1 was also suggested to be a LCSCs marker. Indeed, $ALDH1^+$ LCSCs exhibited enhanced proliferative abilities and self-renewal properties[111,112]. Accordingly, knocking down the ALDH1A3 gene greatly reduced the tumorigenicity and clonogenicity of LCSCs[113]. In addition, $ALDH1^{high}$ LCSCs also showed greater resistance to chemotherapeutic drugs in comparison to $ALDH1^{low}$ cells[112]. Interestingly, the overexpression of the TAZ oncogene induces the formation of LCSCs by activating the

ALDH1 gene[114] ALDH1 also appears to play a key role in chemoresistance as its inhibition leads to the re-sensitization of LCSCs to cisplatin[115].

Of note, additional markers have been used to isolate LCSCs. These include but are not limited to CD47, CD87, CD117, EpCAM, and CK5[116,117].

TSCSCS' BENEFICIAL ROLE IN CANCER MODELING FOR THERAPEUTIC IMPLICATIONS

The conception of CSCs-targeted therapies relies on employing the above-mentioned CSCs' resistant characteristics and markers, which allows for CSCs' isolation, enrichment, characterization, and targeting[118]. CSCs-based therapeutic strategies include selectively targeting the stemness markers, such as the TSCSCs' surface markers, TFs, ABC transporters, and ALDHs[14,119]. As well as, the disrupted signaling pathways that enrich CSCs'-resistant features and contribute to their survival, proliferation, self-renewal, and differentiation. Also, targeting the tumor microenvironment components which acts as a foster niche in protecting CSCs[14,119].

In spite of the significant advances in CSCs' research and the great interest in drug discovery, there are currently few therapeutic approaches that have reached the late clinical stages. Many CSCs-targeting therapeutics performing remarkably *in vitro* and *in vivo* cultures have faced multiple hurdles in clinical trials[14,120]. One major reason behind this is the irrelevance of the preclinical cancer models being used[121-123]. Thus, more relevant CSCs models, that reflect the original tumor behavior of the individual patients, might strengthen the rationale for developing effective CSCs-targeted therapeutic modalities and complement more conventional cancer therapies.

A new era of targeted and personalized anti-cancer therapies has evolved with the three-dimensional (3D) patient-derived organoids (PDOs)[124]. This versatile technique relies on the exclusive ability of SCs to give rise to organ-like structures known as organoids[125]. Sato *et al*[126] established the first organoid model with small intestinal crypt $LGR5^+$ SCs[126]. Subsequently, models of normal and cancer PDOs from multiple tissues were derived successfully[127-134].

The formation of the 3D microscopic organoids from patient tumor tissues is accomplished using specific culturing conditions that are designed to preserve the CSCs component of the patient's tumor [135]. The formed PDOs, hence, recapitulate the structural and functional complexity constituting the originating tumor, mediated by the CSCs' ability for self-renewal and differentiation into multiple cell types[136,137]. PDOs tool allows the modeling of human carcinogenesis in an *in vitro* culture dish[138, 139]. Precisely, the process followed to generate cancer PDOs includes utilizing a tumor tissue sample, surgically isolated from a cancer patient, and dissociating it into single-cell suspension using mechanical dissociation and enzymatic digestion methods. The heterogeneous population of cells obtained, containing TSCSCs, is then cultured in proper culturing conditions to allow the self-organization of cells into functional units or tissue-specific architectures; organ analogs. The suspended culturing system includes the usage of biological or synthetic hydrogel scaffolds that mimic the natural ECM components. In addition to using a specific culturing medium that contains a cocktail of growth factors and inhibitors to imitate the organ stem cell niche, allow the generation of distinct component lineages, and stimulate the long-term expansion of organoids[140,141].

As PDOs are CSCs-based structures and replicate faithfully the heterogeneity and histological characteristics of the original cancers, they gain superiority over other models in terms of mimicking tumor microenvironments, facilitating the formation of ECM, exhibiting adequate proliferation rates with representative cellular morphology, maintaining the expression of 'stemness-related' markers and genes, and demonstrating a realistic individualized drug response[142-144]. This nominates PDOs to be ideal preclinical drug-response models for providing perspectives for testing novel CSCs-targeted therapies and evaluating the potential drug effectiveness in cancer patients (Figure 2).

PDOs technique generally shares several main steps but differs in varying degrees depending on the type of tissue being processed. Scaffold-based techniques are mostly adopted in culturing PDOs, where Matrigel™ is commonly used. The latter is a mixture of heterogeneous and gelatinous proteins secreted by mouse sarcoma cells. It comprises mainly adhesive proteins such as laminin, collagen IV, entactin, and heparin sulphate glycoprotein, which resemble the ECM and provide interactive and structural support to the cells[145-148]. Moreover, the universal organoid medium used in the culturing system adopts the first protocol developed by Sato *et al*[126] which includes advanced DMEM/F12 medium supplemented with epidermal growth factor, Noggin (NOG), and Wnt agonist R-spondin-1[126,127]. Other factors were then added including anaplastic lymphoma kinase 3/4/5 inhibitor A83-01, dihydrotestosterone, fibroblast growth factor-10, fibroblast growth factor-2, prostaglandin E2, nicotinamide (NAM), and p38 inhibitor SB202190, N-acetylcysteine (NAC), B27 supplement and Rho kinase inhibitor Y-27632 to culture PDOs successfully[149].

To date, organoids derivation from multiple human tumors including prostate, colon, bladder, breast, and lung cancers has been described, with varying success rates[133,150-155]. The established PDOs are subjected to tissue-specific genes and lineage markers expression studies to confirm that they represent the original tumor of the patient. Importantly, the cancerous origin of these organoids is confirmed by

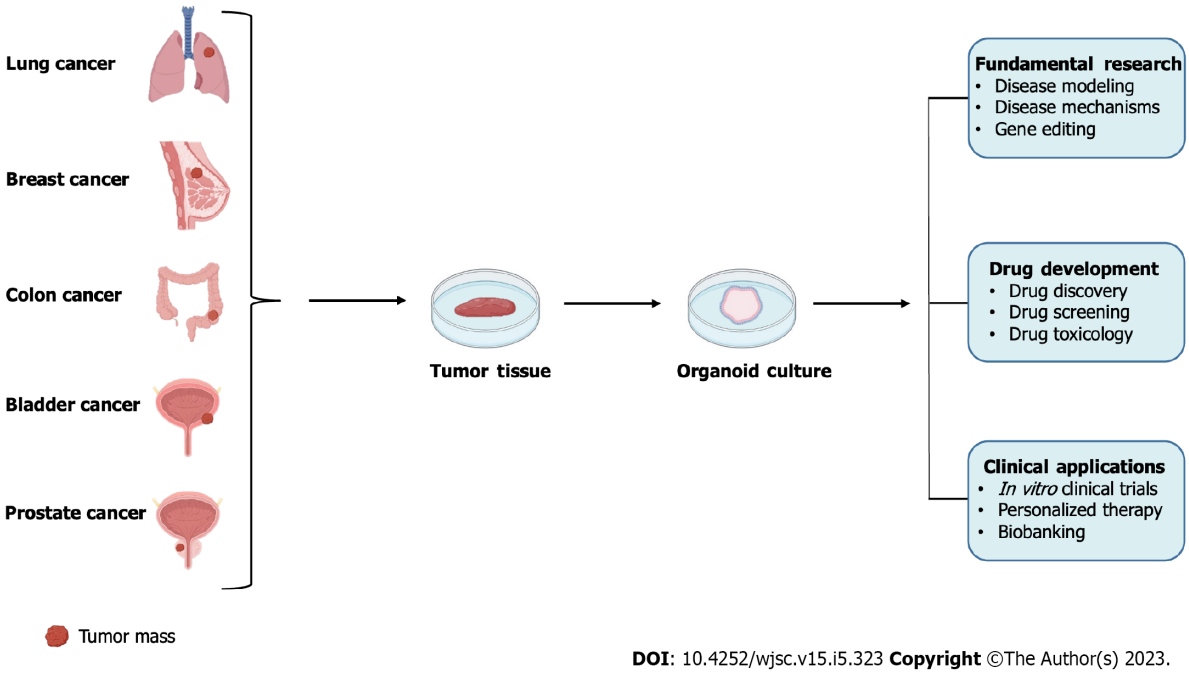

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Figure 2 Schematic presentation of patient-derived organoid applications in cancer research. Patient-derived organoid (PDO) models can be utilized in multiple fields of cancer research including fundamental research, drug development, and clinical application. Cancer PDOs have been used to simulate the tumor tissue *in vitro*, study the disease mechanisms and gene expression patterns, and expose them to different drugs for efficacy screenings and drug discovery validations. Organoids are further used as *in vitro* pre-clinical models for personalized medicine and the generation of 'living' organoid biobanks. PDO culturing system serves as an advanced tool in the implementation and development of precision medicine.

checking for the CSCs markers specific to each tumor tissue. The patient drug response to the therapy of interest can then be evaluated primarily by assessing the organoids' formation efficiency and size.

For example, a study done by Cheaito *et al*[150] established a minimum of 5-factor medium including NAC, NOG, A83-01, B27, and NAM to grow and maintain PC PDOs. Histopathological, transcriptomic, immunofluorescent, and immunohistochemical studies showed that the formed PDOs mimicked the histological architecture and prostate lineage profiles of their corresponding tissue specimens. This was confirmed by the presence of both prostate epithelial lineages, as the organoids stained positive for the luminal- (CK8, AR, and PSA) and basal- (CK5, CK14, and p63) specific markers. In addition, an intermediate cell population, co-expressing luminal CK8 and basal CK5 markers was also detected. Interestingly, CSCs markers, CD44 and CD49f, positive staining demonstrated the existence of putative stemlike cells within the bulk of the PDOs. Furthermore, differential drug response, between different patient samples, was recognized upon treatment with chemo-, radio-, and androgen-deprivation therapies[150]. In another study, Monzer *et al*[151] succeeded in establishing and propagating PDOs that model CC disease. The formed organoids recapitulated the architecture and the characteristics of CC tissues as revealed by the co-expression of the epithelial marker lineage CK19 and the CSC surface marker CD44. The organoids derived from different patients showed to exhibit different responses to Diiminoquinone treatment tested alone or in combination with Fluorouracil (5FU) chemotherapeutic drug. Similarly, Al Bitar *et al's* study showed different responses to individual and combination treatments of radiation and Thymoquinone in CC PDOs[152].

Moreover, Yu *et al*[153] utilized BC PDOs to evaluate chimeric antigen receptor (CAR)-T cell-mediated cytotoxicity against BC. Analysis was done to confirm that the established organoids recapitulate the heterogeneity and the key features of the parental BCs. Based on a set of luminal (CK20, uroplakin II, and GATA3) and basal markers (CK5, P63, and CD44), the formed organoids were classified into luminal or basal subtypes, respectively. All the BC PDOs and their corresponding tumors expressed Ki67 and E-cadherin, confirming their epithelial origin and high proliferative ability. Additionally, the specific surface antigen profiling of each tumor sample was analyzed, and the MUC1 antigen was shown to be highly expressed among all tested antigens, in both the cancer tissues and their derived organoids. MUC1 was then used as a putative target to test the efficacy of second-generation CAR-T cells in BC PDOs[153]. Furthermore, a promising study done by Chen *et al*[154], showed the significance and applicability of using BrC PDOs as pre-clinical models for broader cancer studies, and more specifically as a tool to provide personalized therapy recommendations for patients with advanced refractory disease. This study focused mainly on deriving PDOs from specimens isolated from patients with advanced clinical features, including drug-resistant and metastatic BrC. The histopathological, immunohistochemical, and genomic characteristics were shown to be well inherited by the formed PDOs from the drug-treated as well as treatment-naïve tumors. Distinctive drug

responses were also observed[154]. Furthermore, Kim *et al*[133] demonstrated the distinctive therapeutic responses of LC and normal bronchial PDOs, derived from patient tissues comprising five histological subtypes of LC and non-neoplastic bronchial mucosa. The differential responses to the tested drugs were shown to be affected by the individual genomic alterations profile. The PDOs were also proved to duplicate the tissue architecture and maintain the genomic alterations of the parental lung tumors during long-term expansion *in vitro*[133].

CONCLUSION

In this review, we have discussed briefly some of the CSC features that are known to account for cancer resistance and relapse and make CSCs promising anti-cancer targets. Additionally, we have summarized the updated list of the TSCSC molecular markers in prostate, colon, bladder, and lung tumors that are significant to selectively isolate and therapeutically target the CSCs subpopulation. Besides, we highlighted the advantage of utilizing the CSC-based PDO models to simulate carcinogenesis and predict patient-specific drug responses *in vitro*.

Despite the present challenges[156,157], PDOs are highly credible models that possess more physiological and pathological relevance than traditional ones. This robust method proved to faithfully maintain the histological, genetic, and stemness characteristics of their respective native tissues. Interestingly, the CSCs profile mimicked by the PDOs can serve as a platform for testing CSCs-targeted therapeutics. To our knowledge, there are no clinical trials discussing cancer PDOs in a preclinical context for testing CSC-targeted therapeutics[158].

Indeed, PDOs have prospective applications in patient-specific *in vitro* drug clinical trials and proof-of-concept studies on CSC-targeted therapies and -resistance mechanisms. If remarkable advancements are made, cancer patients will ultimately benefit from this radical technology.

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REVIEW

Advancements in adipose-derived stem cell therapy for skin fibrosis

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Abstract

Pathological scarring and scleroderma, which are the most common conditions of skin fibrosis, pathologically manifest as fibroblast proliferation and extracellular matrix (ECM) hyperplasia. Fibroblast proliferation and ECM hyperplasia lead to fibrotic tissue remodeling, causing an exaggerated and prolonged wound-healing response. The pathogenesis of these diseases has not been fully clarified and is unfortunately accompanied by exceptionally high medical needs and poor treatment effects. Currently, a promising and relatively low-cost treatment has emerged-adipose-derived stem cell (ASC) therapy as a branch of stem cell therapy, including ASCs and their derivatives-purified ASC, stromal vascular fraction, ASC-conditioned medium, ASC exosomes, *etc.*, which are rich in sources and easy to obtain. ASCs have been widely used in therapeutic settings for patients, primarily for the defection of soft tissues, such as breast enhancement and facial contouring. In the field of skin regeneration, ASC therapy has become a hot research topic because it is beneficial for reversing skin fibrosis. The ability of ASCs to control profibrotic factors as well as anti-inflammatory and immunomodulatory actions will be discussed in this review, as well as their new applications in the treatment of skin fibrosis. Although the long-term effect of ASC therapy is still unclear, ASCs have emerged as one of the most promising systemic antifibrotic therapies under development.

Key Words: Adipose-derived stem cell; Cicatrix, hypertrophic; Keloid; Scleroderma, localized; Stromal vascular fraction; Exosomes

Core Tip: Pathological scarring and scleroderma are the most common conditions of skin fibrosis with high medical needs and poor therapeutic effects. Adipose-derived stem cell (ASC) therapy has emerged as a promising treatment for skin fibrosis. Here, we discuss the possible mechanism of skin fibrosis as well as the latest research about the mechanism of ASC therapy and its application in treating these conditions. ASC therapy provides a brand-new insight into the treatment of skin fibrosis.

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INTRODUCTION

Skin fibrosis is characterized by fibroblast proliferation and extracellular matrix (ECM) deposition. In severe cases, it can lead to pathological changes in the skin, such as keloid and hypertrophic scars (HS), systemic sclerosis (SSc), and scleroderma[1,2]. The fact that there are no practical disease-modifying therapies for those diseases and current treatment is mainly toward managing symptoms and relieving complications calls for a new therapy[3,4].

Since 2001, when adipose-derived stem cells (ASCs) were first characterized, ASCs have been broadly studied and applied as the most promising sources of cells with regenerative and multilineage characteristics[5]. In recent years, various ASC derivatives, which are rich in not only ASCs but also other cellular and tissue components, have been seen as possible alternatives to ASCs and have received increasing attention for exploring their potential applications. Due to their immunomodulatory properties and abundance of growth factors[6,7], ASCs and their derivatives have become new remedies in the treatment of skin fibrosis[8-10].

In this review, we discuss the mechanism of skin fibrosis and the mechanism of ASC therapy. We then summarize the application of ASCs and their derivatives in skin fibrosis. Finally, we retrospectively describe the safety of ASC therapy and predict the future of skin fibrosis treatments.

MECHANISM OF SKIN FIBROSIS

Many fundamental studies exploring the molecular mechanisms underlying fibrosis have revealed a large number of genes, molecules, and cell types that may contribute to this problem[11,12].

Keloid and HS

The pathogenesis of keloids and HS is not fully understood due to the complex dynamic process of wound healing. However, among all the factors that stimulate fibroblasts to differentiate into myofibroblasts and produce excessive amounts of collagen and ECM, the role of the inflammatory response is increasingly considered important[1,11,13]. Downregulation of proinflammatory cytokines such as interleukin 6 (IL-6) and IL-8[14] and upregulation of anti-inflammatory cytokines such as IL-10 may reduce scar tissue formation[15]. Additionally, inflammatory cells such as macrophages, T cells, and mast cells, all increase and take part in a variety of biological activities in keloids and HS[1,16]. Although multiple intracellular signaling pathways such as Smad, signal transducer and activator of transcription 3, and extracellular signal-regulated kinase 3, are involved in hypertrophic scar formation, transforming growth factor-β (TGF-β)/Smad is thought to be a driving force[17,18]. Thus, the basic purpose of current prevention and therapy methods is still to reduce inflammatory processes[19].

SSc and scleroderma

SSc is an immune-mediated rheumatic disease that is characterized by excessive collagen from myofibroblasts in the skin and some internal organs, microangiopathy, and impairment of the humoral and cellular immunity system[20,21]. Scleroderma features, without the involvement of internal organs, are similar to SSc[4]. SSc pathogenesis involves early vasculopathy and innate and adaptive immune system dysfunction[12]. Initial vasculopathy and immune system dysfunction are both involved in SSc pathogenesis and cause SSc inflammation and tissue fibrosis[22]. Immune cells, endothelial cells, and fibroblasts interact with each other and release cytokines and growth factors[21]. Workers are convinced that type-1-interferon and interferon-inducible genes play a role in SSc pathogenesis[23]. Additional important factors include platelet-derived growth factor, endothelin 1, insulin-like growth factor 1, and TGF, which is thought to be a major regulator of fibrosis pathways[24]. Combined treatment that targets epigenetic/genetic, vascular, and immunologic defects and progressive fibrosis is urgently needed[12,

21,25].

MECHANISM OF ASC THERAPY

ASCs have long been thought to have immune privileges as mesenchymal stromal cells, which do not induce a severe allogeneic response when injected into another organism[26,27]. However, they have been demonstrated to evoke cellular and humoral responses in vivo, which may lead to the rapid elimination of transplanted cells[27]. However, ASCs function primarily through a "hit-and-run mechanism" with consequently a small effect on therapeutic efficacy, at least in the short or middle term [28,29]. Indeed, most ASCs do not require cell-to-cell contact to function but rather function through paracrine mechanisms that release cytokines, growth factors, and extracellular microvesicles in the surrounding environment[30]. As reported, the therapeutic effect of ASCs and their derivatives depends on paracrine secretion[31-34]. ASC-secreted active substances such as cytokines[35], growth factors[36], chemokines[37], and extracellular vesicles[38], regulate the microenvironment around fibroblasts and themselves[39,40] (Figure 1).

Regulation of the microenvironment

Immunomodulation and anti-inflammatory: After injection, ASCs activate adaptive cellular responses, secreting IL-1, prostaglandin E2 (PGE2), IL-4 and IL-10, and TGF-β, which modulate and stimulate innate immune cells[41]. It was reported that ASCs suppress $CD4^+$ and $CD8^+$ T-cell expansion and differentiation while promoting regulatory T-cell proliferation and enhancing their immunosuppressive activity[42]. Additionally, ASCs secrete immunosuppressive substances such as nitric oxide, PGE2, hepatocyte growth factor (HGF), and indoleamine 2,3-dioxygenase, which downregulate TGF-β in skin fibrosis and attract bone marrow (BM) cells involved in tissue repair[43,44].

Despite their immunomodulatory ability, the anti-inflammatory effects of ASCs have been gaining increasing attention. ASCs can drive anti-inflammatory M2 macrophage polarization and ameliorate macrophage infiltration[34,45]. Additionally, in a rabbit model of HS, ASCs mediated the inhibition of M1-polarized macrophages and defection of inflammation. Moreover, the expression of inflammatory cytokines and proteins such as IL-6 and monocyte chemotactic protein-1, which affect inducible nitric oxide synthase and cyclooxygenase-2, was notably decreased in the treated groups[46-48].

Angiogenic effects: The angiogenic effects of ASCs have been broadly discussed with regard to myocardial infarction, nerve injury, and tissue transplantation[49-52]. The secretion of vascular endothelial growth factor (VEGF) as well as the transcription of angiogenic genes are improved by ASCs [52,53]. ASC transplantation greatly improves revascularization and tissue perfusion in ischemic scars by stimulating endotheliocyte proliferation in blood vessels, hastening the resumption of blood circulation, providing oxygen and nutrition, and improving scar texture[54]. There is also an interplay between ASCs and endothelial precursor cells (EPCs). Growth factors produced by ASCs, such as VEGF, increase the migration and survival of EPCs, while EPC-produced platelet-derived growth factor BB stimulates ASC proliferation and migration[36].

Regulation of fibroblasts

Proliferation and differentiation: Activated dermal fibroblasts change their phenotype into myofibroblasts in response to injury or stress, which increases their expression of α-smooth muscle actin (α-SMA) and contractile ability[55,56]. Previous studies have demonstrated that ASC conditioned medium (ASC-CM) contains abundant growth factors and cytokines, such as IL-10, adrenomedullin, and HGF[7,57]. HGF, proven to inhibit fibroblast differentiation into myofibroblasts, contributes to limiting the profibrotic functions of myofibroblasts[58,59]. It has also been reported that ectodysplasin-A2, insulin-like growth factor binding protein-related protein-1/insulin-like growth factor-binding protein-7 (IGFBP-rp1/IGFBP-7), and thrombospondin-1 are increased in concentration in serum-starved ASC-CM, which could play a role in the inhibition of fibrosis[60]. These ASC-secreted immunosuppressive substances suppress fibrosis by various mechanisms, including reducing the expression of TGF-β1 and collagen and promoting the expression of matrix metalloproteinases (MMP), thus significantly repressing the activity of fibroblasts *in vitro* and *in vivo*[34,61].

Expression of ECM: The synthesis of collagen, hyaluronic acid, and fibronectin by myofibroblasts, in particular, is essential for the prolonged and excessive formation of ECM constituents[56,62]. Inhibition of HS-derived fibroblast (HSF) proliferation and reduction in α-SMA, type I collagen, and type III collagen expression can partly explain the molecular mechanism of the effects of ASCs on HSs[46,63,64]. In another study, ASC-CM reduced the synthesis of collagen and the expression of connective tissue growth factor, fibronectin, and α-SMA[65]. However, in a coculture model of ASCs and normal human dermal fibroblasts, ASCs increased the formation of collagen types I, III, and VI in the ECM[66]. It appears that ASCs could target abnormal fibroblasts and reduce pathological deposition of ECM.

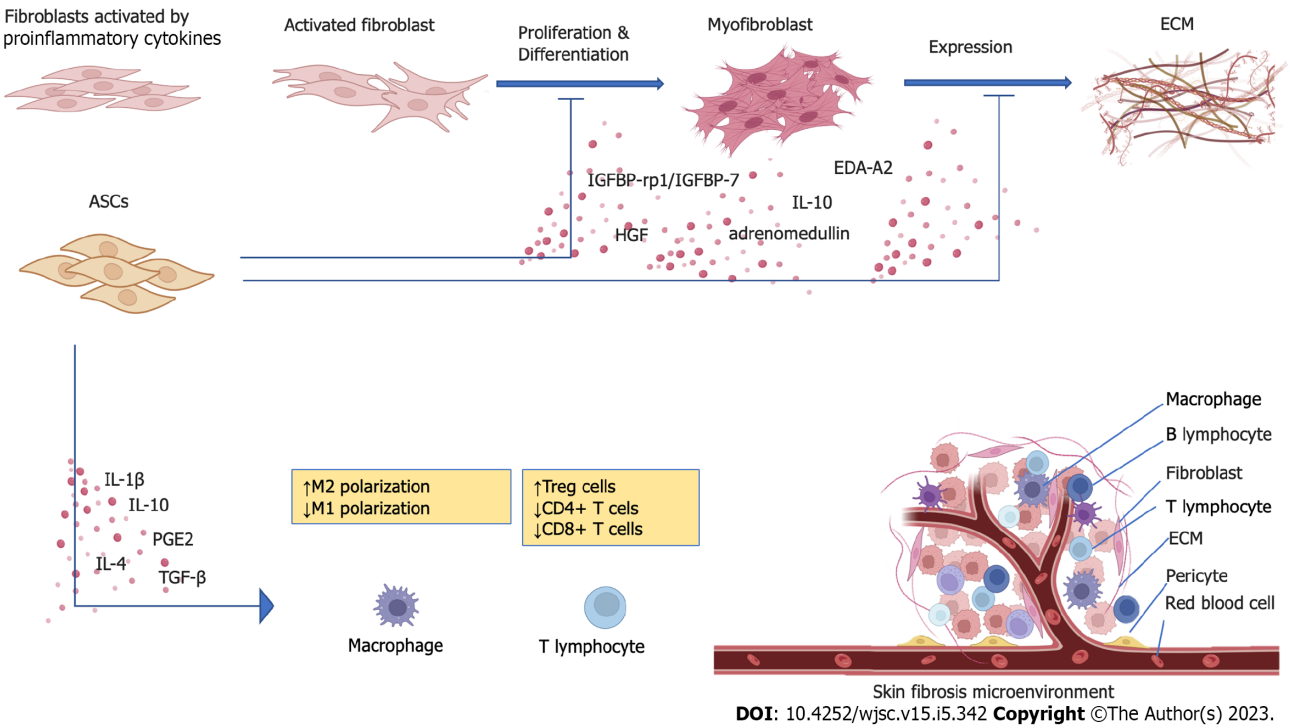

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Figure 1 The brief mechanism of adipose-derived stem cell therapy in skin fibrosis. ECM: Extracellular matrix; ASC: Adipose-derived stem cell; HGF: Hepatocyte growth factor; IGFBP-rp1/IGFBP-7: Insulin-like growth factor binding protein-related protein-1/Insulin-like growth factor-binding protein-7; EDA-A2: Ectodysplasin-A2; IL-10: Interleukin 10; IL-4: Interleukin 4; IL-1β: Interleukin 1β; TGF-β: Transforming growth factor beta; PGE2: Prostaglandin E2. Figure 1 is created with BioRender.com.

ASC THERAPY APPLICATION

ASC therapy, including the application of ASCs and their derivatives, can be roughly divided into ASC-based therapy and stem cell-free therapy. ASC-based therapy is mainly composed of various ASCs and stromal vascular fractions (SVFs), which have been broadly studied and applied in the clinic (Figure 2). Stem cell-free therapy, such as exosomes and ASC-CM, is increasingly popular, with fewer moral and safety concerns.

ASCs

One of the most promising stem cell groups, ASCs, are abundant in adipose tissue, easy to extract, and have few adverse effects. Compared to BM-mesenchymal stem cells, ASCs exert potent anti-inflammatory and remodeling properties with similar therapeutic effects[30].

Intralesional injection of ASCs reduces the formation of scars while improving color quality and scar pliability, potentially leading to an effective and novel anti-scarring therapy[59,67,68]. These studies revealed that ASCs not only inhibited fibroblast proliferation and migration but also reduced the expression of molecules such as TGF-β1 and Notch-1. The antifibrotic effect on fibroblasts was most likely mediated by the inhibition of multiple intracellular signaling pathways[18,65].

As they are inherently heterogeneous, different ASC subgroups have been studied in the hope of finding suitable subgroups for specific diseases.

A subpopulation of ASCs that are positive for $CD74^+$ possesses enhanced antifibrotic abilities both *in vitro* and *in vivo*. Additionally, $CD74^+$ ASC-assisted fat grafts reduce dermal thickness and fibrosis in radiation-induced fibrosis mouse models[69]. Another $CD73^+$ ASC subpopulation has expressed significantly lower levels of procollagen lysyl hydroxylase 1, a potent stimulator of fibrosis, showing better therapeutic effects on wound healing[70].

To modify or enhance some properties of ASCs and overcome the limitations of curative effects of ASCs only, ASCs are coated or activated with small molecule drugs or genetically overexpressing molecules that are involved in fibrosis formation.

After overexpressing MMP-3, ASCs-MMP-3 possess not only the ability of ASCs to accelerate wound healing but also the capability of MMP-3 to reduce scarring[71]. Compared with mASCs alone, migration ability and HGF production are significantly higher in mASCs activated with LMWH, showing higher anti-inflammatory and anti-fibrotic capability, and might be a promising candidate for SSc treatment[72]. IL-10-ASCs have been proven to have the capacity to suppress the development of HS by reducing inflammation during wound healing as well as the proliferation and migration of HSFs that produce ECM[73]. Poly(3-hydroxybutyrate-cohydroxy valerate) loaded with ASCs contains the

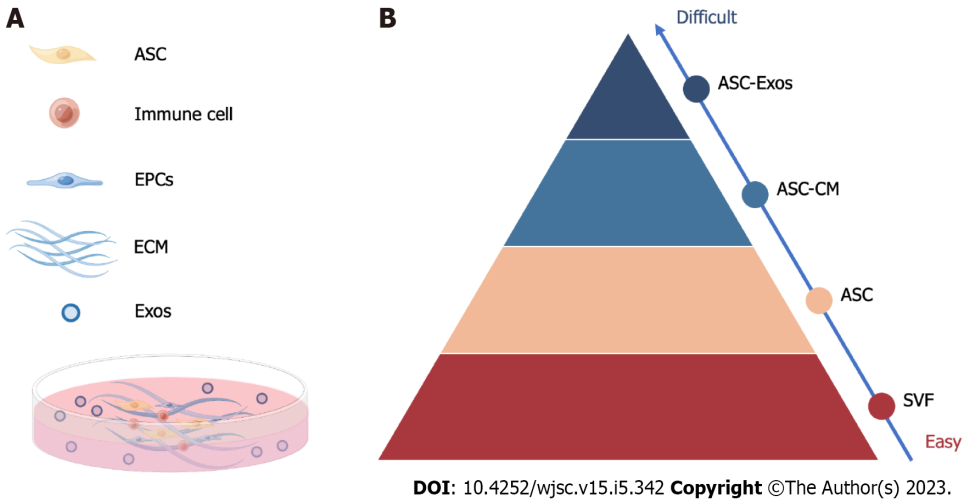

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Figure 2 The culture and classification of adipose-derived stem cells and their derivatives. A: Culture of adipose-derived stem cells (ASC) and their derivatives; B: Classification of ASC and their derivatives. EPCs: Endothelial precursor cells; ECM: extracellular matrix; Exo: Exosomes; ASC: Adipose-derived stem cell; ASC-CM: ASC-conditioned medium; ASC-Exo: ASC exosomes; SVF: Stromal vascular fraction. Figure 2 is created by Figdraw.

bioactive cues required to improve wound healing and scarring[74].

SVF

SVF is an aqueous fraction that contains ASCs, EPCs, endothelial cells, macrophages, smooth muscle cells, lymphocytes, pericytes, and preadipocytes, among other components. The advantages of SVF over ASCs are thought to be in two areas. First, the heterogeneous cellular composition of SVF may be responsible for the superior therapeutic results seen in comparative animal studies. Second, in contrast to ASCs, SVF can be obtained significantly more quickly without the need for cell separation or special cultivation conditions. As a result, the therapeutic cellular product is relatively safe and is only required to meet minimal regulatory requirements[75]. However, it should be emphasized that whereas ASCs are useful for both allogeneic and autologous treatments, SVF is only appropriate for autologous treatments because it contains a variety of cell types that are known to trigger immunological rejection. hASCs have seemed to be more effective than SVF in HS, related to their higher levels of MMP-2 and MMP-2/tissue inhibitors of metalloproteinase-2 ratio, as well as higher expression of TGF-3 and HGF[76]. Whether SVF is indeed superior to ASCs in skin fibrosis treatment needs further research[6,77].

In addition to HS and keloids, SVF is also broadly applied clinically to scleroderma and SSc. SVF gel has superior anti-inflammatory and antifibrotic effects on scleroderma[78]. Moreover, SSc does not impair SVF's ability to heal vascular damage, hence justifying the use of this novel autologous biotherapy[79]. SVF injection is a potentially effective treatment that seems to last for at least one year. Quality of life, Raynaud's phenomenon, finger edema, and hand impairment and discomfort were significantly improved[80-83].

Stem cell-free therapy

The secretome of ASCs, with a focus on exosomes, appears to be a suitable and safe alternative with more effectiveness and fewer adverse effects due to restrictions on the use of stem cells in cell-based treatment. Moreover, the ability to biobank the ASC secretome is a significant benefit of cell-free therapy. In this review, we concentrate on the current understanding of the secretome of ASCs, such as ASC exosomes (ASC-Exos) and ASC-CM, used in skin fibrosis stem cell-free therapy (Figure 3).

ASC-Exo: As one of the components of paracrine signaling, ASC-Exos are small, single membranous secretory organelles rich in proteins, lipids, nucleic acids, and carbohydrate conjugates[26,84,85]. Among other research discoveries, they are thought to have a variety of activities, such as reshaping the ECM and transmitting signals and molecules to other cells. In addition, they are not rejected by the immune system, have homing effects, and the dose is easily controlled[86,87]. Compared to ASCs, ASC-Exos offer a great opportunity to create new cell-free therapeutic techniques that could circumvent the challenges and dangers related to using natural or synthetic stem cells[86,88].

ASC-Exos release miR-29a-3p, which can suppress the expression of several profibrotic, antiapoptotic, remodeling, and methylase genes[89]. ASC-Exos are now a viable new option for the systemic treatment of keloids. They significantly suppress the development of ECM in keloids by decreasing collagen synthesis and impairing the microvessel structure, enhancing the expression of TGF-3 while inhibiting the protein expression of Smad3 and Notch-1[84]. By suppressing the expression of the TGF-1/Smad pathway, ASC-EXOs may prevent keloid fibroblasts from proliferating and migrating and consequently promoting death[90].

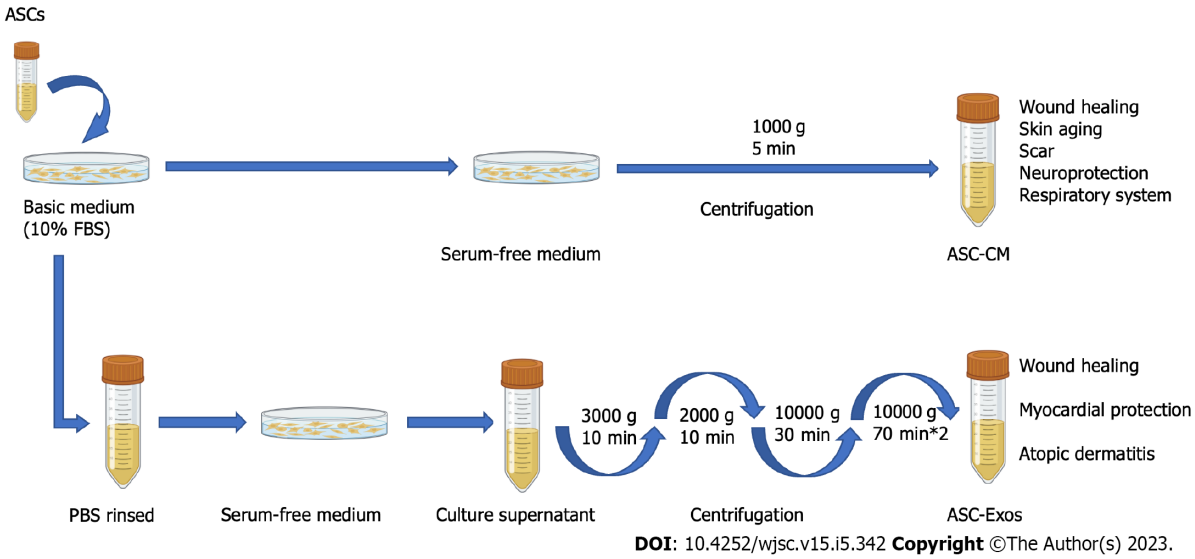

Figure 3 Adipose-derived stem cell conditioned media, exosomes, and adipose tissue extracts synthesis and therapeutic application. ASC: Adipose-derived stem cell; ASC-CM: ASC-conditioned medium; ASC-Exo: ASC exosomes; FBS: fetal bovine serum; PBS: Phosphate-buffered saline. Figure 3 is created with BioRender.com.

In hypertrophic scar fibrosis, ASC-exosomal miR-192-5p targeted IL-17RA to control the Smad pathway, and miR-29a inhibited the TGF-2/Smad3 signaling pathway, which could be responsible for the antifibrotic effects[91,92]. Another postoperative study showed that hASC-Exo therapy inhibited collagen deposition and myofibroblast aggregation *in vivo* and reduced the development of HS[93].

ASC-CM: Active chemicals released by ASCs, such as cytokines, exovesicles, exosomes, DNA, and RNA, are found in ASC-CM and can facilitate tissue healing and control immunity. ASC-CM can lower treatment costs and avoid the safety issues associated with stem cell therapy[94]. One disadvantage of CM over stem cells is the short life of active components. Stem cells can anchor inside a tissue or organ after local administration and function there for a long time, but CM-containing substances such as growth or enzyme factors are rapidly diluted and eliminated by diffusion[95,52].

ASC-CM may reduce collagen deposition and scar formation, inhibiting the p38/mitogen-activated protein kinase signaling pathway can have an anti-scarring effect, and the use of ASC-CM may offer a unique therapeutic approach for the treatment of HS[96]. According to *in vitro* and *ex vivo* experiments, chyle fat-derived stem cell-CM reduced the expression of type I collagen (Col1), type III collagen (Col3), and SMA, which prevents fibrosis in HSFs[63]. ASCs-CM dramatically elevated MMP-1 expression and dose-dependently decreased cell survival, expression of fibrosis markers, tissue inhibitor of metalloproteinases-1, the amount of collagen produced, and the ratio of Col1/Col3. These findings show that ASC-CM efficiently blocks fibrosis-related factors and controls ECM remodeling in HSF[64]. Combining ASC-CM with therapeutic therapies is another development. A histologic study revealed that ASC-CM increased the density of cutaneous collagen and elastin and arranged them in a certain order. A good combination therapy for treating atrophic acne scars and skin rejuvenation is ASC-CM with FxCR[97]. Stronger antifibrotic effects of CD74+ ASC-conditioned media may have resulted from increased production of HGF, FGF2, and TGF-3 and lower levels of TGF-β1[69]. ASC-CM and polysaccharide hydrogels might cross-bind in situ, which could significantly improve the therapeutic results by reducing scar proliferation, offering a promising alternative for the prevention of HS[98].

UPDATES ON THE CLINICAL APPLICATIONS OF ASC THERAPY

To evaluate the effectiveness of ASCs, numerous clinical trials have been carried out; however, they have largely focused on SSCs. More research is required to determine the long-term safety of ASCs, detailed mechanisms of effect, and the capacity to translate experimental results into clinical practice.

ASCs are used to treat secondary-progressive multiple sclerosis in 30 individuals. However, assessments of treatment efficacy revealed a mild tendency toward effectiveness. Establishing the possible therapeutic benefit of this technique would require larger studies and presumably treatment at earlier stages[99].

To compare the effectiveness of an injection of ASC-SVF derived from adipose tissue with placebo in decreasing hand disability in 40 SSc patients. This research demonstrated a gradual improvement with no evidence that the AD-SVF was superior. Given the limitations of this trial, a study with a larger

group of patients is urgently needed to accurately determine the value of ASC-SVF therapy[100]. It was revealed through a randomized controlled trial that regional adipose tissue grafting is beneficial in repairing ischemia digital ulcers in SSc[101].

This study investigates the safety and efficacy of administering autologous SVF cells to SSc patients. Early evaluations at six months suggest a possible efficacy that has to be confirmed in a larger population randomized placebo-controlled trial. Quality of life, Raynaud's phenomenon, finger edema, and hand impairment and discomfort are significantly improved[83]. A sequential 12-mo follow-up showed significant improvement in the vascular suppression score, skin sclerosis, motion and strength of the hands, and finger edema. The decrease in hand discomfort was statistically significant. A benefit was found in daily tasks, housework, and social activities, according to the questionnaire[82].

An open cohort study found that ASCs dramatically reduced the consequences of orofacial fibrosis in SSc. With the inhibition of fibroblast proliferation and important fibrogenesis regulators, including TG-1 and CTGF, ASCs may alleviate skin fibrosis[95].

SAFETY ASSESSMENT

ASCs overcome the ethical issues associated with embryonic stem cells and are therefore considered safe. However, as a stem cell therapy, ASCs still have problems with storage and transport, as well as the risk of inducing tumors and malformations[102]. Further studies on their efficiency are yet needed, taking into account the host environment and patient-related factors. Importantly, a long-term follow-up is needed to supervise cancer recurrence rates in the context of previous malignancy[103].

CONCLUSION

While the underlying mechanism of skin fibrosis is still unclear, ASC therapy plays multiple roles in the treatment of skin fibrosis, with a combination of aesthetic and therapeutic outcomes. Different ASC derivatives show various properties, which might be further explored in clinical trials. In the future, ASC therapy is likely to become an indispensable part of combined treatment in skin fibrosis.

FOOTNOTES

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REVIEW

Modulation of stem cell fate in intestinal homeostasis, injury and repair

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Abstract

The mammalian intestinal epithelium constitutes the largest barrier against the external environment and makes flexible responses to various types of stimuli. Epithelial cells are fast-renewed to counteract constant damage and disrupted barrier function to maintain their integrity. The homeostatic repair and regeneration of the intestinal epithelium are governed by the $Lgr5^{+}$ intestinal stem cells (ISCs) located at the base of crypts, which fuel rapid renewal and give rise to the different epithelial cell types. Protracted biological and physicochemical stress may challenge epithelial integrity and the function of ISCs. The field of ISCs is thus of interest for complete mucosal healing, given its relevance to diseases of intestinal injury and inflammation such as inflammatory bowel diseases. Here, we review the current understanding of the signals and mechanisms that control homeostasis and regeneration of the intestinal epithelium. We focus on recent insights into the intrinsic and extrinsic elements involved in the process of intestinal homeostasis, injury, and repair, which fine-tune the balance between self-renewal and cell fate specification in ISCs. Deciphering the regulatory machinery that modulates stem cell fate would aid in the development of novel therapeutics that facilitate mucosal healing and restore epithelial barrier function.

Key Words: Intestinal stem cell; Epithelial repair; Homeostasis; Regeneration; Self-renewal; Apoptosis

Core Tip: The homeostatic repair and regeneration of the intestinal epithelium upon injury are governed by the Lgr5$^+$ intestinal stem cells (ISCs) located at the base of crypts, which fuel rapid renewal and give rise to different epithelial cell types. We review the current understanding of the intrinsic niche signaling and extrinsic stimulating factors that control homeostasis and regeneration of the ISCs. Deciphering the regulatory machinery that modulates stem cell fate, and formulating strategies for better repair and regeneration would aid in the development of novel therapeutics that facilitate mucosal healing and restore epithelial barrier function.

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INTRODUCTION

Intestinal epithelium serves as the first line of defense against the external environment. As an outward single-layered epithelial structure, the intestinal mucosa withstands continuous mechanical, physico-chemical, and biological insults[1,2]. To counteract intestinal injury and preserve their barrier function, epithelial cells are renewed every 2–5 d in most adult mammals[3]. The epithelial turnover is coordinated by Lgr5$^+$ intestinal stem cells (ISCs) residing at the base of the crypts where they are kept in a multipotent state and produce transit amplifying (TA) progenitor cells. TA cells will undergo several cycles of division before migrating to the villi and ultimately differentiate into multiple lineages[4,5]. The disrupted barrier function and defective mucosal healing are the predominant biological features of intestinal pathology, and particularly, chronic gastrointestinal inflammation such as inflammatory bowel disease (IBD), which is represented by ulcerative colitis and Crohn's disease[6-8]. Current clinical strategies focus on the symptomatic relief and blockade of inflammatory progression[9,10], while better solutions should emphasize the motivation of regenerative response orchestrated by ISCs for complete mucosal healing.

Mucosal healing is an integrated network initiated by a series of biological processes and signals[11]. Intestinal homeostasis is characterized by constant regeneration which demands a fine-tuned balance between ISC proliferation and differentiation[12,13]. In response to diverse insults, the cellular response, combined with the stem cell niche adaptions, synthetically modulates the fate of ISCs to restore homeostasis by replenishment of damaged epithelial cells, or to hasten cell demise by impairment of cell function and vitality[14,15]. Therefore, understanding the cellular response and niche adaptations during injury-induced intestinal regeneration is therefore of importance for ISC biology.

Constant efforts have been made to exploit the regulatory mechanisms of critical components that seal the fate of ISCs. In this review, we give an overview of ISCs and review the adaptations and signals required for homeostasis maintenance. We focus on the cell fate specification and biological alterations of ISCs upon diverse insults and provide insights into intestinal regeneration.

INTESTINAL STEM CELL

Two distinct ISC populations located at the crypts have been proposed: Crypt base columnar (CBC) cells, the active cycling stem cells that facilitate homeostatic self-renewal[16], and +4 cells, the quiescent stem cells reserved for injury-induced repair[17].

CBC cells have been the centerpiece of stem cell research since they were initially identified in 1974 as continuously cycling cells at the base of the crypts[18]. Radionucleotide labeling and autoradiography have been used to state that the cells derived from the crypts migrate upward along the villi to be extruded at the villus tips[19,20]. This conveyor belt mechanism confirms stem cell fueling this rapid self-renewal process resides at the base of the crypts[21]. The generation of Lgr5$^{EGFP-IRES-CreERT2}$ mice reveals that Lgr5, a receptor for WNT signaling-associated R-spondins, is a highly suitable candidate for CBC cell recognition and specification[3]. Single-sorted Lgr5$^+$ stem cells are also able to form these crypt–villus organoids and the Lgr5 hierarchy is maintained in organoids[22]. Recent studies have identified that p27 and Mex3a label the slowly cycling subpopulation of Lgr5$^+$ ISCs based on single-cell transcriptome profiling[23,24].

In addition to Lgr5$^+$ CBC cells, Bmi1$^+$ cells localized at the fourth position of the crypt base and discovered by *in vivo* lineage tracing and transcriptome analyses, are a possible candidate stem cell population[4,25]. Functionally distinct from Lgr5$^+$ ISCs, the quiescent +4 stem cells are considered reserved stem cells that replenish the continuously cycling CBC cells pool when required, and are highly resistant to radiation and insensitive to Wnt signal[17,26]. In face of chemoradiotherapy, Lgr5$^+$

stem cells are vulnerable to chemical- or irradiation-induced injury, due to their predominantly cycling nature[24,27]. Bmi1$^+$ cells quickly revert to ISCs and the *de novo*-generated Lgr5$^+$ ISCs are vital for epithelial regeneration[28]. The evidence summarizes the relationship between active and quiescent stem cells and identifies Lgr5$^+$ stem cells as a substantial contributor to homeostatic regeneration[29].

The identification of new ISC markers and the dedication each subpopulation of ISCs commit to regeneration have improved the understanding of stem cell biology during homeostasis and disease. The emergence of new technologies has promoted the decoding of many key problems in intestinal diseases and tumors[4].

INTESTINAL STEM CELL NICHES

The niche in which ISCs reside can be defined as the microenvironment essential for self-renewal and stemness maintentance[13]. ISCs are strongly linked with adjacent cells of both epithelial and mesenchymal origin. These components, along with their communications, comprise the ISC niche[30,31]. The specific instructive microenvironment offers a native source of signals that fuel ISCs to maintain tissue homeostasis[32]. Various cell types of the niche elaborate typical paracrine signals containing Wnt, R-spondin, Notch, mammalian target of rapamycin (mTOR), bone morphogenetic protein (BMP), epidermal growth factor (EGF) and Hippo, which fine-tune the balance between differentiation and proliferation of ISCs, and ensure the production of an adequate number of cells in homeostatic and injury conditions[1,33] (Figure 1).

Wnt signaling

The canonical Wnt signaling acts as the prominent driver for ISC proliferation. Synchronous Wnt binding to Frizzled and to LRP5/6 suppresses APC-related ubiquitination of β-catenin which mediates its nuclear translocation, the association with lymphoid enhancer binding factor/T cell factor transcription factors, and the succeeding transactivation of Wnt target genes[34-36]. Multiple Wnts such as Wnt2b, Wnt4, and Wnt5a are abundantly expressed in intestinal stroma[30]. A subset of Foxl1$^+$ mesenchymal stromal cells that form a subepithelial plexus around the crypt is a crucial source of intestinal Wnt[37]. Genetic elimination of Foxl1$^+$ cells triggers the loss of Wnt family expression in the epithelium and an abrupt cessation of proliferation of both epithelial stem cell and TA progenitor cell populations, but not Paneth cell[38]. Wnt2b is highly expressed in Gli1$^+$ or αSMA$^+$ subepithelial stromal cells, which is sufficient to restore epithelial integrity when injected into mice which is devoid of Wnt secretion[39]. Gli1$^+$ subepithelial cells are essential contributors to the integrity of the colonic epithelium for Lgr5$^+$ ISC self-renewal in the colon[40]. As a noncanonical Wnt ligand, Wnt5a deficiency causes a failure to develop new crypts at the wound site and limits the proliferation of crypt cells after injury in a transforming growth factor (TGF)β-dependent manner[41]. These findings reveal the essential role of Wnt signal for the stemness and proliferation of ISC and highlight the contribution of stromal cells in the ISC niche.

The R-spondins comprise one of crucial elements of the niche. R-spondins are secretory glycoproteins which firmly cement the capacity of Wnt ligands for the activation of β-catenin-dependent transcription and canonical Wnt signaling, while R-spondins themselves have no intrinsic Wnt signaling activity[29,42]. Overexpressed R-spondins *in vivo* forcefully induce the expansion of ISCs and maintain the epithelial integrity against damage induced by the chemotherapeutic agent 5-fluorouracil, dextran sulfate sodium (DSS), or irradiation[43-45]. Wnt proteins are reported to be insufficient to directly regulate ISC self-renewal, while alternatively grant a fundamental competency through motivating R-spondin ligands to actively motivate ISC[46].

Notch signaling

Notch signaling plays a dominant role in the stem cell niche by preserving the quiescent state of ISCs [47]. Integration between Notch ligands (Notch1–4) and receptors (Jag1–2 and Dll1–4) in adjacent cells is required for Notch activation[48]. Different from Wnt signaling that is mainly generated from a stromal microenvironment, Notch signaling may function *via* neighboring epithelial cells or even stromal subpopulations contacting with ISCs, thus featuring an epithelial niche[49].

Disruption of Notch activity leads to the exhaustion of ISC and differentiation from proliferating TA cells to secretory cells[50]. Simultaneous Notch1/2 deletion recapitulates the global Notch inhibition phenotype of Lgr5$^+$ ISC loss, while the single deletion does not change ISC activity, which suggests the synthetical effect of Notch1/2 in stemness maintenance[51,52].

mTOR signaling

The mTOR signaling is a vital pathway for cellular development and metabolism in mammals. mTOR signaling directly modulates stemness and proliferation of ISCs, functioning as a crucial determinant of cell status within the ISC lineage and modulating differentiation in a nutrient-dependent way[53]. Inhibiting mTOR signaling helps to maintain stemness of ISCs, whereas activation of mTOR facilitates ISCs differentiation and proliferation[54,55]. In the case of caloric restriction, the activity of mTOR

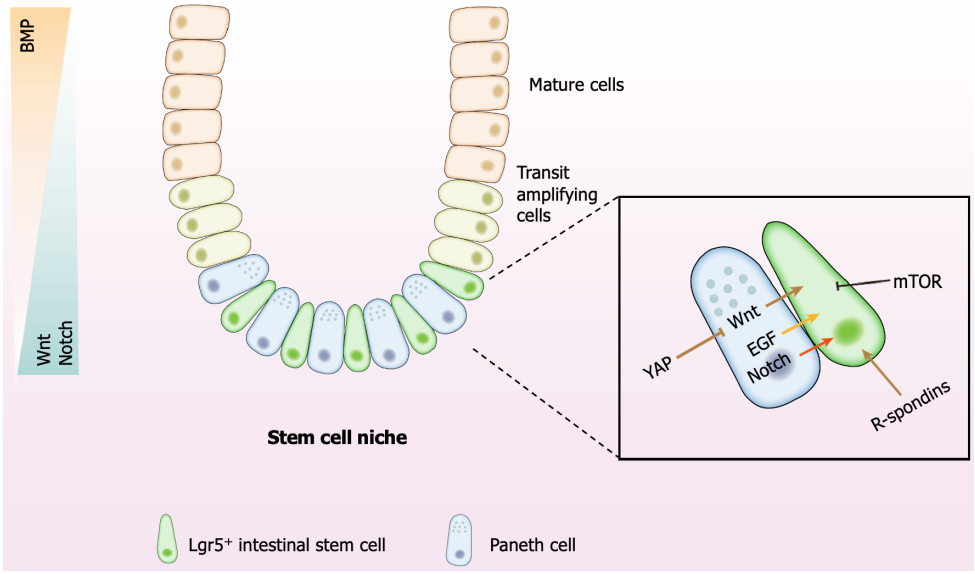

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Figure 1 The intestinal stem cell niche and regulatory signals. The intestinal epithelium consists of crypts and villi. The crypts generate a constant stream of new cells that differentiate and migrate upward into the villi. These Lgr5+ intestinal stem cells reside at the bottom of the crypts and are wedged between Paneth cells, which protect and nurture stem cells. Above the stem cell zone is the lineage-committed progenitor cells, also known as the transit amplifying zone, divided to fuel the rapid epithelial cell turnover. Mature epithelial cells originate from the crypt and move up toward the villus tip. The Wnt and Notch signaling exhibit high activity in the stem cell niche. Activation of the signals decreases along with the increased distance from the crypt bottom. While the BMP activity stands in the opposite direction. WNT, R-spondin, epidermal growth factor, and mammalian target of rapamycin are secreted by Paneth cells or mesenchymal cells. YAP works through the Wnt signaling to maintain the crypt–villus integrity. Besides, Paneth cells provide essential Notch signals to stem cells by expressing Notch ligands. The signaling network in the niche establishes the baseline for self-renewal, fate determination, proliferation, and differentiation of intestinal stem cells. BMP: Bone morphogenetic protein; EGF: epidermal growth factor; mTOR: Mammalian target of rapamycin.

complex 1 is inhibited in Paneth cells, resulting in the paracrine release of cyclic ADP ribose that increases self-renewal of ISCs at the cost of differentiation[56]. In high relevance to diet, the mTOR pathway controls stem cell fate possibly by regulating mitochondrial metabolic states.

BMP signaling

BMP signaling acts as an initiator of differentiation in the crypt. Wnt and BMP signalings are deemed as opposite forces along the crypt–villus axis with counteractive gradients of activity[57]. BMP activity is lower in the bottom and higher towards the top of the villus[58]. To offset the inhibitory effects of BMP signaling on ISC fate, BMP antagonists like Noggin, Gremlin-1, and Gremlin-2 are highly expressed in the crypts, permitting the proliferation of ISCs. The BMP antagonists that enhance ISCs self-renewal are secreted by intestinal subepithelial myofibroblasts and smooth muscle cells[59,60].

EGF signaling

EGF is a vital component of the ISC niche[61]. The EGF receptor is abundantly expressed in CBCs, whereas its ligands are expressed in Paneth cells[62]. The activity of ErbB signaling is monitored by the negative regulation of Lrig1, a transmembrane protein coexpressed with Lgr5 in CBCs[62]. Loss of Lrig1 leads to the activation of receptors and a concomitant rapid expansion of crypts and cell numbers. Blockade of EGF signaling in intestinal organoids drives proliferative ISCs into quiescent state and stops organoid budding[63]. The evidence suggests the requirement of EGF in epithelial regeneration.

Hippo signaling

The Hippo pathway, a highly conserved signaling first described in *Drosophila* as an organ size control pathway, is comprised of a core kinase cascade, Mst1/2 and Lats1/2, which phosphorylate and suppress transcriptional coactivators Yes1 associated transcriptional regulator (YAP) and Tafazzin (TAZ), thereby modulating TEA domain transcription factor 1 (TEAD)-mediated transcriptional activation[64,65]. YAP/TAZ are the core components for stem cell-based regeneration. YAP overexpression in mice accelerates the self-renewal of colonic epithelium, and augments the number of proliferative cells and the cell migration along the crypt-villus axis, as detected by BrdU marker[43]. While YAP depletion causes a significant decrease in crypt proliferation, extensive crypt loss and consequently regeneration failure upon DSS or irradiation[66,67]. Loss of YAP activity contributes to higher sensitivity of ISCs to apoptosis and lower proliferative capacity during regeneration[68]. Moreover, the core Hippo kinases Lats1/2 are essential to maintain ISC activity and their deletion leads to the loss of ISCs[69]. This demonstrates their essential effects on epithelial proliferation and tissue regeneration.

YAP may actively block Wnt signaling and thus apply negative feedback on Wnt signaling *via* β-catenin inhibition[43], indicating the complex interaction between YAP and other niche signals which may need further investigation.

ENDOGENOUS AND ENVIRONMENTAL STIMULI

The intestinal epithelium is exposed to a hostile luminal environment, thus resulting in Lgr5$^+$ ISCs continuously encountering sources of stress to maintain dynamic homeostasis. We summarize the major endogenous and environmental stimuli that influence stem cell regenerative potential (Figure 2).

Endogenous factors

Niche signals: Damage to the crypts can result from infections, chronic inflammation, chemoradiotherapy, or traumatic injury and motivate a series of actions in the stem cell niche[31]. The microenvironment monitors the regenerative response *via* regulating a series of signaling pathways, such as Wnt, Notch, BMP, and Hippo. This has been discussed above.

Extracellular matrix: The state of stem cells largely depends on the properties of the extracellular matrix (ECM)[31]. The integrin complex assists cells in sensing the stiffness of the ECM and directs the fate of ISCs *via* adhesion signaling[70,71]. In this way, the ECM affects cellular behavior, including proliferation and differentiation. ECM stiffness is also a vital endogenous factor of mesenchymal stem cells to differentiate into osteoblasts, myoblasts, or neurons[72]. In particular, YAP/TAZ lay the foundation for ECM stiffness sensing and play a prominent role in intestinal repair and regeneration[73]. High matrix stiffness significantly enhances ISC expansion in a YAP-dependent manner[74]. These clues speculate that ECM sensation is capable of modulating self-renewal in ISCs.

Mitochondrial function: Mitochondrial function emerges as a central player in cell fate determination and extensive control of cellular stress responses, metabolism, immunity, and apoptosis[75]. Mitochondria are the center of energy metabolism and the regulator of stem cell homeostasis[76,77], and oxidative phosphorylation is particularly important to maintain the function of stem cells[78]. For example, pyruvate oxidation in the mitochondria works as a metabolic checkpoint of ISC self-renewal and/or stemness maintenance[77]. Regulators of mitochondrial signals such as ATP, reactive oxygen species (ROS), the mitochondrial unfolded protein response, and AMP-activated protein kinase signaling, will in turn affect stemness and cell cycle progression[75].

Enteric nervous system: In spite of the limited studies into the underlying relationship between the nervous system and intestinal epithelial regeneration, some novel indications are pointed out that the enteric nervous system exerts a potential role with great value. Enteric glial cells are closely connected with the intestinal epithelium and depletion of enteric glial cells will exacerbate DSS-induced injury [79]. The administration of hepatocyte growth factor from neural cells of the enteric nervous system attenuates the hostile effects of DSS[80]. Several reports also unveil a potential effect of the enteric glial cells in mucosal healing through the release of the specific niche factors like glial-derived neurotropic factor, TGF-β1 or 15-deoxy-12,14-prostaglandin J2[41,81,82].

Extrinsic factors

Diet: The biological behavior of stem cells is largely affected by nutritional state[31]. The mTOR signaling is responsible for sensing the nutritional state[83]. Mice fed with a calorie-restricted diet exhibit an augmented function in Lgr5$^+$ ISCs and Paneth cells compared with mice with a normal diet [56,84]. Moreover, the calorie-restricted diet diminishes mTOR activity in quiescent stem cells, improving their resistance to radiation damage and promoting intestinal repair[85]. In contrast, a high-fat diet can increase ISC activity despite decreasing Paneth cells activity. ISCs of high-fat diet mice exhibit higher resistance to irradiation and more efficient organoid budding potential than control mice. The high-fat diet activates Wnt signaling in ISCs dependent on the nuclear peroxisome proliferator-activated receptor δ[86]. Another study also reveals that an obesogenic diet induces ISCs and progenitor cells hyperproliferation, triggers ISC differentiation and cell turnover, and alters the regional characteristics of ISCs and enterocytes in mice[87]. Acute fasting has been shown to lead to transient phosphatase and tensin homolog (PTEN) phosphorylation within quiescent ISCs and render quiescent ISCs functionally poised to contribute to the regenerative response during refeeding[88].

Microorganisms: Microorganisms play an indispensable role in gut homeostasis, but the underlying mechanisms are complicated and elusive. Small molecules and metabolites produced by gut microbiota significantly contribute to the host intestinal development, function, and homeostasis[89]. Lactate from lactic acid-producing bacteria plays a pivotal role in promoting ISC proliferation and epithelial development[90]. Butyrate within the crypts conveys a growth-inhibiting effect on Lgr5$^+$ ISCs *via* Forkhead box O3[91]. The bacterial product muramyl dipeptide has been reported to decrease the level of ROS in ISCs, and promote intestinal organoid growth and tissue repair[92,93]. *Salmonella* can enter

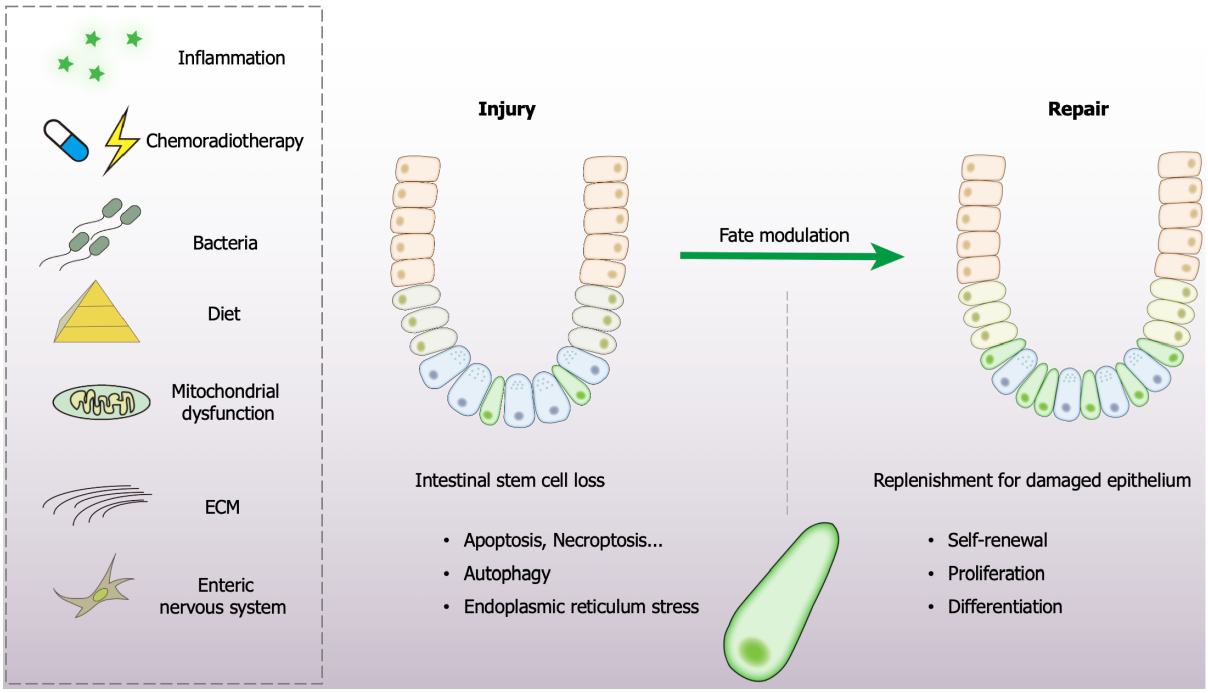

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Figure 2 Modulation of stem cell fate determination and epithelial repair by various sources of stress. Both exogenous (inflammation, chemoradiotherapy, and diet) and endogenous factors (mitochondrial dysfunction, extracellular matrix, and enteric nervous system) have vital effects on stem cell fate modulation. The self-renewal capacity, the balance of proliferation and differentiation are tightly controlled in the process of epithelial repair and regeneration. ECM: Extracellular matrix.

the crypts during infection and cause a significant decrease in Lgr5$^+$ ISCs[94,95]. The enteric pathogen rotavirus specifically invades and deteriorates differentiated cells at villus tips, and then motivates Lgr5$^+$ ISCs, crypt expansion, and hyperproliferation[96]. Gut pathogens are thus distinctive elements capable of tuning the stem cell fate.

Inflammatory signaling: In addition to the local niche signals and physicochemical stimuli, the activation of the immune system is also involved in the interactions between epithelial cells and the niche to guarantee proper initiation and continuation of the regenerative response. Interleukin (IL)-22, which derives from the intestine, contains group 3 innate lymphoid cells that reside in close proximity to intestinal crypts and are upregulated after injury and support subsequent epithelial regeneration[97]. Recombinant IL-22 has been shown to directly target ISCs, thus facilitating the growth of human and mice intestinal organoids and promoting ISC self-renewal[98,99]. A recent study also indicates that the symmetric division of ISCs can be triggered by inflammatory signals to prevent excessive expansion in the process of epithelial repair[100].

Chemoradiotherapy: Intestinal mucosal damage occurs in 40%–60% of patients receiving chemotherapy or radiotherapy[101]. Chemotherapy- or radiotherapy-induced cellular apoptosis can be the primary factor initiating the gastrointestinal syndrome[102,103]. The injury response of intestinal epithelium after chemoradiotherapy has been the most extensively characterized model of Lgr5$^+$ ISC loss and proliferation to date, due to its hypersensitivity to radiation and chemotherapy. Targeting p53-dependent stem cell death is the core strategy for intestinal chemo- or radioprotection[102,104,105]. The Toll-like receptor 4 signaling pathway[106], Slit guidance ligand 2 (Slit2)/ Roundabout guidance receptor 1 (Robo1) signaling[45], gut microbiota[90,107], and dietary components such as green tea derivative (-)-epigallocatechin-3-gallate[108], aspartate[109], pectin[110], and vitamin D[111] have been shown to mitigate the loss of ISCs and alleviate intestinal injury. The deletion of CREPT suppresses the proliferation and differentiation of ISCs and reduces Lgr5$^+$ cell numbers after X-ray irradiation[112]. Therapeutic strategies based on the inhibition of ISC apoptosis without compromising the efficacy of cancer treatment are of great potential.

MODULATION OF STEM CELL FATE

The injury response of intestinal epithelium is critical to restore epithelial integrity upon diverse insults [13]. The immediate response of intestinal damage is the loss of Lgr5$^+$ ISCs, while it is generally adaptive

modulation since the reserved subpopulations are activated to replenish the defects. However, excessive damage may cause ISC depletion and militate against epithelial regeneration. Critical cellular adaptations have been made to restore homeostasis in ISCs.

Apoptosis

As the best-understood form of programmed cell death, apoptosis has been largely clarified in the field of stem cells. Lgr5$^+$ ISCs are more vulnerable to apoptosis than Bmi1$^+$ stem cells are[113]. Considering the critical role of mitochondria in stemness maintenance, regenerative capacity determination, and modulation between self-renewal and cell death programs, mitochondrial function is the vital determinant of stem cell fate[75]. For Lgr5$^+$ ISCs, mitochondrial dysfunction is the major cause of apoptosis. A series of molecules such as Bcl-2, Puma, Survivin, Phosphoribosyl pyrophosphate synthetase 1 (PRPS1), and X-linked inhibitor of apoptosis have been characterized for modulating ISC apoptosis. Other biological processes, including immune response, hormone response, post-translational modification, and signaling such as Hippo and G protein coupled receptor, are crucial to controlling Lgr5$^+$ ISC apoptosis. The pivotal molecules regulating stem cell apoptosis are shown in Table 1. The excavation of novel strategies based on ISC survival is of great significance to epithelial regeneration.

Necroptosis

Necroptosis is also involved in crypt damage. The loss of SETDB1 in ISCs, a histone methyltransferase that induces the trimethylation of histone H3 at lysine 9, triggers Z-DNA-binding protein 1-dependent necroptosis, which irreversibly disrupts the integrity of the epithelial barrier and promotes the progression of IBD[114]. Intestinal organoids lacking ATG16L1 are more prone to initiate tumor necrosis factor (TNF)α-mediated necroptosis, and therapeutic blockage of necroptosis through TNFα or RIPK1 inhibition ameliorates the severity of IBD[115]. TNFα exacerbates necroptosis of differentiated cells and mediates the expansion of LGR5$^+$ ISCs[116]. Therefore, necroptosis inhibitors could be used to promote mucosal healing in IBD patients.

Autophagy

Autophagy is a highly conserved process during evolution in eukaryotes, by which the cytoplasmic materials are degraded inside the autolysosome. Three distinct forms of autophagy, including microautophagy, chaperone-mediated autophagy, and macroautophagy have been described. Autophagy has been demonstrated crucial in modulating the interactions between gut microbiota and innate and adaptive immunity, in host defense against intestinal pathogens, and in maintaining intestinal homeostasis[117].

In the *Drosophila* intestine, autophagy downregulates the sensitivity of differentiated enterocytes to ROS when exposed to commensal bacteria. Mechanistically, the autophagic substrate Ref (2)P/p62 accumulates upon autophagy deficiency, thus inactivating Hippo signaling and leading to stem cell over-proliferation[118]. Autophagy can also protect ISCs against irradiation-induced oxidative stress by preserving mitochondrial health and function. Accordingly, stem cell-based intestinal regeneration after radiotherapy is impaired in mice with Atg5 deficiency. Another recent work has highlighted the role of ATG16L1-dependent autophagy in protecting ISCs from irradiation-induced ROS[92,119]. In a *Drosophila* model, Atg6 deficiency impairs the inhibitory effect of metformin on ISC aging[120]. A recent study has confirmed the role of Atg7 in maintaining epithelial integrity against DNA damage and cell death[121]. With the rapid progress of Lgr5$^+$ ISC isolation and detection, the role of autophagy in ISCs will be further elucidated.

Endoplasmic reticulum stress and unfolded protein response

As the primary organelle for protein folding and quality control, endoplasmic reticulum (ER) is sensitive to multiple intrinsic cellular disturbances and extrinsic environmental changes, which would alter ER homeostasis and cause misfolded protein accumulation, leading to activation of unfolded protein response (UPR)[122]. Previous studies have shown that UPR exerts a significant role in the pathogenesis and progression of IBD[123]. Human genetic studies of IBD have identified primary genetic abnormalities in several genes, including *Xbp1*, *Agr2*, and *Ormdl3*, that encode proteins associated with ER stress[124-126]. More importantly, the control of ISC fate is coordinated by ER stress and UPR. Activation of ER stress leads to the loss of stemness of ISCs in a PERK–eIF2α-dependent manner[127]. XBP1, a stress sensor involved in the UPR, acts as a signaling hub to regulate stem cell function and epithelial DNA damage responses in a p53–DDIT4L-dependent manner[128]. XBP1 is also demonstrated to maintain ISC quiescence and control ISC activity[129]. Intestinal epithelium-specific deletion of glycoprotein 96, an ER-resident master chaperone, causes rapid destruction of stem cell niche, followed by complete eradication of the mucosal layer and epithelial cell death[130]. In summary, UPR is indispensable for stemness maintenance and fate determination of ISCs.

Table 1 Apoptosis regulation of intestinal stem cells

Biological process or signaling	Molecule	Role	Evidence
Mitochondrial dysfunction	Puma	Pro-apoptotic	Puma depletion reduces chemoradiotherapy- induced apoptosis in a p53-dependent manner [102,136]
	Bcl-2	Anti-apoptotic	Bcl-2 is highly expressed in ISCs and alleviating radiation-induced damage[137]
	Survivin	Anti-apoptotic	An essential guardian of ISC during mucosal healing[138]
	PRPS1	Pro-apoptotic	PRPS1 deficiency exhibit resistance against intestinal damage in a manner dependent upon Lgr5+ ISCs[139]
Immune response	IL-22	Anti-apoptotic	IL-22 deficiency led to increased crypt apoptosis, depletion of ISCs[97]
	NOD2	Anti-apoptotic	Nod2 stimulation triggers stem cell survival against oxidative stress-mediated cell death[93]
Hippo	YAP	Anti-apoptotic	Loss of YAP activity results in sensitivity of crypt stem cells to apoptosis and reduced cell proliferation during regeneration[140]
GPCR	β-Arrestin1/2	Anti-apoptotic	βArr reduced the chemotherapy- induced Lgr5+ stem cell apoptosis by inhibiting endoplasmic reticulum stress[141,142]
Hormone	GLP-2	Anti-apoptotic	GLP-2 expanded intestinal organoids and downregulated apoptosis-related genes[143]
	Ghrelin	Anti-apoptotic	Ghrelin treatment accelerated the reversal of radiation-induced epithelial damage and defective self-renewing property of ISCs[144]
Methylation	Mettl14	Anti-apoptotic	Specific deletion of the Mettl14 gene resulted in colonic stem cell apoptosis[145]
	GsdmC	Anti-apoptotic	GsdmC N6-adenomethylation protects mitochondrial homeostasis and is essential for Lgr5+ cell survival[146]
Glycosylation	HYOU1	Anti-apoptotic	HYOU1 glycosylation modulated by FUT2 protects ISCs against apoptosis[147]

ISCs: Intestinal stem cells; PRPS1: Phosphoribosyl pyrophosphate synthetase 1; GPCR: G protein coupled receptor.

FUTURE DIRECTIONS FOR STEM-CELL-BASED THERAPY

Stem-cell-based therapy holds great promise for the complete mucosal healing of gastrointestinal diseases. Related studies have applied exogenous stem cells such as mesenchymal stem cells and placental-derived stem cells for treating intestinal inflammation and injury[131,132], and achieved encouraging outcomes. With the boost of research in the field of ISCs, the intestinal organoid models, especially those of human origin, offer a unique platform to explore the mystery of ISC fate decisions and lineage specification in physiological and pathological conditions[14], and excavate novel strategies to facilitate the regenerative capacity of ISCs. Integration with novel nanomaterials can provide a more effective strategy for facilitating intestinal repair targeting at ISCs, such as grape exosome-like nanoparticles[133], polydopamine nanoparticles[134], and carbon nanoparticles[135]. Thus, one important future direction in the ISCs field is to precisely tune the fate of stem cells for better regeneration.

CONCLUSION

Intestinal epithelial regeneration is a complex network that is based on the function of ISCs. The dynamic balance between stemness and self-renewal is fine-tuned by stem cell niche and various endogenous or extrinsic factors. Great strides have been made in our understanding of the function and fate specification of ISCs in health and disease. In this review, we summarize the different components and signals that function in ISCs in the process of intestinal epithelial injury and repair. Cellular adaptations including apoptosis, necroptosis, autophagy, and UPR have been extensively investigated. Modulating the essential niche signaling or facilitating beneficial elements in the stem cell microenvironment provides novel insights into the regenerative process and opens an avenue for stem cell-based therapies for diseases caused by intestinal epithelial injury.

FOOTNOTES

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REVIEW

Stimulating factors for regulation of osteogenic and chondrogenic differentiation of mesenchymal stem cells

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Abstract

Mesenchymal stem cells (MSCs), distributed in many tissues in the human body, are multipotent cells capable of differentiating in specific directions. It is usually considered that the differentiation process of MSCs depends on specialized external stimulating factors, including cell signaling pathways, cytokines, and other physical stimuli. Recent findings have revealed other underrated roles in the differentiation process of MSCs, such as material morphology and exosomes. Although relevant achievements have substantially advanced the applicability of MSCs, some of these regulatory mechanisms still need to be better understood. Moreover, limitations such as long-term survival *in vivo* hinder the clinical application of MSCs therapy. This review article summarizes current knowledge regarding the differentiation patterns of MSCs under specific stimulating factors.

Key Words: Mesenchymal stem cells; Differentiation; Osteogenic; Chondrogenic; Literature review.

Core Tip: Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating in specific directions. The differentiation process of MSCs depends on diverse specialized external stimulating factors. The results from recent studies have revealed other underrated roles in the differentiation process of MSCs. However, several questions remain to be solved prior to stable and effective clinical treatment. Our review explores the differentiation patterns of MSCs and summarizes the relevant research according to stimulus types. Finally, future prospects are discussed with regard to their clinical applications.

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INTRODUCTION

Mesenchymal stem cells (MSCs), which were originally identified in the bone marrow, are adult stem cells with multilineage differentiation potential. Under specific induction conditions, MSCs could differentiate into bone, adipose, muscle, neural, and endothelial tissue cells[1]. With the development of research, MSCs have been obtained from other tissues, including adipose, peripheral blood, umbilical cord blood, and periodontal membrane tissue[2-5]. Due to their multilineage differentiation potential and rich tissue sources, the application of MSCs in research on regenerative medicine is virtually limitless[6]. However, a specific number of MSCs are necessary for tissue regeneration; hence, there is a requirement for MSC amplification before therapy[7]. The question of how the differentiation of MSCs are controlled *in vitro* and *in vivo* remains unanswered, which has limited the effectiveness of MSCs in the application of regenerative medicine research. Recently, various external stimulus factors, such as biochemical stimuli, hypoxia, physical stimuli, material properties, and exosomes, have been found to have an impact on the differentiation process of MSCs (Figure 1). The purpose of this review is to discuss a variety of recent findings regarding the important external stimulus factors that influence the self-renewal and osteogenic and chondrogenic differentiation potential of MSCs.

BIOCHEMICAL STIMULI

Growth factors, cytokines, and miRNAs are examples of biochemical stimuli that have typically been employed to control the destiny of MSCs. Growth factors and cytokines bind to the corresponding receptors and transfer signals, while miRNAs degrade mRNAs or inhibit the translation of mRNAs to regulate gene expression and thus influence the differentiation fate of MSCs. Numerous studies have examined the effects of various growth factors, cytokines, and miRNAs on the proliferation and differentiation of MSCs into other cellular phenotypes (Table 1).

Growth factors

Growth factors, including fibroblast growth factor (FGF), transforming growth factor (TGF), platelet-derived growth factor, hepatocyte growth factor, granulocyte colony-stimulating factor and bone morphogenetic protein (BMP), are a class of peptides that regulate cell growth and other cell functions by binding to specific cell membrane receptors[8].

FGF-2, also known as basic bFGF, has been the subject of the majority of FGF research to date. In a concentration-dependent manner, bFGF might promote the proliferation of MSCs from several tissue sources, including bone marrow peri-adipocyte cells[9], synovial MSCs[10], adipose-derived stem cells (ADSCs)[11], umbilical cord-derived MSCs[12], and bone MSCs (BMSCs)[13,14]. Ramasamy *et al*[12] reported that cell proliferation increased accordingly with increasing bFGF concentrations in the range of 0-40 ng/mL. However, Ma *et al*[11] and Wang *et al*[14] observed that the proliferation efficiency of cells at 5 ng/mL of bFGF was higher than that at 10 ng/mL. As a result, the use of 5 ng/mL of bFGF appeared to be an appropriate choice to promote the proliferation of different MSCs. In addition to enhancing MSC proliferation, bFGF has been shown to maintain stemness, support cartilage differentiation, and influence osteogenic differentiation[9,10,13]. Intriguingly, Wang *et al*[14] reported that bFGF pretreatment inhibited osteogenic differentiation at the early stage, but promoted it in the medium phase[13]. This finding might indicate that the addition of different growth factors at different phases of osteogenesis induction could successfully promote osteogenic differentiation. Therefore, more studies are needed to clarify the mechanism of action of bFGF at different stages of osteogenic differentiation, as well as to identify the best combination of growth factors to effectively promote the osteogenic differentiation of MSCs.

Previous research has demonstrated the involvement of TGF-β in inducing chondrogenic differentiation[5]. However, while promoting cartilage differentiation, TGF-β also led to early hypertrophic maturation and the eventual formation of nonfunctional fibrocartilage[2,15]. In addition, TGF-β was also found to promote the proliferation of MSCs and their effect on osteogenic differentiation[16,17]. MSC osteogenic differentiation was influenced by TGF-β in a dose-dependent manner. According to research by Xu *et al*[17], low concentrations of TGF-β (1 ng/mL) promoted the osteogenic development of BMSCs, whereas high concentrations (10–50 ng/mL) of TGF-β inhibited osteogenic differentiation. Igarashi *et al*[18] showed that 5 ng/mL of TGF-β regulated the phenotypic differentiation of BMSCs toward osteoblasts but seemed to inhibit osteogenic differentiation at the late stage, suggesting that

Table 1 Growth factors, cytokines, and their effects on the differentiation of mesenchymal stem cells

Factors	Amount/types	Concentration	Cell dource	Results	Ref.
FGF	FGF-2	10 ng/ml	BM-PACs	FGF-2 did not lead to cell differentiation into a chondrogenic lineage	Endo *et al*[9]
	bFGF	5 ng/ml	SMSCs	Promoted SMSCs chondrogenic differentiation	Okamura *et al*[10]
	bFGF	0-40 ng/ml	UC-MSCs	bFGF did not alter osteogenic nor adipogenic differentiation potential	Ramasamy *et al*[12]
	bFGF	20 ng/ml	BMSCs	bFGF pretreatment inhibited osteogenic differentiation of BMSCs at early stage, promoted it in the medium phase, and maintained it in the later stage during osteogenic induction	Wang *et al*[13]
TGF-β	TGF-β3	10 ng/ml	SF-MSCs	Increased the expression levels of COL2A1, SOX9, ACAN, COL10A1	Jia *et al*[15]
	TGF-β	10 ng/ml	ADSCs	Promoted ADSCs chondrogenic differentiation but led to early hypertrophic maturation	Hesari *et al*[2]
	TGF-β1	1, 10, 20 or 50 ng/ml	BMSCs	Low concentration of TGF-β1 (1 ng/ml) promoted osteogenic differentiation of BMSCs while high concentration of TGF-β1 (10 to 50 ng/ml) significantly inhibited osteogenesis	Xu *et al*[17]
	TGF-β	5 ng/ml	BMSCs	Promoted osteogenic differentiation of BMSCs but suppressed the maturation of ostroblastic MSC differentiation at the last stage of osteogenic process	Igarashi *et al*[18]
	TGF-β3	10 μg/L	PDLSCs	Induced chondrogenesis	Choi *et al*[5]
IL	IL-6	100 ng/mL	BMSCs	Promoted BMSCs osteogenic differentiation	Xie *et al*[21]
	IL-17A	5-40 ng/ml	BMSCs	Promoted the neuronal-associated gene expression of BMSCs	Chen *et al*[24]
	IL-17	50 ng/mL	Mouse MSCs	Enhanced the osteogenic differentiation of mMSCs	Liao *et al*[22]
	IL-6	100 ng/mL	hMSCs	IL-6/soluble IL-6R promoted chondrogenic differentiation of MSCs	Kondo *et al*[20]
	IL-17A	50 ng/ml	BMSCs	Inhibited osteogenic differentiation of BMSCs	Wang *et al*[23]
	IL-22	10 ng/ml	MSCs	Upregulated osteogenic and adipogenic transcription factors	El-Zayadi *et al*[25]

FGF: Fibroblast growth factor; FGF-2/bFGF: Basic fibroblast growth factor; TGFβ: Transforming growth factor β; IL: Interleukin; BMSCs: Bone mesenchymal stem cells; BM-PACs: Bone marrow peri-adipocyte cells; ADSCs: Adipose-derived stem cells; hMSCs: Human mesenchymal stem cells; SMSCs: Synovial mesenchymal stem cells; UC-MSCs: Umbilical cord-derived mesenchymal stem cells; SF-MSCs: Synovial fluid-derived mesenchymal stem cells; PDLSCs: Periodontal ligament stem cells; COL2A1: Collagen type II alpha 1 chain; SOX9: Sex-determining region Y-box 9; ACAN: Aggrecan protein; COL10A1: collagen type X alpha 1 chain.

additional cellular signals were necessary for the osteogenic differentiation of some types of MSCs. Therefore, it is crucial to determine how to prevent hypertrophy during TGF-β promoted cartilage differentiation.

Cytokines

The fate of MSCs might be influenced by many cytokines, such as interleukin (IL), tumor necrosis factor (TNF) and interferons (IFN). Studies have previously examined how various cytokines affected osteogenic differentiation. IL-10, IL-11, IL-18, and IFN-γ promoted osteogenesis, while TNF-α, TNF-β, IL-1α, IL-4, IL-7, IL-12, IL-13, IL-23, IFN-α and IFN-β inhibited osteogenesis[19]. In this article, we focus on recently discovered cytokines such as IL-6, IL-17, and IL-22 that have the potential to affect the fate of MSCs.

MSCs both produced IL-6 and reacted to it. Furthermore, the gradual reduction in IL-6 secretion by MSCs during chondrogenic differentiation suggested that IL-6 was one of the distinguishing characteristics of undifferentiated MSCs[20]. Nevertheless, the addition of exogenous IL-6 was found to be

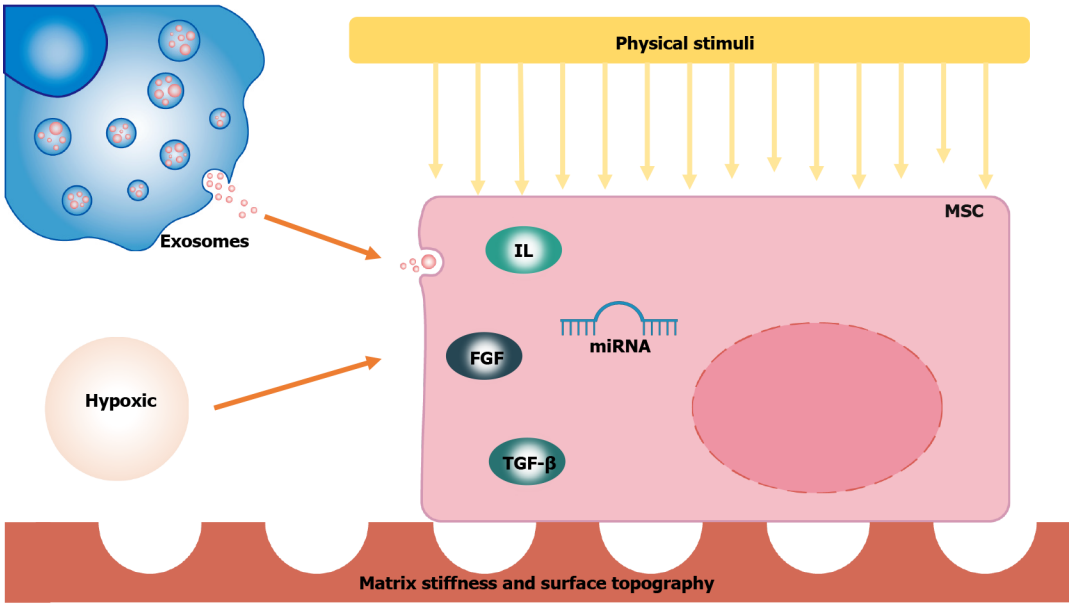

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Figure 1 Overview of stimulating factors in differentiation of mesenchymal stem cells. MSC: Mesenchymal stem cell; IL: Interleukin; FGF: Fibroblast growth factor; TGF-β: Transforming growth factor-β.

effective in promoting the osteogenic differentiation and chondrogenic differentiation of MSCs[20,21]. In contrast to previous studies, Xie *et al*[21] discovered that IL-6 secretion by BMSCs increased rather than decreased with osteogenic differentiation. The effect of IL-17A on the osteogenic differentiation of MSCs also seemed to be contradictory. Liao *et al*[22] reported that IL-17A inhibited the osteogenic differentiation of MSCs as well as pre-osteoblast cell lines. However, the study by Wang *et al*[23] showed the opposite. The appearance of these phenomena might be due to different microenvironments and cellular sources. Additionally, different concentrations of IL-17A have been shown to promote neuronal differentiation, with the best effect at 20 ng/mL[24]. The effect of IL-22 on the proliferation and differentiation of MSCs was first reported by scholars in 2017, which showed that IL-22 alone could upregulate the levels of osteogenic and lipogenic transcription factors but needed to be combined with IFN-γ and TNF to promote the proliferation of MSCs[25].

Cytokines must bind to specific receptors to transmit signals. The amount of the relevant receptor for cytokines appeared to be the rate-limiting element regulating the differentiation of MSCs[20]. Therefore, more studies are required to determine how cytokines affect the growth and differentiation of MSCs. Moreover, a fresh approach will be to look for factors that may raise the number of cytokine receptors on the surfaces of MSCs.

miRNAs

Small non-coding RNAs (approximately 20–25 nucleotides) called miRNAs are a subclass that could bind to complementary target sites in mRNA molecules to inhibit translation or decrease mRNA stability, which controls gene expression[26]. In this case, miRNAs could regulate the expression of key genes during the differentiation of MSCs in specific lineages to influence the direction of differentiation of MSCs (Table 2).

The osteogenic differentiation of MSCs was found to be regulated by micro RNA-1286[27], micro RNA-223-3p[28], micro RNA-346-5p[29], micro RNA-21[4] and micro RNA-130a[30], whereas the chondrogenic differentiation of MSCs was found to be regulated by micro RNA-130b[31], micro RNA-218[32], micro RNA-495[33] and micro RNA-30a[34]. In addition to this, some miRNAs also exhibited roles in regulating the adipogenic differentiation[30], endothelial differentiation[26], neuronal differentiation[35], and myocardial differentiation[36,37] of MSCs.

In conclusion, investigating the impact of biochemical stimuli on the growth and differentiation of MSCs has aided our understanding of the patterns of the aberrant differentiation of MSCs in diseased situations and aided in identifying novel therapeutic targets. It appears to be a promising avenue to examine the impact of the combination of diverse biochemical stimuli on the fate of MSCs, since distinct biochemical stimuli in the microenvironment in which MSCs are positioned function in a compound manner. Additionally, since the functions of cytokines and growth factors are dependent on binding to the appropriate receptors and some studies have indicated that receptor expression might be the rate-limiting factor, it would be preferable to determine methods to boost receptor expression as opposed to raising cytokine and growth factor concentrations.

Table 2 Micro RNA and their effects on the differentiation of mesenchymal stem cells

Amount/types	Expression	Cell source	Results	Ref.
micro-RNA-1286	Over expression	hMSCs	↓ Osteogenic differentiation	Zhou *et al*[27]
micro-RNA-223-3p	Low expression	BMSCs	↑ Osteogenic differentiation	Long *et al*[28]
micro-RNA-346-5p	Over expression	BMSCs	↓ Osteogenic differentiation	Zhang *et al*[29]
micro-RNA-21	Over expression	hucMSCs	↑ Osteogenic differentiation	Meng *et al*[4]
micro-RNA-130a	Over expression	BMSCs	↑ Osteogenic differentiation ↓ adipogenic differentiation	Lin *et al*[30]
micro-RNA-130b	Low expression	BMSCs	↑ Chondrogenic differentiation	Zhang *et al*[31]
micro-RNA-218	Over expression	SDSCs	↑ Chondrogenic differentiation during the eraly stage	Chen *et al*[32]
micro-RNA-495	Over expression	hMSCs	↓ Chondrogenic differentiation	Lee *et al*[33]
micro-RNA-30a	Over expression	BMSCs	↑ Chondrogenic differentiation	Tian *et al*[34]
micro-RNA-145	Low expression	ADSCs	↑ Endothelial differentiation	Arderiu *et al*[26]
micro-RNA-124	Over expression	ADSCs	↑ Neuronal differentiation	Mondanizadeh *et al*[35]
micro-RNA-10-5p	Low expression	BMSCs	↑ Myocardial differentiation	Li *et al*[36]
micro-RNA-499a-5p	Over expression	BMSCs	↑ Cardiomyogenic differentiation	Neshati *et al*[37]

↑: Increase; ↓: Decrease; hMSCs: Human mesenchymal stem cells; BMSCs: Bone mesenchymal stem cells; hucMSCs: Human umbilical cord mesenchymal stem cells; SDSCs: Synovium-derived mesenchymal stem cells; ADSCs: Adipose-derived stem cells.

PHYSICAL STIMULI

In addition to the previously mentioned biochemical stimuli, physical stimuli such as electromagnetic fields (EMF), microgravity (MG), fluid shear stress (FSS), and hydrostatic pressure (HP) could also have an impact on the proliferation and differentiation of MSCs (Table 3). EMF, a non-invasive biophysical therapy, is a combination of electric and magnetic fields and has been widely used in the treatment of bone diseases[38,39]. Exposure to sinusoidal EMF (1mT,15Hz,4h/d) promoted the proliferation and osteogenic and chondrogenic differentiation of BMSCs[40]. In contrast, Wang *et al*[41] found that EMF also promoted the osteogenic differentiation of MSCs but did not inhibit their proliferation under the same parameters. With the exception of 75 Hz square EMF, Asadian *et al*[42] discovered that EMFs of various frequencies and waveforms (25, 50 Hz square, and sinusoidal waveform EMFs) enabled the suppression of BMSC growth. This might imply that MSCs from different sources had different sensitivities to EMFs. Distinct EMFs had different responses to MSCs. It is crucial to investigate the most appropriate EMF parameters for the proliferation or directed differentiation of MSCs from various sources. For instance, MSCs exposed for a brief period of time to low-amplitude and low-frequency pulsed EMF could be encouraged to differentiate into chondrogenic cells[43], while sinusoidal EMF at 1 mT, 15 Hz, 4 h/d was favorable for MSCs to differentiate into osteogenic cells[40,41], and higher-frequency EMF could also encourage MSCs to differentiate into neuronal cells[42].

Another independent factor influencing the destiny of MSCs has been identified as MG. Most of the research was thus for only conducted in a simulated MG (SMG) environment produced by a clinostat or rotating vessel, since examining the proliferation and differentiation patterns of MSCs in an actual MG environment led to some technical and budgetary challenges[44]. Quynh *et al*[45] found that SMG inhibited the proliferation of human umbilical cord MSCs by blocking the cell cycle; in contrast, a study by Nakaji-Hirabayashi *et al*[46] revealed a proliferative effect. The various SMG action times could be responsible for this circumstance. Shorter SMG treatments appeared to inhibit osteogenesis[47-49] and promote endothelial cell differentiation[48], neuronal differentiation[44,48], and adipogenic differentiation[48,49]. However, extended SMG decreased the potential for chondrogenic differentiation in MSCs[50] and encouraged their differentiation toward osteogenesis[46,48]. Different SMG action times had different effects on the cytoskeleton and could even lead to the aforementioned changes through different signal transduction pathways. In this regard, further studies are needed to determine the appropriate SMG treatment time in regulating the specific lineage differentiation of MSCs.

FSS refers to the mechanical force caused by the friction of fluid flow on the apical cell membrane. It has been demonstrated that the proliferation and differentiation of MSCs are significantly influenced by the strength, timing, and rate of FSS. Jing *et al*[51] discovered that the proliferation of BMSCs could be effectively induced by 0.06 dyn/cm^2 of FSS stimulation, but as the intensity of the FSS increased, cell proliferation gradually decreased or was even inhibited. Meanwhile, Zhao *et al*[52] revealed that FSS

Table 3 Physical stimuli and their effects on the differentiation of mesenchymal stem cells

Physical stimuli	Parameters	Cell source	Results	Ref.
EMF	1 mT, 15 Hz, 4 h/day	BMSCs	BMSCs pretreated with EMF exhibited stronger osteogenic and chondrogenic differentiation potential and weaker adipogenesis capacity	Tu *et al*[40]
	25, 50, 75Hz square and sinusoidal waveform EMF	BMSCs	EMF induced BMSCs differentiation to neuron cells in all treatment groups	Asadian *et al*[42]
	1 mT, 15 Hz, 4 h/day	Rabbit MSCs	EMF enhanced the osteogenic potential of MSCs	Wang *et al*[41]
	PEMF	MSCs	Brief exposure to low amplitude PEMFs enhanced the ability of MSCs to produce and secrete paracrine factors capable of promoting cartilage regeneration	Parate *et al*[43]
SMG	30 g for 72 h or 10 days	Adult rat MSCs	A shorter period of SMG promoted MSCs to differentiate into endothelial, neuronal and adipogenic cells. In comparison, a longer period of SMG promoted MSCs to differentiate into osteoblasts	Xue *et al*[48]
	10 rpm, 72 h, 0.001 G	BMSCs	Inhibited osteogenic differentiation of MSCs	Liu *et al*[47]
	30 rpm clinorotation, 3 d	Adult rat MSCs	Promoted the neuronal differentiation of rat MSCs	Chen *et al*[44]
	7 rpm, 21 d	hMSCs	Lowered the chondrogenic potential of hMSCs	Mayer-Wagner *et al*[50]
Microgravity	0.001 G	hMSCs	microgravity-cultured hMSCs showed a better ability to differentiate into osteoblasts and adipocytes compared to cells cultured under natural gravity conditions	Nakaji-Hirabayashi *et al* [46]]
Spare microgravity		hMSCs	Spare microgravity reduced the osteogenic differentiation of hMSCs and shifted the osteogenesis of hMSCs into adipogenesis, even during ostergenic induction	Zhang *et al*[49]
FSS	0.375 dyn/cm^2, 2 h/d	BMSCs	Promoted osteogenesis-related genes and proteins in BMSCs	Jiang *et al*[54]
	0.06 dyn/cm^2, 6 h/d	BMSCs	Proper FSS stimulation obviously enhanced BMSCs osteogenesis, while the expressions of osteogenic genes decreased with higher intensity of FSS	Jing *et al*[51]
	0.5, 0.8 Pa, 3 h/d	MSCs	Promoted MSCs ostegenesis	Jiao *et al*[55]
	3-7 dynes/cm^2	hMSCs	Enhanced osteogenic differentiation	Zhao *et al*[52]
	4.2 dynes/cm^2	hMSCs	FSS could lead to the osteogenic differentiation of hMSCs	Liu *et al*[53]
	ΔSS from 0 dyn/cm^2 to 10 dyn/cm^2	MSCs	Fast ΔSS (0–0') profits the chondrogenic differentiation, while Slow ΔSS (0–2') advances osteogenic differentiation	Yue *et al*[57]
	ΔSS from 0 dyn/cm^2 to 10 dyn/cm^2	MSCs	Fast ΔSS (0–0') profits the chondrogenic differentiation, while Slow ΔSS (0–2') advances osteogenic differentiation	Lu *et al*[56]
HP	10 MPa, 1 Hz, 4 h/d, 5 d/w, 3 w	BMSCs	HP promoted BMSCs chondrogenic differentiation	Steward *et al*[60]
	0-0.5 MPa, 0.5 Hz	hMSCs	HP promoted the differentiation of the hMSCs toward osteogenesis	Huang *et al*[59]
	270 kPa, 1 Hz, 1 h/d, 5 d/w, 3 w	BMSCs	HP promoted chondrogenic differentiation of BMSCs	Luo *et al*[64]
	100 psi	ADSCs	HP significantly increased osteogenic differentiation of AMSCs	Ru *et al*[65]
	90 kPa, 1 h	BMSCs	HP promoted chondrogenic differentiation of BMSCs	Zhao *et al*[61]
	90 kPa, 1 h	BMSCs	HP promoted the expression of marker genes for early osteogenic differentiation and chondrogenic differentiation of the BMSCs	Zhao *et al*[62]

BMSCs: Bone mesenchymal stem cells; EMF: Electromagnetic field; PEMF: Pulsed electromagnetic field; ADSCs: Adipose-derived stem cells; SMG: Simulated microgravity; hMSCs: Human mesenchymal stem cells; hucMSCs: Human umbilical cord mesenchymal stem cells; FSS: Fluid shear stress; ΔSS: Rate of fluid shear stress; HP: Hydrostatic pressure.

regulated cell proliferation in a rate- and time-dependent manner, with high FSS (9–20 dyn/cm^2) and the continuous effect of low FSS both inhibiting MSC proliferation, but the intermittent effect of low FSS (1–9 dyn/cm^2) appeared to have little or no effect. Liu *et al*[53] shown that FSS (4.2 dyn/cm^2) could promote the proliferation of MSCs implanted on 3D poly(lactic-co-glycolic acid) scaffolds. Although the

effects of FSS on the proliferation of MSCs were differently stated, its promotion of osteogenic differentiation[52-55] seemed to be consistent. Regarding how the rate of FSS (ΔSS) affects MSCs, it was observed that quick ΔSS (From 0 dyn/cm^2 in 0 min) was more beneficial for MSCs' chondrogenic development, whereas slow ΔSS (From 0 dyn/cm^2 in 2 mins) encouraged their osteogenic differentiation [56,57]. Clearly, more research is required to confirm the impact of FSS on MSC proliferation, as well as the appropriate stimulus parameters for osteogenic differentiation and MSC proliferation.

HP, unlike other physical stimuli, applies homogeneous tension without causing cellular deformation [58]. Physiological load (0.1-10 mPa) did not affect the proliferation of MSCs[59,60], whereas a load of 90 kPa effectively promoted the proliferation of MSCs[61,62], a possibility that resulted from the initiation of the cell cycle by HP[62]. Studies conducted in the past have indicated that HP at low loads (1–50 kPa) has an osteogenic impact on MSCs, whereas HP at physiological loads efficiently promoted chondrogenic differentiation[63]. This concept was also supported by several recent research works[60,64]. Some investigations, however, discovered a facilitative effect of physiological loading on MSCs' osteogenic differentiation[59,65], and a chondrogenic effect of low loading on MSCs[61,62]. Additionally, the study by Zhao *et al*[62] discovered that HP (70 kPa) could not only stimulate RhoA activation, which in turn promoted the expression of early osteogenic differentiation genes in BMSCs, but could also upregulate Rac1 and downregulate RhoA, which further promoted cartilage development in BMSCs. These findings suggested that further studies are needed to determine the effects of different loads of HP on the spectral differentiation of MSCs and their complex mechanisms.

Overall, physical stimuli did influence MSCs' proliferation and differentiation to varying degrees, but there is still no consensus on the parameters that are most conducive to the proliferation and specific lineages' differentiation of MSCs. Cell heterogeneity, various stemness potentials, culture conditions, and techniques that simulated physical stimulation might all be contributing factors in this issue. Therefore, more studies are needed to determine the appropriate parameters of physical stimuli that promote the differentiation of MSCs. In fact, the actual microenvironment in which cells were exposed was multifactorial. Therefore, some studies are now starting to consider the effect of compound factors [50,55,61,66] on the behavior of MSCs. Compound factors could have synergistic effects that increase the benefits for MSCs or counteract the drawbacks of a single factor. This might emerge as a new trend.

HYPOXIA

In most studies, MSCs were cultured under atmospheric oxygen tension (20%-21% O_2)[67]. However, MSCs in different ecological niches encounter oxygen concentrations that are significantly lower than 20% (Table 4). For instance, the O_2 concentration that MSCs experienced varied from 1% to 5%[68] in adipose tissue and from 1% to 7%[69] in bone marrow. As a result, MSCs from different tissue sources were in a hypoxia microenvironment *in vivo*. Hypoxia activated various signaling pathways within a cell, which could lead to either cell death or cell adaptation[70]. Theoretically, culturing MSCs at physiological oxygen concentrations facilitated their proliferation, differentiation, and the secretion of cytokines and growth factors. Ciapetti *et al*[71] discovered that hypoxic circumstances greatly boosted BMSCs' proliferation and colony-forming capacity, as well as the expression of genes relevant to bone, such as alkaline phosphatase and osteocalcin, supporting the above idea. In contrast, in a study by Xu *et al*[72], hypoxia inhibited the osteogenic differentiation of BMSCs by activating the Notch pathway. Therefore, we focus on the effect of hypoxia on the behavior of MSCs and try to explain the contradictory findings in different studies.

The two primary techniques used nowadays to create *in vitro* hypoxic settings are anaerobic chambers [73] and simulation utilizing different chemicals[74]. In a study by Elabd *et al*[75], moderate hypoxia (5% O_2) circumstances promoted the chondrogenic and adipogenic differentiation of BMSCs but had no effect on proliferation or osteogenic differentiation. At the same oxygen concentration, Lee *et al*[76] showed that hypoxia promoted MSC proliferation and increased the chondrogenic differentiation potential. The proliferation of MSCs was also effectively promoted at a 5.5%-6.5% O_2 concentration simulated by 10 μM $CoCl_2$ and 4.0 mmol/L Na_2SO_3[74]. In contrast, Yu *et al*[77] demonstrated that a 50 M $CoCl_2$-simulated hypoxia environment appeared to prevent the growth of MSCs. Consistently, the osteogenic differentiation of MSCs was promoted in hypoxia environments simulated using different concentrations of $CoCl_2$[74,77]. Cicione *et al*[78] investigated the changes in the trilineage differentiation potential of BMSCs under severe hypoxia (1% O_2) and showed that the trilineage differentiation potential of BMSCs was inhibited to different degrees. Additional research demonstrated that the activation of the Notch pathway may be responsible for the suppression of the osteogenic differentiation of MSCs by severe hypoxia (1% O_2)[3,72]. In addition, Kim *et al*[79] found that hypoxia could inhibit the osteogenic differentiation of ADSCs by upregulating insulin-like growth factor binding-protein-3. Hypoxia has also been shown to encourage the tendon[73] and neural[80] differentiation of MSCs.

Compared to the laboratory culture environment (20%-21% O_2), hypoxia is more representative of the oxygen concentration in the ecological niche of MSCs. Varied oxygen concentrations had extremely different impacts on MSCs. Moderate hypoxia environment enhanced MSCs' proliferation, osteogenic differentiation, and chondrogenic differentiation. The differentiation capability of all three lineages of

Table 4 Hypoxia and their effects on the differentiation of mesenchymal stem cells

Conditions	Cell source	Results	Ref.
Hypoxic culture (5%O_2)	BMSCs	↑ Chondrogenic differentiation; ↑ adipogenic differentiation	Elabd *et al*[75]
Hypoxic culture (5.5%-6.5%O_2)	Balb/c mouse clonal MSCs	↑ Osteogenic differentiation	Kim *et al*[74]
Hypoxic culture (50 μM $CoCl_2$ simulation)	Mice MSCs	↑ Osteogenic differentiation	Yu *et al*[77]
Hypoxic culture (5%O_2)	ADSCs	↑ Chondrogenic differentiation	Lee *et al*[76]
Hypoxic culture (1%O_2)	PBMSCs	↑ Osteogenic differentiation	Yang *et al*[3]
Hypoxic culture (1%O_2)	BMSCs	↓ Osteogenic differentiation; ↓ adipogenic differentiation; ↓ chondrogenic differentiation	Cicione *et al*[78]
Hypoxic culture (1%O_2)	BMSCs	↑ Neuronal differentiation	Wang *et al*[80]
Hypoxic culture (1%O_2)	BMSCs	↓ Osteogenic differentiation	Xu *et al*[72]
Hypoxic culture (2%O_2)	ADSCs	↑ Tenocyte differentiation	Yu *et al*[73]
Hypoxic culture (2%O_2)	ADSCs	↓ Osteogenic differentiation	Kim *et al*[79]
Hypoxic culture (2%O_2)	BMSCs	↑ Osteogenic differentiation	Ciapetti *et al*[71]

↑: Increase; ↓: Decrease; BMSCs: Bone mesenchymal stem cells; MSCs: Mesenchymal stem cells; ADSCs: Adipose-derived stem cells; PBMSCs: Peripheral blood mesenchymal stem cells.

MSCs was, however, somewhat hindered under severe hypoxia. The contradictory behavior in the aforementioned research might potentially be connected to the cell source of MSCs and whether they were differentiated under hypoxia conditions. In view of current studies generally focusing on hypoxia exposure either in the phase of expansion or differentiation, which have not been fully grasped, further research is necessary to comprehend the effects on MSCs specifically in these two culture forms.

MATRIX STIFFNESS AND SURFACE TOPOGRAPHY

Two crucial material physical characteristics that have a significant impact on MSC behavior are matrix stiffness and surface topography. Matrix stiffness is a passive mechanical parameter that the cell can not directly sense. By exerting traction pressures on the cytoskeleton through focal adhesion, cells might deform the extracellular matrix (ECM), reflecting matrix stiffness[81]. Materials with ECM properties are currently being designed to simulate the actual microenvironment of cells. The ECMs of different native tissues, such as bone, cartilage, nerves, or blood vessels, are composed of micro- and nanoscale topographic patterns[82]. As a result, an increasing number of researchers have begun to look into how the substrate surface topography affects MSC behavior. Size and surface roughness are the two most fundamental parameters of surface topography[83], and the effects of these two factors, as well as substrate stiffness, on the proliferation and differentiation of MSCs are also mainly explored here.

Matrix stiffness

Stiffness is one of the most common metrics in assessing a material's mechanical properties[81], and it is typically expressed in terms of Young's modulus. Matrix stiffness has been shown in many studies to affect the proliferation and differentiation of MSCs. MSCs exhibited higher proliferative behavior under a higher substrate stiffness, and Winer *et al*[84] found that MSCs inoculated in 250-Pa polyacrylamide gels that mimicked the elasticity of bone marrow and adipose tissue exhibited cell cycle arrest, but these arrested cells re-entered the cell cycle when a stiff substrate was present[84]. In comparison to lower-stiffness gels, higher-stiffness matrices could increase the number of cells by a factor of 10[85]. With fibronectin-coated polyacrylamide hydrogels, Sun *et al*[86] controlled the mechanical environment of BMSCs and discovered that BMSCs' proliferation increased with increasing stiffness. However, as opposed to firmer substrates, Lin *et al*[87] discovered that MSCs cultivated on softer substrates had greater cell proliferation rates. Gelma hydrogels with different concentrations not only had different hardness, but also showed different porosity as well. Moreover, the pore size also seemed to be one of the influencing factors for the proliferation and differentiation of MSCs. Indeed, many studies have focused on the effect of matrix stiffness on the direction of differentiation of MSCs. MSCs exhibited the upregulation of biomarkers matching tissue stiffness on polyacrylamide gels of different stiffness, such as neurogenic (0.1-1 kPa, brain), myogenic (8-17 kPa, muscle), and osteogenic (25-40 kPa, bone) markers

[88]. BMSCs could be driven to develop into an osteogenic phenotype and expressed increased quantities of bone-derived biomarkers including Runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and bone-bridging proteins when grown on polyacrylamide hydrogels (62-68 kPa) [86]. Rowlands *et al*[85] found that the osteogenic differentiation of MSCs occurred mainly on polyacrylamide gels of 80 kPa stiffness and that RUNX2 was also expressed at high levels. This might be due to the fact that the 80 kPa collagen I coating could well simulate the microenvironment of the bone tissue. Without an induction medium, the stiffness of the hydrogel itself had a substantial impact in controlling MSC differentiation early on, with softer substrates encouraging the adipogenic differentiation of MSCs, while harder substrates encouraged the osteogenic differentiation of MSCs[89]. However, this effect seemed to be gradually attenuated by biochemical effects in the culture medium, implying that the effects of different factors on the differentiation behavior of MSCs might occur at different stages of differentiation. On 22 kPa gels, as opposed to softer matrices, MSCs produced larger quantities of ALP, which was consistent with the effect of matrix stiffness on osteogenic fractionation shown in the previous work[90]. Although more disagreement has emerged regarding the effect of softer matrices on the differentiation fate of MSCs, such as adipogenic differentiation[84,90-92], myogenic differentiation[85,88], neurogenic differentiation[88], and endothelial differentiation[87], there seems to be a consensus on the osteogenic role of harder matrices for MSCs. The Stiffer matrix enabled cells to produce more cytoskeletal tension and sent differentiation signals *via* transmembrane proteins such as integrins[81,85], which promoted osteogenic differentiation. Furthermore, the nuclear localization of Yes-associated protein (a key mediator of mechano-transduction) and RUNX2 could be impacted by the substrate stiffness[89,90].

Surface topography

Zhao *et al*[93] produced nanotubes of various sizes and micro- and nano-hybrid topographies with ECM-like micro/nanostructures and examined their effects on the proliferation and osteogenic differentiation of MSCs. They discovered that larger-sized nanotubes hindered the early proliferation of MSCs, but the micro- and nano-morphology group had a greater cell number. Additionally, they discovered that MSC osteogenic differentiation might be induced by micro/nanotopographies, even in the absence of osteogenic inducers[93]. Similar results were obtained by Chen *et al*[94], who discovered that the micron/submicron hybrid topography of Ti surfaces promoted osteogenic and chondrogenic differentiation in the early stages of induced differentiation. By introducing nanoengineered topographic glass substrates with different surface roughness, Qian *et al*[95] investigated the impact of surface morphology on the osteogenic differentiation of MSCs. They found that surface roughness could replace the osteogenic inducer dexamethasone and worked in concert with ascorbic acid and β-glycerophosphate to jointly promote the osteogenic differentiation of MSCs[95]. In the past, it was generally agreed that surface roughness seemed to have a positive effect on osteogenic differentiation[95-97]. The osteogenic differentiation of MSCs, however, was more strongly influenced by the nanopore size than by the surface roughness, according to several recent studies[83,98]. The frequent coupling of size and surface roughness in many studies makes it difficult to state the degree of influence of each factor on the behavior of MSCs[83]. Moreover, the methods used to prepare rough surfaces in these studies differ, such as randomly rough surfaces produced by treatments such as mechanical polishing, acid etching, *etc*., where cells form focal attachments that differ from those seen on surfaces of the same roughness [98]. Therefore, more research is required to demonstrate how size and surface roughness affect MSC proliferation and differentiation, respectively. Through a variety of pathways, including the control of adhesion, cytoskeletal tension, and nuclear localization of transcription factors[95], MSCs appeared to be able to detect and respond to the surface topography, which in turn influenced their behavior such as proliferation and differentiation. At this stage, it has been reported that micro- and nano-surface topographies inhibit the proliferation of MSCs and promote osteogenic differentiation to some extent. However, there is no detailed elaboration on their respective effects on MSCs in terms of size and surface roughness.

EXOSOMES

Various cells jointly create the microenvironment by secreting functional molecules, which leads to the sharing of stimuli between multiple cell lineages[99]. In addition to the ECM and growth factors, exosomes were considered to be an important component of the microenvironment[100]. Exosomes are small vesicles with a diameter of 30-150 nm that are released by cells through cytosolic action. The released exosomes could interact with target cells and translocated proteins, lipids, mRNAs and miRNAs to the cytoplasm of target cells[101]. Crosstalk existed between MSCs-osteoblasts and monocytes-macrophages and researchers used this to regulate bone homeostasis[99]. In vitro, BMSCs' behaviors were influenced variably by cell-conditioned media produced by variously polarized macrophages[102]. Previous studies had suggested that cytokines were the main contributors to the function exercised by macrophages. However, Song *et al*[103] found that lipopolysaccharide (LPS)-activated macrophage-derived exosomes inhibited the osteogenic differentiation of BMSCs by

mediating inflammatory stimuli. Therefore, the effect of exosomes secreted by monocytes-macrophages on the differentiation of MSCs should be considered (Table 5).

According to Liu *et al*[104], miR-21a-5p found in M1 macrophage-derived exosomes directed BMSCs toward an osteoblastic fate during the early stages of osteogenesis[104]. In their investigation of the effects of MO, M1, and M2 macrophage-derived exosomes on BMSCs, Xia *et al*[105] discovered that M1 macrophage-derived exosomes efficiently enhanced the proliferation, osteogenic differentiation, and adipogenic differentiation of BMSCs, but M2 macrophage-derived exosomes were harmful to the proliferation of BMSCs and, curiously, all three hindered the chondrogenic differentiation of BMSCs. Xiong *et al*[106] noticed that miRNA-5106, enriched in M2 macrophage-derived exosomes, promoted the osteogenic differentiation of BMSCs by suppressing the expression of salt-inducible kinase 2 (SIK2) and SIK3, which was consistent with the role of M2 macrophage-derived exosomes in promoting osteogenesis in a study by Li *et al*[107]. Kang *et al*[108] demonstrated that M0 and M2 macrophage-derived exosomes were positive for BMSC osteogenesis while M1 macrophage-derived exosomes lowered BMP expression and inhibited the osteogenic differentiation of BMSCs[108]. Despite being enriched in distinct miRNAs, primary extraction M2 macrophages[109] and RAW264.7 mouse monocyte-macrophage leukemia cell[107] derived exosomes both showed osteogenesis-promoting and lipogenic differentiation-inhibiting effects. Current research has indicated the impact of exosomes produced from monocytes[110], osteoclasts[111], and osteoblasts[112] on BMSCs, in addition to exosomes released by macrophages. Ekström *et al*[110] found that exosomes released from LPS-stimulated monocytes could be ingested by MSCs and encouraged the osteogenic differentiation of MSCs. Liang *et al*[111] showed that osteoclast-released exosomes promoted osteogenic differentiation and facilitated osteogenic mineralization by inhibiting Rho GTPase activating protein 1. This might imply that active osteoclasts release large amounts of extracellular vesicles during the resorption phase, promoting the osteogenesis of MSCs for better stabilization and bridging the transition between bone resorption and formation. The addition of osteoblast exosomes could further enhance the expression of RUNX2 and osterix, thereby promoting osteogenic differentiation, and, in addition, osteoblast exosomes could even alter adipocyte ECM-mediated lineage differentiation[112].

Exosomes, one of the recently identified microenvironment components, have unique benefits, such as a nano size, non-toxicity, low immunogenicity, biocompatibility, and versatility of use, drawing widespread attention[113]. The current work appeared to demonstrate the beneficial influence of M2 macrophage-derived exosomes on the osteogenic differentiation of MSCs. As for MO and M1 macrophage-derived exosomes, further research is required to understand their impacts on MSC differentiation and the processes at play. At the same time, research has been conducted progressively on the influence of exosomes released by cells in the same microenvironment as BMSCs on the differentiation of BMSCs, which might represent a new avenue.

CONCLUSION

MSCs play important roles in pathological and physiological processes because of their self-renewal, migration, and pluripotency. Especially due to their multi-differentiation potential, MSCs have been considered as a new therapeutic agent in regenerative medicine. Since the detailed mechanisms involved in these regulation processes has not been fully revealed, research on intrinsic and extrinsic factors regulating MSCs' differentiation may provide new methods in manipulating the cell fate of MSCs. Here, we discussed multiple chemical and mechanical factors affecting the osteogenic and chondrogenic differentiation of MSCs, including typical differentiation promoting patterns, cell environmental factors, and other interesting research areas, such as material morphology and exosomes. After sensing these differentiation-stimulating factors, MSCs from various sources are able to differentiate into specific cell lineages. With the rising demand for MSCs in clinical treatment, noble strategies have been developed that aim at inducing the stable and directional differentiation of stem cells, and further providing efficient methods of MSC regulation in basic research and clinical application.

Meanwhile, there is much more to discover in stem cell research. Due to some limitations of MSCs, such as homing efficiency and long-term survival *in vivo*, most of the research has achieved its results at the cellular level in vitro. Moreover, discrepancies remain between single-factor experiments and synergistic effects by multiple factors. At present, extensive research on factors stimulating MSCs' differentiation has promoted our understanding of cell functional alterations. However, mechanisms involved in manipulating MSCs' cell fate have so far been incomplete. With the deepening of stem cell research alongside technology improvements, the synergistic effect of multiple factors inducing MSC differentiation is increasingly likely to be clarified, as well as providing new patterns in clinical stem cell therapy.

Table 5 Exosomes of different cell sources and their effects on the differentiation of mesenchymal stem cells

Source and kind	Specific cargo	Target	Results	Ref.
M1 macrophages-EVs	miRNA-21a-5p	BMSCs	↑ Osteogenic differentiation	Liu *et al*[104]
M0 macrophages-EVs		BMSCs	↓ Chondrogenic differentiation	Xia *et al*[105]
M1 macrophages-EVs		BMSCs	↑ Osteogenic differentiation; ↑ adipogenic differentiation; ↓ chondrogenic differentiation	
M2 macrophages-EVs		BMSCs	↓ Chondrogenic differentiation	
M2 macrophages-EVs	miRNA-5106	BMSCs;SIK2 and SIK3	↑ Osteogenic differentiation	Xiong *et al*[106]
M2 macrophages-EVs	miRNA-690	BMSCs	↑ Osteogenic differentiation; ↓ adipogenic differentiation	Li *et al*[107]
M0 macrophages-EVs		MSCs	↑ Osteogenic differentiation	Kang *et al*[108]
M1 macrophages-EVs	miRNA-155	MSCs	↓ Osteogenic differentiation	
M2 macrophages-EVs	miRNA-378a	MSCs	↑ Osteogenic differentiation	
M2 macrophages-EVs	miRNA-26a-5p	BMSCs	↑ Osteogenic differentiation; ↓ adipogenic differentiation	Bin-bin *et al*[109]
Macrophages-EVs		BMSCs	↓ Osteogenic differentiation	Song *et al*[103]
Monocytes-EVs		MSCs	↑ Osteogenic differentiation	Ekström *et al*[110]
Osteoclasts-EVs	miRNA-324	BMSCs	↑ Osteogenic differentiation	Liang *et al*[111]

↑: Increase; ↓: Decrease; EVs: Extracellular vesicles; MSCs: Mesenchymal stem cells; BMSCs: Bone mesenchymal stem cells; SIK2/SIK3: Salt-inducible kinase 2/3.

FOOTNOTES

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REVIEW

Cell transplantation therapies for spinal cord injury focusing on bone marrow mesenchymal stem cells: Advances and challenges

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Abstract

Spinal cord injury (SCI) is a devastating condition with complex pathological mechanisms that lead to sensory, motor, and autonomic dysfunction below the site of injury. To date, no effective therapy is available for the treatment of SCI. Recently, bone marrow-derived mesenchymal stem cells (BMMSCs) have been considered to be the most promising source for cellular therapies following SCI. The objective of the present review is to summarize the most recent insights into the cellular and molecular mechanism using BMMSC therapy to treat SCI. In this work, we review the specific mechanism of BMMSCs in SCI repair mainly from the following aspects: Neuroprotection, axon sprouting and/or regeneration, myelin regeneration, inhibitory microenvironments, glial scar formation, immunomodulation, and angiogenesis. Additionally, we summarize the latest evidence on the application of BMMSCs in clinical trials and further discuss the challenges and future directions for stem cell therapy in SCI models.

Key Words: Spinal cord injury; Bone marrow derived mesenchymal stem cells; Neuroprotection; Axon; Myelin; Inhibitory microenvironment

Core Tip: In this work, we review the specific mechanism of bone marrow-derived mesenchymal stem cell (BMMSC) in spinal cord injury (SCI) repair mainly from the following aspects: Neuroprotection, neuronal circuit, axon sprouting and or regeneration, myelin regeneration, inhibitory microenvironment, glial scar formation, immunomodulation, and angiogenesis. Additionally, we also summarize the latest evidence on application of BMMSC in clinical trials and further discuss the challenges and future directions for stem cell therapy in SCI models.

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INTRODUCTION

Spinal cord injury (SCI) is a serious neurological disorder that often results in paralysis during the reproductive years, causing temporary or permanent changes in normal motor, sensory, and autonomic functions, with significant impacts on individuals, families, and socioeconomic systems[1]. It has been reported that more than 27 million patients worldwide experience long-term disability due to SCI[2], with 541 cases per 100000 people[3]. Complex pathophysiology and time sensitivity in particular limit the therapeutic effects of SCI[4]. In incomplete SCI, there is less hemorrhage in the gray matter and no change in the white matter 3 h after injury; 6-10 h after injury, the hemorrhagic foci gradually expand, and the neurological tissue becomes edematous, which gradually subsides after 24-48 h. As the degree of incomplete SCI differs between mild and severe injuries, milder injuries only have small foci of necrosis in the center, and most of the nerve fibers are preserved. Severe injuries may have foci of necrosis and softening in the center of the spinal cord and are replaced by gliosis or scarring, and only a small portion of the nerve fibers are preserved. Most posttraumatic tissue degeneration is caused by multiple secondary injuries, including blood-spinal cord barrier (BSCB) disruption, free radical formation, ion imbalance, apoptosis, demyelination, and inflammatory response (Figure 1). Spontaneous recovery occurs within a limited time window because the subacute phase of SCI is thought to be detrimental to axonal regeneration and functional recovery[5]. Currently, clinical treatment includes surgical decompression, stabilization of the spinal cord, relief of spasticity and rehabilitation care, which consists primarily of supportive care and injury management. Frustratingly, the effectiveness of these treatments is limited because they are not effective in stimulating repair of the injured spinal cord.

CLINICAL CHARACTERISTICS AND PATHOLOGY OF SCI

The spinal cord consists of the gray and white matter, which contains nerve cell bodies and ascending and descending fibers. Thus, the different locations and levels of SCI can cause various degrees of disability, from partial loss of motor or sensory function to complete paralysis below the injured location. The resulting lifelong devastating deficits associated with impaired mobility (weakness or paralysis), sensation and autonomic dysfunction, and disabled neurological conditions lead to permanent and severe impacts on patients' daily lives and their caregivers.

Pathophysiology following SCI comprises interrelated multicellular, multimolecular interactions and multiphasic events[6]. Classically, the pathophysiology of SCI is divided into two phases: Primary injury and secondary injury. SCI commonly occurs after sudden trauma because of direct and immediate mechanical insult to the spinal cord from vertebral fractures and dislocation with features of bone fragments and spinal ligament tearing. This was accompanied by extensive bleeding with further compression and interruption of the spinal cord blood supply. Thus, the primary injury mainly includes compression, contusion, shear, laceration, and acute stretching. This is followed by disruption of the neural parenchyma, shearing of the axonal network, destruction of the glial membrane, and vascular disruption[7,8]. Secondary injuries consist of ischemia, spinal cord ischemia-reperfusion injury, vascular dysfunction, edema, excitotoxicity, formation of free radicals, glial and neuronal apoptosis, and the inflammatory response[7]. This secondary damage is divided into three phases: The acute phase, which is accompanied by vascular and cell membrane damage and the secretion of different proinflammatory factors, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), with microglial activation. The subacute phase is accompanied by edema and vascular damage, inflammatory cytokine and glutamate secretion, astrogliosis, and demyelination for a few days. The chronic phase has symptoms such as the formation of a cavity in the spinal cord[8]. Based on further study of the spinal cord

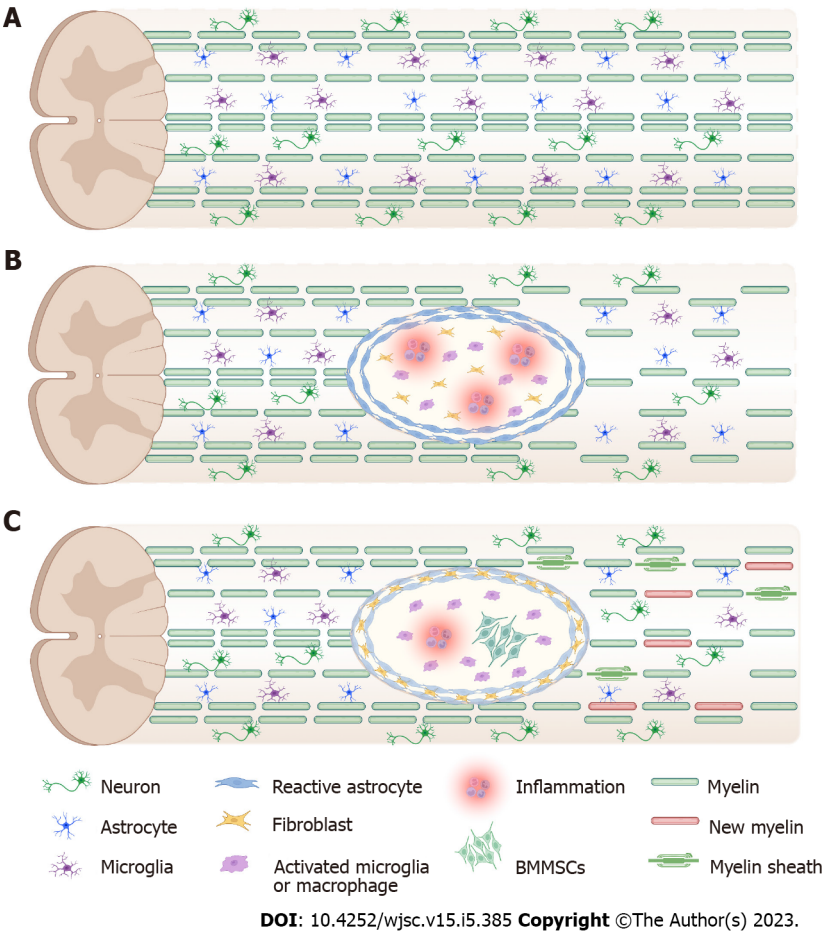

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Figure 1 Pathophysiology of spinal cord injury and related mechanisms of bone marrow-derived mesenchymal stem cell in spinal cord injury. A: The normal spinal cord contains axons wrapped in the myelin sheath, neuron bodies, microglia, fibroblasts, and astrocytes; B: Secondary injuries following primary injury include glial and neuronal apoptosis, axon rupture, inflammatory response, ischemia, spinal cord ischemia-reperfusion injury, vascular dysfunction, edema, inhibitory microenvironments, excitatory toxicity, and free radical formation; C: Bone marrow-derived mesenchymal stem cell promote the mechanism of spinal cord injury repair, including neuroprotection, axon growth, myelination, immune regulation, microenvironment regulation, inhibition of scar formation, and promotion of angiogenesis. BMMSCs: Bone marrow-derived mesenchymal stem cells.

microenvironment, pathophysiological changes are divided into tissue, cellular and molecular levels. The tissue level involves hemorrhage and ischemia, glial scar formation, demyelination and remyelination[9]. The cellular level involves the activation of astrocytes, the differentiation of endogenous neural stem cells, oligodendrocyte progenitors and microglia, and the infiltration of macrophages. The molecular level involves the expression of neurotrophic factors and their pro-peptides, cytokines, chemokines, and ion imbalance. There is an imbalance between promoting and inhibiting growth molecules in the microenvironment of SCI, where growth inhibitors occupy the dominant position.

THE THERAPEUTIC POTENTIAL OF STEM CELLS

Cell transplantation has come to the forefront in SCI regenerative strategies due to its potential neuroprotective effects. Many cell types have been widely investigated in SCI treatment, including oligodendrocyte precursor cells, Schwann cells, olfactory ensheathing cells, neural stem cells, and mesenchymal stem cells (MSCs)[10,11]. MSCs have the capacity for self-renewal and multilineage differentiation potential and can differentiate into osteoblasts, adipocytes, and chondrocytes[12]. Moreover, MSCs express surface markers (CD105, CD73 and CD90) and do not show expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, or human leukocyte antigen type DR surface molecules[13]. MSCs mainly exist in bone marrow and can also be isolated from other tissues and organs, such as umbilical cord, adipose tissue and muscle. Among these, bone marrow-derived MSCs (BMMSCs) are the most widely studied cell type in SCI application due to their low immunogenicity, easy isolation, few ethical concerns and reduced tumorigenesis risks[14]. According to the current research progress at home and abroad, BMMSC-based treatment has extraordinary prospects in the field of SCI.

In this review, we will summarize the applications of BMMSCs in SCI based on the most frequently proposed mechanisms: Neuroprotection, neuronal circuit, axon sprouting and/or regeneration, myelin regeneration, inhibitory microenvironments, glial scar formation, immunomodulation, and angiogenesis. A better understanding of these mechanisms could allow the identification of more targeted therapies.

NEUROPROTECTIVE EFFECTS OF BMMSCS

Neurons are postmitotic, without the ability to proliferate, and strategies developed to promote neuronal protection and regeneration have long-term benefits. Neuroprotective measures are crucial not only for the preservation of further injury for optimal neuron survivability but also for the restoration of injured nerve cells during pathological progression. BMMSCs reestablish the injured spinal cord *via* neuroprotection, neural regeneration, and remyelination in SCI[15]. MSCs can release growth and neurotrophic factors, including brain-derived neurotrophic factor (BDNF)[16], vascular endothelial growth factor (VEGF)[17], glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), fibroblast growth factor (FGF), neurotrophin-3, and epidermal growth factor, which can enhance regeneration and repair in damaged tissues[18,19]. miR-22, regulated by Gasdermin D mRNA, plays a role in inhibiting the occurrence of pyroptosis[20]. miR-22-modified BMMSCs suppress pyroptosis-mediated inflammation and neuronal injury during SCI[20]. Moreover, MSC-derived exosomes enhance the survival of neurons and repair of nerve fibers by inhibiting Nod1 inflammasome activation, suppressing pyroptosis in pericytes, preserving the integrity of the BSCB[21], inhibiting neuronal apoptosis through the Wnt/beta-catenin signaling pathway[22] and eventually improving functional recovery. The overexpression of miR-338-5p in exosomes was shown to profoundly increase the expression levels of neurofilament 200 and growth-associated protein-43 and decrease those of myelin-associated glycoprotein (MAG) and glial fibrillary acidic protein (GFAP), which provided neuroprotective effects through the cannabinoid receptor 1/Rap1/Akt pathway after acute SCI[23]. cAMP-mediated Rap1 activation plays an important role in apoptosis reduction and neuronal survival induced by the PI3K/Akt pathway[23].

Despite the inhibitory environment in the adult mammalian central nervous system, neuronal-intrinsic mechanisms are sufficient to support significantly extensive axonal growth and synapse formation after SCI, resulting in the formation of new neuronal circuits that restore electrophysiological activity and behavior[24]. When stem cells are cotransplanted with a supportive fibronectin matrix containing growth factors, axons form connections with host axons over significantly long distances. Even when crossing the inhibitory white matter, they elongate rapidly at a rate of 1-2 mm per day[24]. In addition, axons from the host spine regenerate into neural stem cell grafts, and this bidirectional growth contributes to the recovery of hindlimb mobility[24]. BMMSCs were initially thought to be similar to neural stem cells in their ability to multidirectionally differentiate into neurons and glial cells, but this theory was gradually discarded. In fact, the mechanism of action of BMMSCs may be cell fusion or trans-differentiation rather than cell differentiation[25]. In summary, transplanted BMMSCs after SCI exhibit significant autocrine and paracrine activities, which in turn stimulate the proliferation and differentiation of other cells and themselves, promoting tissue repair and functional recovery[26].

AXON GROWTH

The axon is a unique cellular structure that maintains communication between neurons[27]. An important cause of persistent dysfunction after SCI is axonal disruption, and therefore promoting axonal regeneration and plasticity is very promising. However, previous studies have shown that the percentage of injected MSCs aggregating in the CNS is 0.75%-18.5%[28] and 6.7%[29], and thus it is conceivable that only a fraction of the cells reach the site of SCI. The Nakano *et al*[30] found that bone marrow stromal cell transplantation *via* cerebrospinal fluid was effective in acute, subacute and chronic spinal cord injuries in rats, and although the transplanted cells did not survive more than 7 d, a large number of axons crossed longitudinally through the astrocyte-deficient connective tissue. Transplantation *via* the CSF is a more clinically preferred modality because it does not cause secondary injury to preserved spinal cord tissue. From another perspective, Okuda *et al*[31] demonstrated that transplantation of BMMSC sheets after SCI prompted Tuj-1-positive axons to span a specially designed cell sheet without requiring a scaffold, offering a permissive microenvironment for damaged axons[31].

BMMSC therapy has been shown to play a positive role in rodent models of SCI[32], but evidence of its long-term therapeutic efficacy and effectiveness in human clinical trials is limited. Gene modification of MSCs, for example, by overexpression of neurotrophic or growth factors, is one of the ways to enhance their well-known beneficial effects. Overexpression of IL-13, an inducer of M2 microglia/macrophages, in BMMSCs significantly ameliorates axonal retraction caused by axonal-attacking macrophages[33]. In addition, the combination of neurotrophic factors and physical stimuli is often used to enhance the effects of BMMSCs. Cografting stromal-derived factor-1-overexpressing BMMSCs with

neural stem cells (NSCs) at day 9 after SCI resulted in better axonal density enhancement than BMMSCs alone[34]. Furthermore, physical factor therapy has also shown a synergistic effect, as low-intensity pulsed ultrasound-optimized BMMSCs transplanted one week after SCI showed a good promotion effect on axonal regeneration[5].

Although the transplanted cells can reach the site of injury, they cannot survive long enough to integrate with the host spinal cord tissue, thus showing that these cells do not act as scaffolds for tissue repair. The use of biomaterials can provide transplanted cells with an environment closer to their physiological state, maintaining and regulating stem cell properties and protecting them from the harsh local microenvironment. Permissive bridging material allows nerve fibers with regenerative growth potential or collateral sprouting to pass through nonpermissive physical spaces[35]; therefore, tissue-engineered grafts loaded with cells and growth factors have become popular as bridging therapy for SCI. After spinal cord injuries in rats and dogs, NT-3/fibroin-coated gelatin sponge scaffolds were shown to continuously release NT-3 for up to 28 d, maintain the cell activity of MSCs, promote axonal regeneration, and attenuate the inflammatory response[36]. The multichannel poly lactic-co-glycolic acid scaffolds implanted with Schwann cells and BMMSCs were shown to effectively connect the injury gap of rats with complete SCI, and the cell combination strategy promoted the survival and neuron-like characteristics of BMMSCs and finally facilitated axonal regeneration and functional recovery[37]. The short half-life and rapid clearance challenges posed by the innate immune system have hampered the popularity of extracellular vesicle therapy[38]. Wang *et al*[39] synthesized an F127-polycitrate-polyethyleneimine hydrogel (FE) with sustainable and long-term extracellular vesicle release (FE@EVs), and its *in situ* administration after SCI inhibited the inflammatory response and promoted myelination and axonal regeneration. There is no shortage of biomaterials in clinical trials for cancer, but no clinical trials on biomaterials for SCI repair have been registered on the ClinicalTrials.gov website. Possible reasons for this include inconsistency between injury models from preclinical studies and actual injury models in the clinic (thoracic SCI models are often used in preclinical studies, but cervical SCI is more common in humans[40]), unpredictable residual degradation products in the body, and potentially low payloads [41]. Caution is needed in drawing conclusions about axonal regeneration because the current consensus is that it refers to regrowth of axons after transection[42], whereas there is a significant degree of axonal preservation in incomplete spinal cord injuries, and the best model for exploring axonal regeneration is complete SCI.

REMYELINATION

Demyelination in traumatic SCI can lead to loss of function, and poor myelination of preserved nerve fibers may lead to permanent functional impairment. Myelin loss is accompanied by oligodendrocyte apoptosis, and replacement of lost oligodendrocytes and myelin improves conductivity and protects axons from degeneration[43]. The process of transplanted cells producing myelin around axons that have lost myelin sheaths is a mechanism that enhances recovery after SCI[43]. Conditioned medium from MSCs not only improves the survival of oligodendrocytes *in vitro* in culture but also increases levels of Olig2, a transcription factor that plays a key role in the differentiation of oligodendrocytes, in SCI[44]. Intravenous infusion of MSCs during the chronic phase in a severely injured SCI model promotes remyelination of axons[45]. BMMSCs can act as catalysts for the differentiation of NSCs into oligodendrocytes by regulating Id2 and Olig1/2[46]. However, the genetically engineered cells showed a more satisfactory therapeutic effect than the original cells. BMMSCs secrete various trophic factors, including VEGF, insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), basic FGF, GDNF [47,48], and certain extracellular matrix molecules, such as laminin and type IV collagen[49]. Given the positive effects of IGF-1 on oligodendrocyte differentiation and survival during normal development [50], IGF-1-overexpressing BMMSCs better protect the integrity of myelin sheaths[51]. Similarly, GDNF is a potential target for axon enhancement and is directly involved in axonogenesis and dopaminergic neuronal differentiation *via* the Ras/MARK pathway and P13K signaling pathway[52].

In addition to the abovementioned cytotrophic factors, myelination-related graft factors can also be used as targets for genetic engineering. Silencing Nogo-66 receptor expression was shown to promote neurite outgrowth after BMMSC differentiation and increase myelinated nerve fibers after SCI[53]. There are benefits from the effects of neuroprotective drugs themselves, which have antioxidant effects, inhibit intracellular calcium overload, regulate γ-aminobutyric acid receptors and inhibit apoptosis, and combining these drugs can also improve the therapeutic effect of BMMSCs. Currently, the combination of BMMSCs and propofol, a neuroprotective anesthetic, has dramatically increased the number of myelinated and nonmyelinated fibers after SCI[54]. Transplanted cells with the ability to myelinate do not always promote functional recovery, and sufficient myelination needs to occur to achieve significant results[55].

IMMUNOMODULATION

The inflammatory response is a critical component in the secondary injury cascade, which can persist for weeks to months after SCI, during which microglia/macrophages and leukocytes are recruited to the injury site. A certain inflammatory response in the injured spinal cord is required for clearing neurotoxic cellular debris and limiting tissue damage. However, macrophage clearance can promote regeneration after SCI. In addition, overactivation of the inflammatory response can aggravate tissue damage[56]. In the acute phase of SCI, these cells produce proinflammatory cytokines such as IL-6, IL-β, and TNF-α. Reactive oxygen species (ROS), matrix-metalloproteinase (MMP), and inducible nitrous oxide synthase are released by neutrophils, macrophages and microglia, which can exacerbate the inflammatory response[57]. Microglia/macrophages have been regarded as an important cell type in the innate and adaptive immune responses after SCI[58]. Homeostatic macrophages are the main phenotype in the normal spinal cord, but resting macrophages are activated into different phenotypes. Some macrophages produce proinflammatory cytokines that aggravate inflammation and inhibit axon regeneration, while other macrophages produce anti-inflammatory cytokines that promote functional recovery, such as IL-10 and transforming growth factor-β (TGF-β)[59]. Therefore, immunomodulatory therapeutic approaches are mainly focused on inducing macrophage polarization from the proinflammatory phenotype to the anti-inflammatory phenotype following SCI.

Many studies have shown that BMMSC transplantation after SCI can exert therapeutic effects by attenuating detrimental inflammation or enhancing beneficial inflammation. Yagura *et al*[60] found that human BMMSC implantation significantly increased CCL5 expression and enhanced macrophage polarization. Similarly, another study analyzed the tissue expression levels of IL-1β, TNF-α, and Toll-like receptor 4 (TLR4) in a rat SCI model after intravenous BMMSC injection[61]. This study demonstrated that BMMSCs could attenuate spinal cord inflammation by inhibiting the TLR4-mediated signaling pathway and decreasing the expression levels of IL-1β and TNF-α. Similar situations were observed in another study that investigated the efficacy of grafted BMMSCs after SCI[62]. Interestingly, most recent studies have focused on the anti-inflammatory roles of exosomes derived from BMMSCs in SCI treatment[20,63,64]. For example, Sheng *et al*[63] confirmed that the application of BMMSC-derived exosomes promoted the phagocytosis ability of macrophages by upregulating the expression of MARCO, an important phagocytic receptor. In addition to modulating the phagocytic capacity of macrophages, BMMSC-derived exosomes could also affect the balance of macrophage polarization through the nuclear factor-kappaB pathway[65].

BMMSC transplantation exerts immunoregulatory effects following SCI mainly by inducing the formation of anti-inflammatory immune cells, modulating the expression levels of TLRs, and inhibiting the inflammatory response in the injured spinal cord, thereby promoting functional recovery[61]. Immediately after SCI, the organism enters an immunosuppressive state due to shock and stress. The first cells to mobilize at the injured site are the myeloid cells of the innate immune system, such as neutrophils and macrophages, which phagocytose debris. Then, adaptive immune cells such as B- and T-lymphocytes are recruited to the injured spinal cord[66,67]. As previously described, microglia, which participate in the clearance of apoptotic and myelin debris, are resident immune cells[68]. In the acute stage after SCI, microglia extend their processes toward the injured site and are biased toward the activated subtype, which leads to a further loss of neurons and promotes scar formation[69]. During this period, although microglia have beneficial effects on tissue recovery by clearing cellular and myelin debris, they have also been reported to aggravate secondary tissue damage and axonal retraction[67,70]. Homeostatic microglia in the injured spinal cord may play a protective role in tissue repair. Therefore, therapeutic strategies for targeting immunomodulation should be directed at modulating cytokine levels and other factors in the microenvironment and balancing activated/homeostatic microglia levels.

IMPROVING THE INHIBITORY MICROENVIRONMENT

In mammals, damaged axons in the spinal cord are unable to regenerate at the lesion site and to reestablish synaptic connections with their destination due to “natural barriers” and diminished intrinsic regenerative capacity[71,72]. This barrier consists of a lumen and a nonpermissive environment. Nogo-A, MAG and oligodendrocyte myelin glycoprotein (OMgp) are well-defined myelin breakdown products[73], but knockdown of the MAG and OMgp genes did not lead to neuronal growth after SCI, suggesting that Nogo-A plays a dominant role in inhibiting axonal regeneration, while MAG and OMgp play secondary roles[74]. In addition, several types of cells, including astrocytes, glial cells, and microglia/macrophages, migrate to the center of the injury, leading to the formation of glial scars and impeding the progression of axonal growth cones[75]. Glial scarring does not always play a negative role; on the one hand, it initiates the injury repair process, limits lesion expansion and inhibits the spread of the inflammatory response, and on the other hand, it acts as a physical barrier to nerve regeneration[76].

Few oligodendrocyte markers were found to be expressed in SCI centers transplanted with BMMSCs, while the number of axons was significantly increased, indicating that the transplanted cells provided a suitable environment for the regeneration and neural differentiation of endogenous neural stem cells [77]. BDNF in overexpressed BMMSCs further promotes axonal remyelination by affecting oligodendrocytes[78]. Zhao *et al*[79] found that the association of BDNF-overexpressing BMMSCs with platelet-rich plasma promoted more axonal remyelination and oligodendrocytes, probably due to astrocyte migration to the lesion region and increased graft BDNF-BMSCs, which could provide a favorable substrate for stabilizing regenerating axons in an inhibitory environment. However, damaging properties of the microenvironment (such as increased ROS) result in extensive stem cell death and dysfunction, severely impairing stem cell therapy for SCI.

INHIBITING GLIAL SCAR FORMATION

The spinal injury scar is generally classified into two components: The lesion core and the lesion border [80]. The lesion core is primarily composed of stromal-derived fibroblasts and inflammatory immune cells and is commonly regarded as the fibrotic scar. The lesion border is formed by microglia and reactive astrocytes, which surround the core and are generally considered the glial scar. The glial scar (mainly astrocytic) strongly upregulates the expression of intermediate filament proteins such as vimentin, GFAP, and chondroitin sulfate proteoglycans (CSPGs)[9]. At the acute stage after SCI, the glial scar separates healthy tissue from inflammatory cells and limits the spread of inflammation. However, the glial scar creates a long-lasting physical and molecular barrier that hinders axon regeneration and outgrowth during the chronic period[81]. Over the past decade, accumulating evidence has attributed the failure of axon regeneration to diminished intrinsic neuronal plasticity and the presence of glial scars and myelin-associated growth inhibitors.

Numerous experimental studies in SCI animal models have shown that BMMSC transplantation can suppress glial scar formation. Okuda *et al*[31] explored whether BMMSC sheets are permissive for injured nerve fiber elongation and the extension of astrocyte processes. They found that GFAP-positive astrocyte processes penetrated into the BMMSC-transplanted site, which is an indicator of a permissive environment for axon outgrowth[31]. Another study showed that transplantation of human BMMSCs in an SCI rat model largely reduced the inflammatory reaction and the expression of collagen type IV, one of the markers of fibrotic scars[82]. The application of biomaterials to improve the survival rate and efficacy of implanted cells has attracted attention in recent years. Some biomaterials are primarily used as scaffolds to support the growth of BMMSCs[83,84], while a few biomaterials are capable of modulating the harsh microenvironment following SCI[85,86]. For instance, Li *et al*[84] investigated the efficacy of a ROS-responsive hydrogel cotransplanted with BMMSCs in a rat transection SCI model. They performed double immunofluorescence staining to identify the formation of two distinct scars: The glial scar was labeled by GFAP, and the fibrotic scar was labeled by platelet-derived growth factor receptor-β. These results revealed that this BMMSC-encapsulated hydrogel could significantly alleviate the formation of both glial and fibrotic scars at the injury site[84]. In addition, CSPGs, which are enriched in glial scars and secreted by reactive astrocytes, are potent inhibitors of axonal outgrowth. Takeuchi *et al*[87] found that SCI mice treated with chondroitinase ABC, a kind of CSPG-digesting enzyme, can promote axon regeneration and improve functional outcome. Indeed, another study explored the efficacy of chondroitinase ABC plus BMMSCs in the repair of SCI. Notably, the application of chondroitinase ABC combined with BMMSCs significantly reduced GFAP expression at the injury site, and the scar tissue area was much smaller than that in the model group[88].

To date, the specific molecular mechanisms of scar formation have been widely studied. TGF-β initiates signaling after binding to transmembrane type I and type II receptors, and then the type I receptor leads to the recruitment and phosphorylation of Smad2 and Smad3 proteins[89]. After the formation of a heteroprotein complex together with the co-Smad protein SMAD4, this complex can translocate into the nucleus, where it acts as a transcription factor to regulate target gene expression [90]. There is a growing body of evidence to suggest that the TGF-β/Smad signaling pathway plays an important role in collagen deposition[91]. Studies have shown that administration of BMMSCs after SCI can inhibit scar formation by downregulating TGF-β and collagen expression[92,93]. Furthermore, studies have shown that the activation and proliferation of astrocytes can be suppressed after inhibiting the JAK/STAT3 or JNK/c-Jun pathway, thus reducing scar formation and promoting functional recovery after SCI[94]. Kim *et al*[95] suggested that acute transplantation of BMMSCs can modulate astrogliosis through the MMP2/STAT3 pathway.

In summary, BMMSC transplantation can suppress glial scar formation and provide a favorable environment for axon regeneration after SCI. However, there has been some debate in the field on the role of scar formation in recovery following SCI[96,97]. Although the glial scar has an important protective role in separating healthy tissue from pathology after injury, it has been acknowledged that scar formation has an inhibitory role, as it is associated with failed axon regrowth. Recent evidence suggests that the phenotypes of reactive glial cells are considered the key regulators of the dual nature of the spinal injury scar[98]. In addition, the opposing roles of the scar matrix cannot be ignored, which

contains beneficial molecules that are required for the formation of scar borders and inhibitory molecules such as CSPGs, tenascin, ephin B2, and slit proteins[99]. Therefore, therapeutic strategies for targeting the spinal injury scar should be directed at reducing scar formation or blocking inhibitory molecules associated with the scar.

PREVENTING BLOOD VESSEL LOSS AND IMPROVING ANGIOGENESIS

After SCI, immediate loss of spinal vascular support occurs, which induces local hypoxia around the injury epicenter, followed by a series of molecular cascades that lead to increased microvascular permeability and BSCB disruption. Angiogenesis, which is the process of forming new vasculature, plays an important role in the proliferation phase of wound healing[100,101]. Therefore, stabilizing the provisional vessels and forming a permanent vascular network are necessary for tissue repair following SCI. Angiogenesis is a multistep process that requires endothelial proliferation and differentiation, crosstalk among endothelial cells and extracellular matrix components, and the interplay of multiple proangiogenic and antiangiogenic factors[102]. VEGF, which is an important proangiogenic factor, is upregulated after hypoxia stimulus and induces blood vessel morphogenesis by binding to the VEGF receptor. Other critical angiogenesis-related proteins include FGF, TGF-β, angiopoietin-1, and angiopoietin-2[103].

Accumulating evidence has documented that BMMSC engraftment can promote angiogenesis and vascular stability in the treatment of different diseases, especially ischemic diseases, such as myocardial infarction and cerebral infarction[104-109]. Similarly, enhancement of angiogenesis by implanted BMMSCs has been demonstrated after SCI in animals[108]. In addition, *in vitro* studies also confirmed that BMMSC transplantation promotes vascular formation and vasoprotection[110,111]. In general, there is a close relationship between angiogenesis and enhanced functional recovery following SCI. Emerging evidence has shown that improved angiogenesis and BSCB integrity can promote motor function recovery[112,113]. A recent study found strong correlations between the level of angiogenesis and the number of surviving BMMSCs at the injury site. This study demonstrated that the expression of occludin and ZO-1 was significantly upregulated, which indicates the maturation and sealing of newly formed vasculature[114]. BMMSCs can produce FGF and VEGF-A, which can enhance the proliferation, migration, and vascular tube formation of microvascular endothelial cells[115]. BMMSCs also secrete specific factors, including IGF-1, HGF, VEGF, NGF, and TGF-β1, which can provide a favorable environment for angiogenesis after SCI[116]. For instance, Cantinieaux *et al*[117] investigated the efficacy of conditioned medium from BMMSCs in SCI treatment in a rat model and found that blood vessels displayed larger diameters in the conditioned medium-treated group, indicating enhanced regional blood perfusion at the lesion epicenter.

BMMSC engraftment can promote revascularization, enhance blood supply and increase BSCB integrity, which will attenuate secondary injury and promote axon growth, thereby improving functional recovery following SCI. Improved functional outcome after SCI is closely related to successful revascularization. First, a well-vascularized injury site can provide a regeneration-permissive microenvironment for the transplanted cells to survive. Additionally, blood vessels may act as a scaffold to guide transplanted cell migration and axon sprouting after injury. Emerging evidence has demonstrated a significant interaction between vascular regrowth and nerve repair. For example, some neurotrophins, such as NGF and NT-3, can control the sympathetic innervation of blood vessels, and VEGF-A, secreted by neurons and glial cells, can enhance vascular regrowth. To date, treatments based on revascularization for SCI include gene modulation, proangiogenic factor administration, cell therapy and biomaterial application. However, many aspects of the process of blood vessel formation remain unclear, and the therapeutic effect is limited. Therapeutic strategies for targeting angiogenesis after injury should be focused on the identification of combined strategies.

CLINICAL TRIALS OF BMMSCS FOR SCI PATIENTS

During past decades, cell transplantation has been regarded as a promising therapy after SCI. There have been not only many animal and preclinical studies but also a considerable number of clinical studies, and several systematic reviews/meta-analyses have proven the effects of cell transplantation in patients with SCI[118-122]. Among them, MSC transplantation is the most widely used and promising therapeutic approach for treating SCI. MSCs are mainly derived from adipose tissue and bone marrow sources and are accordingly divided into adipose tissue-derived MSCs and BMMSCs. The main stem cell type used in clinical trials to treat SCI is the BMMSC, which has lower immunogenicity and a widely available source and has been proven to be overall safe, well tolerated and valid in SCI patients, with a particular effectiveness in chronic and complete injuries.

After assessing the relevant literature, we found 38 clinical studies containing 1090 participants that provided overall evidence of the safety and efficacy of BMMSC cell transplantation for SCI patients, which was mainly represented by ASIA score improvement in at least one segment, and both sensory

and motor improvements were observed according to different previous studies. Some studies have shown that up to 70% of patients with complete cervical SCI and 33% of patients with thoracic SCI could recover at least one spinal cord level within 1 year after injury by spontaneous recovery[123,124]. Chhabra *et al*[125] indicated that most of the spontaneous neurological recovery in AISA subjects was likely to occur within the zone of partial preservation, which was less likely related to cell therapy. Due to the complicated process of neuroregeneration, minor therapeutic effects at the anatomical/histological level were difficult to detect in clinical trials, which might be ignored. Some novel assessments may provide further insights into the recovery of neuroregeneration after SCI in future clinical research. Furthermore, an increasing number of studies have tended to certify the same efficacy on bladder and gastrointestinal functions by slightly improved maximum capacity and decreased bladder pressure and residual urine volume, which are still unsatisfactory.

Considering the various therapy effects, there were related issues: Ambiguities in the selection of patients, timing of intervention, injection doses and routes of stem cell transplantation in different clinical trials. The optimal dose of cell transplantation has not yet been determined, and cell numbers between 10^6-10^8 seemed to be more beneficial[126-128]. Transplant routes included intrathecal, scaffold-loaded, intralesional, venous, arterial, and subdural administration, with intrathecal injection as the most widely used.

However, there were still some potential adverse events (AEs) observed, such as neuropathic pain, muscle spasm, and fever. Some of these AEs were slight and without further injury, while other more potentially serious AEs required a longer follow-up visit. Most of the current studies have a small number of samples, are low quality, lack control groups, and represent single-arm, early-stage clinical trials with the main purpose of evaluating the safety of stem cells. Nonetheless, prospective, well-designed randomized trials in larger cohorts with extensive follow-up are still awaited to confirm and update the findings.

CONCLUSION

Based on the results of previous studies, the effects that can be achieved with a single BMMSC treatment are limited, and combination therapy is an important future development direction. Combination therapy using various molecules or factors (including gene modulation, *etc.*) can enhance the effect of cellular therapy and achieve multi-effective and superimposed effects. In addition, most preclinical studies are currently designed with observation periods of 4 and 8 wk, and longer observation periods are important for the clinical translation of stem cell therapy to explore and address certain adverse effects when possible. Finally, given the current encouraging preclinical trial results, some treatments have been translated into clinical practice. BMMSC transplantation has been shown to be safe in SCI patients, and partial efficacy has been seen in some cases, but most clinical studies are still in phases I and II, and the results of phase III trials have extraordinary implications for the clinical translation of stem cell therapy for SCI. In conclusion, although many problems and challenges remain, researchers have been working to optimize preclinical studies and actively translate them to the clinic, and these efforts will pave the way for the field of SCI.

FOOTNOTES

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REVIEW

Different priming strategies improve distinct therapeutic capabilities of mesenchymal stromal/stem cells: Potential implications for their clinical use

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Abstract

Mesenchymal stromal/stem cells (MSCs) have shown significant therapeutic potential, and have therefore been extensively investigated in preclinical studies of regenerative medicine. However, while MSCs have been shown to be safe as a cellular treatment, they have usually been therapeutically ineffective in human diseases. In fact, in many clinical trials it has been shown that MSCs have moderate or poor efficacy. This inefficacy appears to be ascribable primarily to the heterogeneity of MSCs. Recently, specific priming strategies have been used to improve the therapeutic properties of MSCs. In this review, we explore the literature on the principal priming approaches used to enhance the preclinical inefficacy of MSCs. We found that different priming strategies have been used to direct the therapeutic effects of MSCs toward specific pathological processes. Particularly, while hypoxic priming can be used primarily for the treatment of acute diseases, inflammatory cytokines can be used mainly to prime MSCs in order to treat chronic immune-related disorders. The shift in approach from regeneration to inflammation implies, in MSCs, a shift in the production of functional factors that stimulate regenerative or anti-inflammatory pathways. The opportunity to fine-tune the therapeutic properties of MSCs through different priming strategies could conceivably pave the way for optimizing their therapeutic potential.

Key Words: Mesenchymal stromal/stem cells; Mesenchymal stromal/stem cell therapeutic properties; Mesenchymal stromal/stem cell paracrine effects; Mesenchymal stromal/stem cell priming; Pro-inflammatory priming; Hypoxic priming, 3D culture priming

Core Tip: Mesenchymal stromal/stem cells (MSCs) have demonstrated promising therapeutic results in the field of regenerative medicine. However, due to their heterogeneity, the application of MSCs in clinical trials has shown moderate or poor efficacy. Here, we review data on the principal priming approaches for enhancing the therapeutic potential of MSCs. We found that different priming strategies can modify MSC properties and, in this case some therapeutic effects on different disease models can be obtained in relation to dose and/or combination of the priming factors used. The production of priming type-specific functional factors in MSCs could pave the way toward implementing new MSC-based therapies.

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INTRODUCTION

Mesenchymal stromal/stem cells (MSCs) are multipotent adult stem cells involved in the homeostasis of tissue regeneration and, because of their therapeutic potential, have been extensively investigated in various clinical conditions[1-6]. Though MSC treatment was initially thought to promote tissue regeneration thanks to MSC multipotency of differentiation[7-9], recent evidence has revealed that the efficacy of MSC-based therapies is, at least in part, linked to the production of functional paracrine factors. These cells are able to secrete numerous products, *e.g.*, growth factors, cytokines, chemokines, and extracellular vesicles (EVs), which can regulate many pathophysiological processes, such as fibrosis, immune dysregulation, angiogenesis, and stimulation of tissue resident stem cells, in order to coordinate both tissue regeneration and functional recovery[10-12]. In injured tissue, MSC engraftment is limited because they undergo cell death, and their beneficial effects are exerted through secretion of various functional factors that not only enhance the function of resident cells, but also attract immune and progenitor cells, contributing to the coordination of tissue repair[13,14]. Therefore, considering the importance of the paracrine component in mediating MSC functions, there is growing interest in the molecular basis of MSC secretion involved in the therapeutic function of these cells.

Over the years, a large number of tissues, including placenta, adipose, umbilical cord, dental pulp, bone marrow, synovial membrane, liver and others, have been used as a source of MSCs[15-20]. It is quite clear that MSCs derived from all these sources possess a wide variety of functional effects, which they apply physiologically to their own original tissue, regulating homeostasis and regeneration. Interestingly, these effects may be useful for therapeutic applications of MSCs[3,21]. Currently, there are 1487 clinical trials registered at clinicaltrials.gov aimed at studying MSC therapeutic efficacy in the treatment of several clinical disorders, including lung, liver, kidney, orthopedic, cardiovascular, neurodegenerative, and immune diseases. In different clinical settings, MSC-therapies have been tested, showing tolerable safety, and demonstrating therapeutic benefits, and this has led to regulatory approvals of some MSC-based therapeutic products in several countries. In 2012, Cartistem, a MSC product based on the use of umbilical cord-derived MSCs for the treatment of traumatic or degenerative osteoarthritis, was approved by Korea's Ministry of Food and Drug Safety[22]. Moreover, Remestemcel-L, based on the use of bone marrow-derived MSCs (BM-MSCs), has been investigated in a phase 3 clinical trial in patients with steroid-refractory acute graft-versus-host disease (GVHD)[23]. Recently, due to the immunomodulatory properties of Remestemcel-L, which are able to work against cytokine storm linked to several inflammatory conditions, this therapy has also been tested for the treatment of coronavirus disease 2019-associated multisystem inflammatory syndrome[24]. The increasing interest in the clinical applications of MSCs as a cellular therapy has also been evidenced by the burgeoning of several companies that sell MSC therapies to United States clinics[25]. However, this has highlighted that in some cases the propensity for economic gain has outweighed the clinical advantages, despite the lack of solid scientific evidence that supports the broad use of MSCs in treating various human disorders. Indeed, in many clinical trials it has been shown that MSCs have moderate or poor efficacy, and the results from some studies are controversial[26-31]. In particular, due to both the inconsistent criteria used for the MSC identity across studies, and MSC heterogeneity, which depends on the different MSC origin[32] and the diverse harvesting and culture strategies[33], the clinical results obtained after MSC therapy are frequently variable. This makes it very difficult to obtain reliable conclusions regarding MSC therapeutic efficacy. Thus, while MSCs demonstrate a good margin of safety as cellular treatment, they have usually been therapeutically ineffective in humans[21].

These issues have underscored the urgent need to optimize the clinical use of MSCs or enhance MSC therapeutic effects. After determining the most appropriate cell source to use both in terms of invasiveness for cell isolation and cell yield, specific standardized production methods are needed to

ensure MSC therapeutic abilities and, therefore, their clinical efficacy. MSCs can be considered a key regulatory component in the tissue stem cell niche and, starting with the physiological role that these cells play in regulating tissue regeneration following injury[3,4,6,34-39], specific priming strategies can be understood and adapted for MSC clinical application. In this regard, much attention has been paid to the opportunity of MSC pre-conditioning to prime the cells before their clinical use. In this case, the therapeutic properties of MSCs can be modulated by pre-treatment of cells with hypoxia, cytokines, as well as growing MSCs under three-dimensional (3D) culture. In those instances, in response to MSC priming, the phenotype of MSCs was switched toward an anti-inflammatory, pro-trophic and more regenerative potential, which results in an enhanced therapeutic function of the cells[3,40-45].

In this review, we summarize the principal priming methods aimed at improving MSC efficacy as a therapeutic product. We would also like to highlight the fact that specific priming strategies can be considered more suitable for some types of diseases, leading to new therapeutic approaches that could be used to develop more powerful and predictable MSC therapies.

THE SECRETION OF PARACRINE FACTORS MEDIATE THE THERAPEUTIC FUNCTION OF MSCs

The secretion of functional products is central to MSC-based therapy, as demonstrated in numerous studies. Indeed, individual components of MSC secretome, such as functional proteins and EVs, are involved in the regulation of various biological processes, including angiogenesis, immunoregulation, wound healing, and tissue repair/protection[14,46-49]. Among the MSC-derived functional products, exosomes (EXOs), belonging to EVs, are anuclear particles ranging from 50 to 200 nm in size that are constitutively released from the endosomal compartment of MSCs. They contain a plethora of functional protein and other molecules, including microRNAs (miRNAs), which mediate several MSC properties [15,50,51]. EXOs are key components of intercellular communication, because they are released into the intercellular space where they exert local paracrine or distal systemic effects[52]. In fact, EXOs are able to regulate numerous biological processes, including angiogenesis[53], cell proliferation[54], and the activation/inhibition of immune cells[55]. Interestingly, EXO content can be changed by various priming stimuli[3,40,55]. Recently, it has been revealed that EXO-derived miRNAs play a critical role in mediating EXO effects[56]. MiRNAs are 19-22-nucleotide-long non-coding RNAs that regulate mRNA translation, and are involved in many cellular processes[56,57]. Therefore, even if some therapeutic functions of the MSCs are mediated by cell-to-cell contact, the secretion of paracrine factors can be considered the main mechanism by which MSCs elicit functional responses in target cells[3,40,58,59]. In many *in vitro* and *in vivo* disease models, MSC-derived products have been identified as responsible for therapeutic effects[60-63]. For example, promising preclinical therapeutic effects have been obtained using MSC-derived EVs. In particular, regarding BM-MSC-derived EVs, Haga *et al*[64] found that these functional factors were able to reduce hepatic injury by modulating cytokine expression in a mouse model of fulminant hepatic failure. Reis *et al*[65] demonstrated that the administration of EXOs in a rat model of gentamycin-induced kidney injury, was able to improve the kidney injury score. Moreover, it has been shown that EXOs derived from umbilical cord-derived MSCs were able to accelerate wound healing in a rat skin burn model[66], and EXOs derived from BM-MSCs overexpressing hypoxia-inducible factor (HIF)-1α accelerated bone regeneration and angiogenesis in a rabbit model of steroid-induced avascular bone necrosis[67].

MSCs can also secrete a number of cytokines/chemokines that control both the innate and adaptive immune responses, resulting in immunoregulation and the induction of tolerance[68]. Indeed, it has been shown that MSCs can produce both anti- and pro-inflammatory factors which, depending on their ratio, regulate the pro- or anti-inflammatory activity of MSCs[69]. In this case, final immunoregulatory properties may be affected by cell culture conditions that can prime/enhance MSC properties[3,70,71]. MSCs also have the ability to roll and adhere to post-capillary venules, and migrate to injured tissues, contributing to tissue repair/regeneration[72]. In this case, once MSCs reach the site of the injury, these cells put in place an active regulation by producing paracrine factors that impact tissue survival/repair, and activate tissue resident stem cells[3,73,74]. The secretion of various soluble factors has also been found to be responsible for the pro-angiogenic and anti-apoptotic effects of MSCs[75]. Though not well understood, the beneficial effects of conditioned media (CM) derived from MSCs have been clearly demonstrated by various experimental findings, supporting the concept of paracrine effects[76]. Several preclinical studies have tested the efficacy of CM in different diseases models. MSC-derived CM has been shown capable of improving cell viability and reducing inflammation in both *in vitro* and *in vivo* models of lung ischemia/reperfusion injury (IRI)[59,77]. Moreover, it has been demonstrated that BM-MSC-derived CM was able to reduce lung inflammation and edema in a mouse model of lipopolysaccharide-induced lung injury[78], and to improve renal tissue pathology in a mouse model of cisplatin-induced kidney injury[79]. Youdim *et al*[80], in a rat model of fulminant hepatic failure, found that the CM derived from BM-MSCs reduced leukocytic infiltrates and hepatocellular death. The CM derived from the same cells, in a mouse model of antigen-induced arthritis, was also able to reduce joint swelling, cartilage loss, and tumor necrosis factor (TNF)-α secretion[81]. In a rat model of lung fibrosis

and hypertension, using CM derived from adipose MSCs (AdMSCs), demonstrated the ability of secretome to reduce collagen deposition and improve lung blood flow[82]. In a rabbit model of surgical bone lesion, Linero and Chaparro[83] found that the CM produced from AdMSC cultures induced bone regeneration.

THE SECRETION OF MSC PARACRINE FACTORS CAN BE MODULATED BY VARIOUS PRIMING STRATEGIES

Given the heterogeneity of results supporting the efficacy of MSCs in the treatment of different human disorders, there is a need to improve the therapeutic properties of MSCs, and the best way might be that of preconditioning/priming. Though this approach has been widely used in the field of immunology, has also been effectively applied to MSCs[3,84,85]. Among commonly used priming strategies, leading approaches can be attributed to three main categories: (1) MSC priming with inflammatory molecules; (2) MSC priming with hypoxia; and (3) MSC priming with 3D cultures. These priming signals activate potential MSC mediators, including surface receptors and ligands, signalling molecules that induce survival/growth, regulatory molecules such as miRNAs, and transcription factors, which can modify the MSC phenotype[86-89], with a consequent boosting of MSC therapeutic functions (Figure 1).

Priming with inflammatory molecules

Numerous studies have revealed that the immunosuppressive properties of MSCs are not intrinsically possessed, but require priming of MSCs by inflammatory factors[90-92]. Depending on the inflammatory conditions, it has been demonstrated that MSC phenotypes can be polarized into MSC type 1 (with pro-inflammatory properties) and MSC type 2 (with immunosuppressive properties)[93,94]. Several strategies have been implemented to modulate/enhance the secretion of functional molecules in MSCs. As shown in Figure 2, the treatment of MSCs with inflammatory cytokines, including interferon-gamma (IFN-γ), interleukin (IL)-1α/β, IL-25, IL-6, TNF-α, and IL-17 enhanced the immunomodulatory properties of MSCs[40,95-112]. These treatments increase the production/secretion of functional factors, including hepatocyte growth factor (HGF), transforming growth factor-β, IL-6, prostaglandin E2 (PGE2), leukemia inhibitory factor, granulocyte colony-stimulating factor, IL-10, macrophage inflammatory protein-1α, indoleamine 2,3-dioxygenase (IDO), intercellular adhesion molecule, programmed death ligand 1-2, monocyte chemoattractant protein (MCP)-1, monokine induced by IFN-γ, induced protein 10, and macrophage inflammatory protein-1β, which in turn confer more paracrine immunomodulatory properties to MSCs (Figure 2). It has been demonstrated that CM enriched with the above-described factors was able to inhibit T cell proliferation/activation, reduce the secretion of inflammatory mediators, and induce monocyte polarization towards anti-inflammatory the M2 phenotype[40,102,105-112]. It has been shown that the treatment with inflammatory cytokines was also able to improve the immunomodulatory capabilities of EXOs, and these effects appear to be mediated by specific miRNAs, such as miR-21, miR-23a, miR-26b, miR-125b, miR-130b, miR-140, miR-146a, miR-203a, miR-223, miR-224, and miR-320a[40,109,111,113].

Priming with hypoxia

Differently from inflammatory cytokines, hypoxic treatment of MSCs seems to stimulate primarily the secretion of functional factors involved in the processes of angiogenesis and tissue proliferation/regeneration (Figure 2). Hypoxic preconditioning was able to promote angiogenic potential of MSCs *via* the activation of the HIF-1α-GRP78-Akt axis, and the overproduction of vascular endothelial-derived growth factor (VEGF) and HGF[114]. Lee and Joe[115] demonstrated that hypoxia priming induces an increase in HIF-1α expression and consequent VEGF production, improving the ability of MSCs to stimulate migration and tube formation of human umbilical vein endothelial cells (HUVECs). Moreover, Bader *et al*[116] found that hypoxic preconditioning induces the anti-apoptotic and pro-angiogenic effects of MSCs compared with untreated cells. In particular, Bcl-xL, BAG1, and VEGF were overexpressed after hypoxic priming, enhancing HUVEC proliferation and migration. Hypoxic MSCs are also able to produce numerous factors related to tissue remodelling, including matrix metallopeptidase 1 (MMP1), MMP2, and MMP9[117-119], as well as crucial factors such as IL-8 and MCP-1, involved in the chemotaxis and activation of innate immune responses[120,121]. Also with regard to EVs, hypoxic priming has been shown to have important effects. Xue *et al*[122] discovered that EXOs derived from hypoxia-treated MSCs were able to increase migration and tube formation of HUVECs through the PKA signalling pathway. Moreover, Ge *et al*[123] demonstrated the efficacy of hypoxic MSC-derived EXOs in enhancing angiogenesis. In particular, they showed that hypoxic EXOs containing miR612 promoted, through HIF-1α activation, the production of VEGF in human brain microvascular endothelial cells, inducing proliferation, migration, and angiogenic activities of these cells.

Priming with 3D culture of MSCs

Various *in vitro* strategies have been applied for the production of MSCs, with improved therapeutic

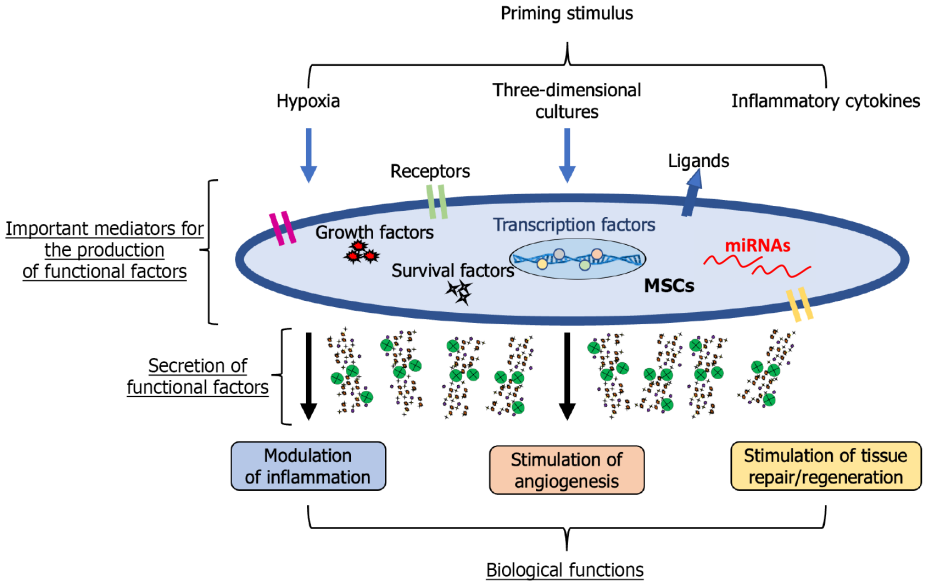

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Figure 1 Potential mechanisms mediating mesenchymal stromal/stem cell-primed therapeutic properties. Mesenchymal stromal/stem cells (MSCs) can be primed through different signals, including hypoxia, three-dimensional cultures, and inflammatory cytokines to obtain a therapeutic phenotype. The potential mediators of this new phenotype comprise a plethora of regulatory molecules within MSCs, including surface receptors and ligands, signalling molecules inducing survival/growth, regulatory molecules such as microRNAs, and transcription factors regulating several pathways. Thus, primed MSCs can modulate inflammation, stimulate angiogenesis, and promote tissue repair/regeneration. MSCs: Mesenchymal stromal/stem cells; miRNAs: MicroRNAs.

properties, and priming with inflammatory factors may impact the expression of HLA-DR, thus altering allogeneic therapeutic possibilities[124-126]. MSC priming through 3D culture techniques, which allows for the generation of MSC spheroids, strictly recapitulates the *in vivo* MSC niche and enhances the phenotypic profile of MSCs, increasing both trophic and immunomodulatory functionalities. MSC spheroid action is exerted by the paracrine secretion of functional factors that possess anti-inflammatory, angiogenic, anti-fibrotic, anti-apoptotic, and mitogenic properties (Figure 2)[127]. Recently, through omics approaches, such as RNA sequencing and analysis of DNA methylation, it has been demonstrated that, compared with conventional 2D culture, MSC spheroids were able to modify their transcriptome profile by overexpressing genes that can regulate proliferation/differentiation, as well as immunomodulatory and angiogenic processes[128]. Concerning immunomodulatory and regenerative effects, 3D culture of MSCs seems to have more intermediary functions than the above-mentioned priming strategies (priming with inflammatory molecules or hypoxia) (Figure 2). 3D MSC spheroids have been shown to be capable of secreting multiple functional factors. For example, it has been found that various regenerative and immunomodulatory factors, such as stromal cell-derived factor-1α, growth-related oncogene α, MCP-1/3; IL-4, IL-10; epidermal growth factor (EGF), leukemia inhibitory factor, placental growth factor-1, VEGF-A/D, HGF, insulin like growth factor 1, TNFAIP6, STC1, platelet-derived growth factor B, transforming growth factor-β, PGE2, and IDO were up-regulated in 3D MSC spheroids compared with those of the MSCs cultivated under conventional 2D conditions[43,44,59,73,128-131] (Figure 2). The paracrine effects of 3D MSC appear to be also mediated by EVs. In particular, EXOs derived from MSC 3D cultures have been shown to have higher yields and enhanced activity. Indeed, compared with 2D cultures, EXOs isolated from CM of MSC spheroids were able to inhibit T cell proliferation and stimulate angiogenesis *in vitro*[44], as well as attenuate inflammation and periodontitis *in vivo*[132], and stimulate tissue regeneration in both *in vitro* and *in vivo* models[133].

THERAPEUTIC PROPERTIES OF PRIMED MSCs IN PRECLINICAL MODELS

Principal priming strategies to treat chronic immune-related disorders

By virtue of their immunomodulatory properties, MSCs are being studied to treat numerous chronic conditions, including GVHD and inflammatory bowel disorders, in order to attenuate inflammation and induce tissue recovery (Table 1). As already mentioned, treating MSCs with inflammatory factors enhances their immunomodulatory properties, and renders these cells able to inhibit T cell proliferation/activation and induce monocytes toward an anti-inflammatory phenotype. This quality makes these cells more clinically effective when applied to chronic inflammatory-related diseases (Figure 2). Indeed, several experimental studies have demonstrated that the treatment of MSCs with inflammatory factors, such as IFN-γ, IL-1β, and IL-25, enhanced MSC therapeutic effects in *in vivo* models of chronic

Table 1 Representative priming strategies of mesenchymal stromal/stem cells and their application in preclinical studies

MSCs	Dose	Priming treatments	Study model	Observed therapeutic effects	Ref.
AMSCs	1×10^5 MSCs/5×10^5 PBMCs	IFN-γ	*In vitro* model of T cell activation and monocyte M1/M2 polarization	Regulation of T cell activation/anergy and induction of M2-like polarized phenotype in monocytes	[40]
BM-MSCs	0.5×10^6 MSCs/mouse	IFN-γ	*In vivo* model of chronic colitis	Attenuation of inflammation and colitis	[96]
BM-MSCs	NA	IFN-γ; TNF-α	*In vitro* model of MLR	Inhibition of allogeneic MLR	[97]
CB-MSC-derived EVs	NA	IFN-γ	*In vivo* model of acute kidney injury and *in vitro* model of T cell activation	Regulation of T cell activation and amelioration of kidney injury with unprimed MSCs only	[100]
BM-MSCs and CB-MSCs	1×10^6 MSCs/mouse	IFN-γ	*In vivo* model of GVHD	Reduction of the symptoms of GVHD	[101]
BM-MSCs	1×10^4 MSCs/2×10^3 macrophages	IFN-γ; LPS; TNF-α	*In vitro* model of monocyte M1/M2 polarization	Induction of monocyte polarization toward an anti-inflammatory M2 phenotype	[102]
UC-MSCs	1×10^6 MSCs/mouse	IFN-γ; TNF-α	*In vivo* model of GVHD	Reduction of the symptoms of GVHD	[103]
BM-MSCs	2.5×10^5 MSCs/5×10^5 macrophages	IFN-γ; IL-1β	*In vitro* model of monocyte M1/M2 polarization	Induction of monocyte polarization toward an anti-inflammatory M2 phenotype	[105]
BM-MSC-derived CM	NA	IFN-γ; IL-1α/β; TNF-α	*In vitro* model of LPS-injured microglial cells	Reduction in the secretion of inflammatory factors	[106]
AdMSCs; BM-MSCs; CB-MSCs.	NA	IFN-γ	*In vitro* model of T cell activation	Suppression of T cell proliferation	[110]
BM-MSCs	NA	IFN-γ; spheroids	*In vitro* model of T cell activation	Suppression of T cell activation and proliferation	[112]
BM-MSCs	2×10^6 MSCs/mouse	IFN-γ	Autoimmune encephalomyelitis	Attenuation of pathologic manifestations	[134]
BM-MSCs	1×10^6 MSCs/mL	IFN-γ	*In vitro* model of T cell activation and *in vivo* model of colonic wounds	Regulation of T cell activation and acceleration of healing of colonic mucosal wounds	[135]
UC-MSCs	2×10^6 MSCs/mouse	IL-1β	*In vivo* model of chronic colitis	Attenuation of inflammation and colitis	[98]
UC-MSCs	1×10^6 MSCs/mouse	IL-1β	*In vivo* model of sepsis	Increase in survival rate	[109]
MSC-derived EVs	40 μg/mouse	IL-1β	*In vitro* model of monocyte M1/M2 polarization and *in vivo* model of sepsis	Induction of monocyte M2 polarization and amelioration of sepsis	[111]
AdMSC-derived CM	20 μL/rat	TNF-α	*In vivo* model of wound healing	Acceleration of wound closure and angiogenesis	[99]
BM-MSCs	1.6×10^6 MSCs/mouse	TNF-α	*In vivo* model of peritonitis	Attenuation of inflammatory responses	[136]
BM-MSCs	5×10^6 MSCs/rat	IL-25	*In vivo* model of chronic colitis	Attenuation of inflammation and colitis	[95]
BM-MSCs	1×10^6 MSCs/mL	IL-6	*In vivo* model of liver fibrosis	Reduction of liver injury and fibrosis	[104]
BM-MSCs	3.91×10^4 MSCs/3.91×10^6 T cells	IL-17	*In vitro* model of T cell activation	Suppression of T cell proliferation/activation and Th1 cytokines	[108]
AdMSCs	5×10^5 MSCs/mouse	Hypoxia	*In vivo* model of hindlimb ischemia	Improvement of angiogenesis	[114]
BM-MSC-derived CM	100 μL/mouse	Hypoxia	*In vivo* model of wound healing	Acceleration of skin wound healing	[120]
BM-MSCs	2.5×10^5 MSCs/mouse	Hypoxia	*In vivo* model of pancreatic islet transplantation	Reversion of impaired glucose tolerance	[121]
BM-MSCs	5×10^5 MSCs/mouse	Hypoxia	*In vivo* model of hindlimb	Improvement of angiogenesis	[139]

			ischemia		
AdMSCs	5 × 105 MSCs/mouse	Hypoxia	*In vivo* model of hindlimb ischemia	Improvement of functional recovery and neovascularization	[140]
AdMSC-derived CM	NA	Hypoxia	*In vivo* model of partial hepatectomy	Enhanced liver regeneration	[142]
AdMSCs	2 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of acute kidney injury	Improvement of angiogenesis and inhibition of ROS generation	[145]
AdMSC-derived CM	100 μL/mouse	Hypoxia	*In vivo* model of acute kidney injury	Improvement of renal function and reduction of inflammation	[146]
BM-MSCs	1 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of lung IRI	Attenuation of pathologic lung injury score by inhibiting inflammation and generation of ROS and anti-apoptotic effects	[147]
BM-MSCs	NA	Hypoxia	*In vivo* model of radiation-induced lung injury	Improvement of antioxidant ability	[148]
BM-MSCs	1 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of myocardial infarction	Improvement of angiogenesis and function	[150]
BM-MSCs	1 × 10^6 MSCs/mouse	Hypoxia	*In vivo* model of myocardial infarction	Prevention of apoptosis in cardiomyocytes	[151]
BM-MSC-derived EVs	1 μg of EVs/mouse	Hypoxia	*In vivo* model of myocardial infarction	Reduction of cardiac fibrosis	[152]
BM-MSC-derived EVs	50 μg of EVs/rat	Hypoxia	*In vivo* model of cardiac IRI	Reduction of IRI and improvement of cardiomyocyte survival	[153]
BM-MSC-derived EVs	200 μg of EVs/20 g	Hypoxia	*In vivo* model of myocardial infarction	Improved cardiac repair by amelioration of cardiomyocyte apoptosis	[154]
BM-MSCs	1 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of cerebral ischemia	Enhanced angiogenesis and neurogenesis	[157]
BM-MSC-derived CM	100 μg of CM/kg	Hypoxia	*In vivo* model of traumatic brain injury	Improved neurogenesis, motor and cognitive function	[158]
UC-MSCs	1 × 10^5 MSCs/rat	Hypoxia	*In vivo* model of spinal cord injury	Increase in axonal preservation and decrease of apoptosis	[159]
PMSC-derived CM	100 μL/mouse	Hypoxia	*In vivo* model of scar formation	Reduction of scar formation	[162]
BM-MSCs	5 × 10^6 MSCs/rat	Hypoxia	*In vivo* model of partial hepatectomy	Enhanced liver regeneration	[164]
DP-MSCs	N.A.	Hypoxia	*In vivo* model of dental pulp injury	Regeneration of dental pulp with a rich vasculature	[167]
AF-MSC-derived CM	N.A.	Hypoxia	*In vivo* model of wound healing	Acceleration of skin wound healing	[168]
AMSC-derived CM and EVs	200 μL CM and 5 μg EVs/1 × 10^5 PBMCs, and 100 μL CM and 5 μg EVs/1 × 10^4 HUVECs	3D cultures/spheroids	*In vitro* model of T cell activation and HUVEC cells	Induction of angiogenesis and inhibition of T cell proliferation	[44]
AMSCs	250 μL CM/ 1.5 × 10^5 alveolar epithelial cells	3D cultures/spheroids	*In vitro* model of lung IRI	Attenuation of IRI side effects by improving the efficacy of in vitro EVLP	[59]
AMSC-derived CM	50 μL CM/ 1 × 10^4 liver cells	3D cultures/spheroids	*In vitro* model of liver IRI	Attenuation of IRI side effects by inhibiting inflammation and apoptosis	[131]
BM-MSCs	3 × 10^6 MSCs/mouse	3D cultures/spheroids	*In vivo* model of peritonitis	Production of anti-inflammatory cytokines	[137]
BM-MSCs	1.5 × 10^6 MSCs/mouse	3D cultures/spheroids	*In vivo* model of peritonitis	Attenuation of inflammatory responses	[138]
CB-MSCs	1 × 10^7 MSCs/mouse	3D cultures/spheroids	*In vivo* model of hindlimb ischemia	Improvement of survival and angiogenesis	[141]
AdMSCs	2 × 10^6 MSCs/rat	3D cultures/spheroids	*In vivo* model of acute kidney injury	Reduction of apoptosis and tissue damage, promotion of vascularization, and amelioration of renal	[143]

				function	
UC-MSC-derived EVs	200 μg of EVs/mouse	3D cultures/spheroids	*In vivo* model of acute kidney injury	Attenuationof pathological changes and improvement of renal function	[144]
BM-MSCs	2×10^6 MSCs/rat	3D cultures/spheroids	*In vivo* model of myocardial infarction	Promotion of cardiac repair	[155]
BM-MSCs	5×10^5 MSCs/rat	3D cultures/spheroids	*In vivo* model of myocardial infarction	Stimulation of a vascular density and improvement of cardiac function	[156]
AdMSCs	1×10^7 MSCs/mouse	3D cultures/spheroids	*In vivo* model of hindlimb ischemia	Improvement of angiogenesis	[163]
AdMSCs	2×10^6 MSCs/rabbit	3D cultures/spheroids	*In vivo* model of disc degeneration	Induction of disc repair	[169]
BM-MSCs	NA	3D cultures/spheroid	*In vivo* model of bilateral calvarial defects	Induction of bone regeneration	[170]
SMSCs	NA	3D cultures/spheroid	*In vivo* model of osteochondral defects	Induction of cartilage regeneration	[171]

MSCs: Mesenchymal stem cells; BM-MSCs: Bone marrow-derived mesenchymal stem cells; AMSCs: Amnion-derived mesenchymal stem cells; UC-MSCs: Umbilical cord-derived mesenchymal stem cells; AdMSCs: Adipose-derived mesenchymal stem cells; CB-MSCs: Cord blood-derived mesenchymal stem cells; WJ-MSCs: Wharton's Jelly-derived mesenchymal stem cells; PMSCs: Placenta-derived mesenchymal stem cells; AF-MSCs: Amniotic fluid derived mesenchymal stem cells; SMSCs: Synovial derived mesenchymal stem cells; EVs: Extracellular vesicles; CM: Conditioned medium; NA: Not available; GVHD: Graft-versus-host disease; IRI: Ischemia-reperfusion injury; 3D: Three-dimensional; IFN: Interferon; TNF: Tumor necrosis factor; IL: Interleukin; MLR: Mixed lymphocyte reactions; LPS: Lipopolysaccharide; HUVEC: Human umbilical vein endothelial cell.

colitis[95,96,98]. Rafei *et al*[134], in a mouse *in vivo* model of autoimmune encephalomyelitis, found that treatment with allogeneic MSCs primed with IFN-γ reduced clinical signs in a dose-dependent manner. In this study the authors showed that, though the priming treatment induced the increase of CCL2 and MHCI/II expression in IFN-γ-primed MSCs, it inhibited manifestations of autoimmune encephalomyelitis while keeping their immunogenicity low. The use of IFN-γ- or TNF-α-primed MSCs has also been shown to attenuate symptoms of GVHD[101,103]. In these cases, in the first study it was shown that therapeutic effects of MSCs were mediated by overproduction of IDO induced through the IFN-γ-JAK-STAT1 pathway[101]. In the second study, the therapeutic function of MSCs was activated by TNF-α, which induced overexpression of Chi3 L1 and consequent suppression of T-helper 17 cells[103]. Recently, it has been revealed that the priming of MSCs with IL-1β relieved the side effects of sepsis[109,111]. In particular, Song *et al*[109] demonstrated that IL-1β makes MSCs more effective in inducing macrophage polarization toward an anti-inflammatory M2 phenotype, and this effect was mediated, at least in part, through overproduction of EXOs containing miR146a. Similar results on M2 macrophage polarization were also obtained by Yao *et al*[111], who revealed the ability of IL-1β to stimulate the production of MSC-derived EXO containing miR21. The therapeutic efficacy of MSCs primed with IFN-γ was also found in an *in vivo* model of colonic wounds. Particularly, García *et al*[135] showed that these cells were able to enhance healing of colonic mucosal wounds in both immunocompromised and immunocompetent mice. Similar results were also obtained using MSCs primed with TNF-α, which were able to accelerate wound closure and angiogenesis in an *in vivo* model of wound healing[99]. The priming with inflammatory cytokines seems to also be effective for the treatment of chronic liver diseases. Indeed, treatment with IL-6 improved the ability of MSCs to reduce liver injury[104]. The study reported that in a mouse *in vivo* model of liver fibrosis, treatment with IL-6-primed MSCs reduced both fibrosis and apoptosis, and improved liver functions[104]. Moreover, TNF-α-primed MSCs were also able to attenuate inflammation in an *in vivo* model of peritonitis[136]. In this study, the authors demonstrated that TNF-α induced the overproduction of the anti-inflammatory factor TSG-6, generating a mechanism that reduces inflammation in an *in vivo* model of zymosan-induced peritonitis[136]. Interestingly, in a similar experimental model, Bazhanov *et al*[137] found that after intraperitoneal injection MSCs formed 3D aggregates, and stimulated the production of anti-inflammatory cytokines, such as IL-10 and PGE2. In this regard, Bartosh *et al*[138] showed that the priming of MSCs with 3D culture decreased inflammation in an *in vivo* model of peritonitis[138]. In particular, the authors suggest that MSC spheroids overexpressed TSG-6, and these cells were more effective than conventional MSCs as therapy for diseases characterized by unresolved inflammation.

Overall, the above-mentioned studies suggest that treatment with pro-inflammatory cytokines or the 3D culture of MSCs represents promising priming strategies for enhancing the MSC immunoregulatory phenotype, making these cells more suitable for clinical disorders related to exacerbated immune responses (Figure 2).

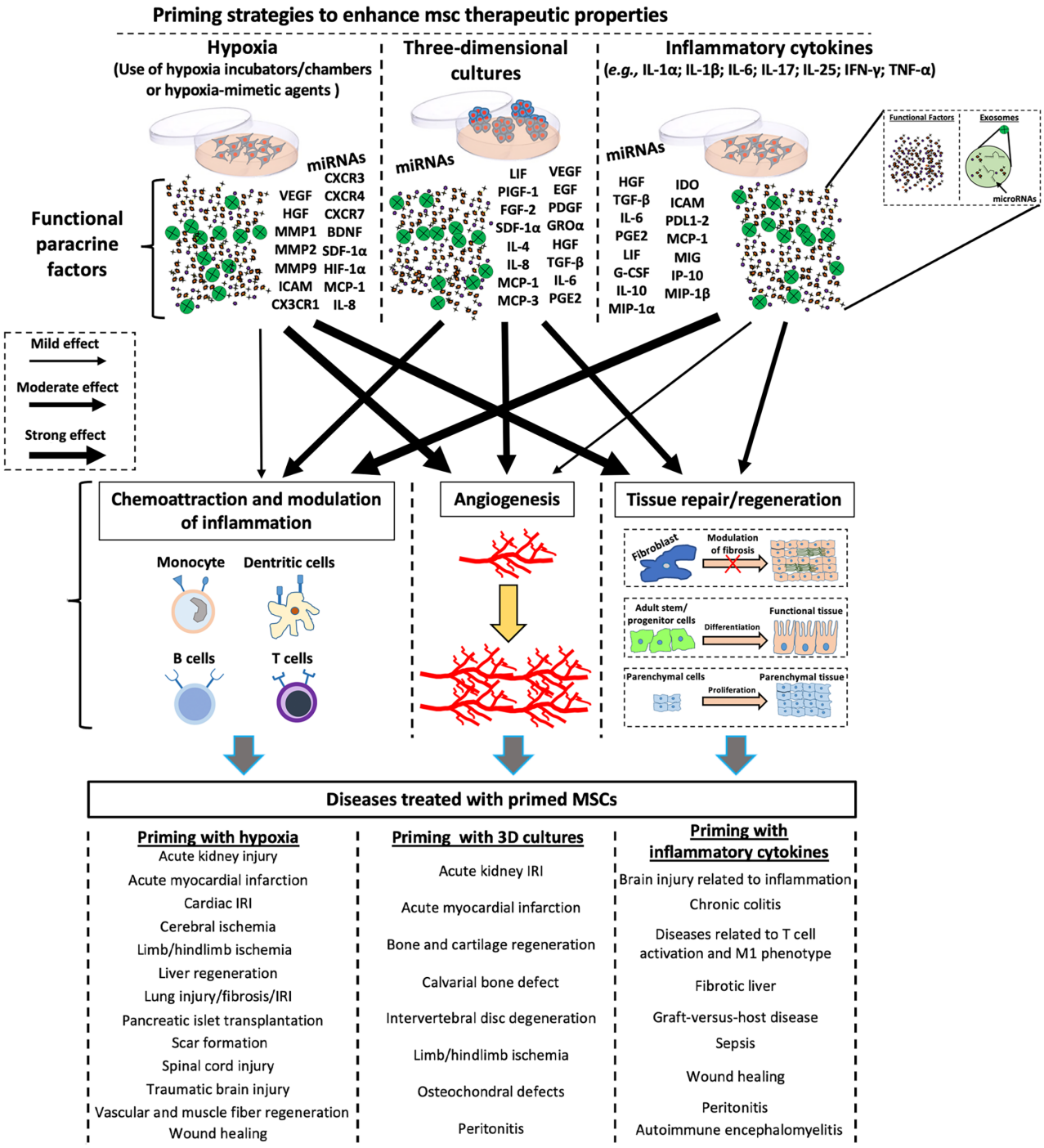

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Figure 2 Schematic representation of the molecular effects after priming of mesenchymal stromal/stem cells. Mesenchymal stromal/stem cells (MSCs) can be primed through various stimuli, including hypoxia, three-dimensional cultures, and pro-inflammatory cytokines to enhance their therapeutic potential. Each priming method induces the production of specific factors (*e.g.*, trophic factors, angiogenetic factors, chemokines, cytokines, and exosomes containing both proteins and microRNAs), which induce the activation of biological processes such as angiogenesis, tissue repair/regeneration, chemoattraction, and modulation of inflammation. Each priming strategy seems to stimulate the production of functional factors in a different way, thus eliciting different responses. miRNA: MicroRNA; VEGF: Vascular endothelial-derived growth factor; CXCR: Chemokine receptor; HGF: Hepatocyte growth factor; MMP: Matrix metallopeptidase; BDNF: Brain-derived neurotrophic factor; SDF: Stromal cell-derived factor; HIF: Hypoxia-inducible factor; ICAM: Intercellular adhesion molecules; MCP: Monocyte chemoattractant protein; IL: Interleukin; LIF: Leukemia inhibitory factor; PIGF: Placental growth factor; EGF: Epidermal growth factor; FGF: Basic fibroblast growth factor; PDGF: Platelet-derived growth factor; GRO: Growth-related oncogene; TGF: Transforming growth factor; PGE2: Prostaglandin E2; IDO: Indoleamine 2,3-dioxygenase; PDL1-2: Programmed death ligand 1-2; MIG: Monokine induced by interferon-gamma; G-CSF: Granulocyte colony-stimulating factor; IP-10: Induced protein 10; MIP: Macrophage inflammatory protein; IRI: Ischemia/reperfusion injury; MSCs: Mesenchymal stromal/stem cells; 3D: Three-dimensional.

Main priming strategies for treating acute injury

Priming strategies for MSCs have been considered a crucial tool for enhancing their therapeutic effects, making these cells more suitable for application in the field of regenerative medicine[3,85]. However,

while the priming of MSCs with pro-inflammatory cytokines potentially represents the principal strategy modulating inflammation in chronic immune-related disorders (or, in any case, conditions in which the inflammation is exacerbated), the priming of MSCs with hypoxia is thought to represent the more appropriate priming strategy for boosting MSC effects for the stimulation of tissue function recovery after acute injury (Figure 2). This has been demonstrated in numerous study models, and on different organs (Table 1). For example, hypoxia pre-conditioning significantly improved blood flow recovery in mouse models of hindlimb ischemia. Rosová *et al*[139] demonstrated that hypoxic MSCs better migrate to the injured site compared with non-hypoxic MSCs, thus speeding up the restoration of blood flow. The authors demonstrated that the observed effects were likely mediated by the HGF-cMET axis. It has been shown that hypoxia helps MSCs to better integrate in the damaged tissue. Han *et al*[140] revealed that hypoxic priming enhanced survival and proliferation of transplanted MSCs, thus improving the regeneration of hindlimb ischemic tissues. After MSC treatment, the authors observed inhibition of apoptosis and promotion of neovascularization and, as they showed the increased expression of the normal cellular prion protein upon hypoxia pre-conditioning, they identified this prion as a potential target for MSC therapy. In a similar manner, Lee *et al*[115] recently identified GRP78 as new potential target for the development of functional MSCs. GRP78 has been shown to be induced by hypoxia, thus increasing transplanted-MSC survival and proliferation in a mouse model of hindlimb ischemia. Moreover, the authors found that the HIF-1α-GRP78-Akt axis regulates the suppression of cell death signals, and increases angiogenic cytokine secretion, thus strongly improving tissue recovery from the damage[114]. Recently, it has been found that mild hypoxia can be induced in MSCs when they are cultured as spheroids. Various studies have clearly demonstrated that 3D culture conditions induce hypoxia in the core of the spheroid, thus stimulating the production of both growth and pro-angiogenic factors, which in turn stimulate the fast recovery of damaged tissues in mouse models of hindlimb ischemia[141,142]. Interestingly, it has also been shown that the CM derived from MSCs primed by 3D culture attenuated injury and inflammation in two IRI *in vitro* models of both lung and liver[59,131]. 3D pre-conditioning has been shown to also be effective for other type of diseases, such as acute kidney injury (AKI). Xu *et al*[143] found that 3D pre-conditioned MSCs, when transplanted in mice with AKI, are more viable than the 2D cultured cells, and exhibit higher paracrine secretions, as evidenced by the increased levels of VEGF and TSG-6. Furthermore, the authors show that the paracrine secretion, which also includes basic fibroblast growth factor, insulin like growth factor, and EGF, significantly improved renal function and reduced tissue apoptosis, thus speeding up the regeneration of renal tissues upon injury[143]. Recently, the secretome of 3D MSCs transplanted for the treatment of AKI was furtherly investigated. For example, Cao *et al*[144] found that the paracrine effect on AKI was mediated not only by soluble factors, such as anti-inflammatory cytokines, but also by EXOs, whose production is increased after 3D pre-conditioning. Furthermore, by using a cisplatin-inducing AKI model in mice, the authors showed that the increased number of EXOs upon 3D culture enhanced the renoprotective and anti-inflammatory efficacy of MSCs[144]. Treatment of AKI with MSC therapy has been implemented in recent years by defining new protocols of MSC pre-conditioning. Along with 3D culturing, hypoxia priming has been used for the treatment of IRI-inducing AKI in animal models, and Zhang *et al*[145] demonstrated that hypoxia priming enhanced angiogenic and antioxidative MSCs properties in a rat model of renal IRI. In addition, in the same model, the authors found that transplanted MSCs attenuated renal apoptosis by reducing cleaved caspase3 activation. Notably, hypoxia also enhanced MSC therapeutic potential in a cisplatin-induced mouse model of AKI. Overath *et al*[146] found that hypoxic conditions increased the efficacy of transplanted MSCs in attenuating renal damage upon injury both by reducing creatinine and N-GAL serum levels, and decreasing pro-inflammatory cytokine release. MSC hypoxia pre-conditioning has also been found to be strongly effective for the treatment of IRI in the lung. For example, MSC infusion in lung perfusates demonstrated that hypoxic MSCs quickly migrate from the pulmonary artery to the lung tissue, where they attenuate parenchymal damage by reducing oxidative stress, inflammation, and apoptosis, and by stimulating cell proliferation and survival[147]. In a similar manner, MSC hypoxia has been found to have important effects also for radiation-induced lung injury (RILI). A mouse model of RILI was recently established by exposing the lungs of mice to irradiation, thus generating tissue damage. Upon irradiation, the authors demonstrated that hypoxic MSCs reside for longer in the injured tissue compared with normoxic MSCs. In addition, Li *et al*[148] showed that hypoxia-primed MSCs enhanced cell viability and proliferation, as well as anti-oxidative and anti-apoptotic capabilities in lung parenchymal cells. Finally, the authors highlighted the role of HIF-1 in modulating resistance to lung hypoxic stress induced by RILI, thus promoting tissue repair and regeneration upon injury.

The use of MSCs as cellular therapy has also been shown to be effective for the treatment of acute myocardial injury in several preclinical models (Table 1). Also in this case, to ameliorate the therapeutic effects of MSCs various priming strategies have been evaluated. In particular in myocardial infarction (MI), it has been widely believed that tissue injury is related to ischemia and the hypoxic environment. Therefore, the *in vitro* hypoxic condition was tested to improve MSC therapeutic effects in MI animal models[149]. In a mouse model of MI, it was found that intramyocardial injection of hypoxia-preconditioned MSCs reduces infarct size, influences heart remodelling by modulating vasculogenesis, and improves heart functions, promoting cell survival[150,151]. Of note, expression analysis in hypoxic MSCs has revealed an increase in expression of pro-survival and pro-angiogenic factors, including HIF-

1α, ANGPT1, VEGF, Flk-1, Bcl-2, Bcl-xL, and these proteins can act in a paracrine manner on MI, inducing functional recovery[150]. It has also been observed that hypoxic MSCs influence the expression of specific miRNAs that can be secreted through EVs. In particular, Feng *et al*[152] demonstrated that after hypoxic treatment of MSCs an increase of miR22 was observed in EXOs, and this miRNA was considered responsible for targeting Mecp2, with beneficial effects on survival of cardiomyocytes exposed to ischemia. Similarly, EVs derived from hypoxic MSCs overexpressing miR26 were able to reduce the damage from ischemia/reperfusion in a rat model[153]. In the same way, in an MI mouse model the intracardial injection of hypoxic-preconditioned MSC-derived EXOs was able to positively regulate cardiomyocyte proliferation and survival, and this effect was ascribable to the overexpression of miR125b[154]. In addition to the use of hypoxia priming, the use of 3D culture has also been shown to be effective in the improvement of MSC therapeutic effects on the treatment of acute myocardial injury. You *et al*[155], in an acute MI rat model, found that treatment with 3D-primed MSCs resulted in a retention of MSCs at the epicardium, where MSCs exerted cardiac protection/repair, and functional recovery. Moreover, in the same animal model, Wang *et al*[156] revealed that 3D MSCs were able to stimulate vascular density and improve cardiac function after MI.

Over the last decade, MSCs have also been intensively studied for their potential use in the treatment of neurological acute injury, including cerebral ischemia, traumatic brain injury, and spinal cord damage. For example, in an *in vivo* model of cerebral ischemia, it has been shown that hypoxic-preconditioned MSCs enhanced angiogenesis and neurogenesis after ischemia[157]. In an *in vivo* model of traumatic brain injury, Chang *et al*[158] demonstrated that the priming of MSCs with hypoxia improved their therapeutic function, and resulted in an amelioration of neurogenesis, and motor and cognitive functions. Moreover, in a rat model of spinal cord injury, hypoxic MSCs were also able to increase axonal preservation and decrease apoptosis[159].

Principal priming strategies for stimulating tissue regeneration

MSCs are involved in tissue homeostasis, which is necessary for physiologically coordinating regeneration/repair of tissue, also after injury[3,6,36]. Thus, the use of MSCs in regenerative therapies is garnering great interest due to their potentially numerous clinical applications.

In the complex process of cutaneous wound healing, a central role is played by fibroblasts, which contribute, through the interaction with surrounding cells, to the production of ECM, glycoproteins, adhesive molecules, and various growth factors[160]. Recent evidence suggests that CM produced by primed MSCs from different sources, such as bone marrow[120], adipose tissue[160], amnion fluid[161], and placenta[162] enhanced the migration and proliferation of fibroblasts *in vitro*, and accelerated wound healing in *in vivo* models (Table 1). In all these cases, hypoxia treatment represented the chosen priming strategy for driving MSCs in increasing secretion of various angiogenic factors, cytokines, and chemokines. Therefore, the priming of MSCs with hypoxia might well represent the main approach to improving the therapeutic effects of MSCs to be applied in the stimulation of tissue regeneration (Figure 2). This idea has also been supported by other studies (Table 1). Indeed, in both hepatectomized mouse and rat models, it has been demonstrated that hypoxic MSCs produce crucial functional molecules, including HGF and VEGF, which were considered responsible for the induction of liver regeneration[163,164]. Kuo *et al*[165] showed that systemic infusion of MSCs restored liver function and promoted liver regeneration in rodents. In this regard, in a rat massive hepatectomy model, Yu *et al*[164] found that hypoxia-conditioned MSCs secreted significantly more VEGF than normoxia-conditioned cells, and the infusion of primed MSCs promoted proliferation of hepatocytes and liver regeneration. Several studies have focused on the signalling pathways up-regulated by MSC during liver regeneration. Lee *et al*[163] using a partially hepatectomized mouse model, found that treatment with hypoxic MSC-derived CM increased the viability of hepatotoxic hepatocytes, and enhanced liver regeneration through JAK/STAT3 signalling. These data were also confirmed by Lee *et al*[166], who confirmed the activation of JAK/STAT3 signalling induced by MSC CM during mouse liver generation. Hypoxic MSCs that secrete high level of VEGF were also able to regenerate pulp-like tissues and vasculature similar to the native pulp in a rat model of pulp repair[167]. HGF and VEGF produced by hypoxic MSCs were considered by Chang *et al*[158] to be responsible for improvement of neuronal proliferation. Moreover, Zhilai *et al*[159] demonstrated that both HGF and VEGF produced by hypoxia-primed MSCs facilitated axonal survival in a rat model of spinal cord injury. Han *et al*[140], in a murine hindlimb ischemia model, found that the expression levels of EGF, VEGF, fibroblast growth factor, and HGF were significantly higher in ischemic tissue treated with hypoxic MSCs, where an improvement of neovascularization was observed. The efficacy of hypoxic MSCs was also tested in reducing scar formation and inducing wound healing in various *in vivo* models[120,162,168].

Despite the fact that the principal MSC priming strategy used for both *in vitro* and *in vivo* regeneration experiments was hypoxia treatment, 3D culture of MSCs as priming strategy has also been investigated in tissue regeneration (Figure 2). In fact, MSC spheroids have also shown therapeutic abilities with regard to both bone and cartilage regeneration. In particular, it has been found that treatment with MSC spheroids was effective in inducing disc repair in an *in vivo* model of disc degeneration, bone regeneration in an *in vivo* model of bilateral calvarial defects, and cartilage regeneration in an *in vivo* model of osteochondral defects[169-171].

CONCLUSION

The therapeutic effects of MSCs have been demonstrated in both *in vitro* and *in vivo* studies. Nevertheless, due to their heterogeneity related mainly to tissue source, which can impact MSC functional properties[85,172], the application of MSCs in clinical trials has shown moderate or poor efficacy. MSCs are considered key regulators of tissue repair and, in this case, different stimuli are crucial in modulating the functional properties of these cells. In fact, it is believed that inflammation and low oxygen levels are essential signals for triggering MSC activity in a suitable manner. Moreover, it has recently been shown that different priming approaches can eliminate the functional heterogeneity of MSCs[173]. Therefore, specific priming strategies have been implemented to improve the regenerative and immunomodulatory properties of MSCs. In this review, we have explored data regarding the principal priming approaches used to enhance the therapeutic potential of MSCs. The above-mentioned data underscore that several factors play a role in the ability to modify MSC properties. Moreover, some therapeutic effects, on different disease models, can be obtained in relation to dose and/or combination of the priming factors used.

Several diseases have in common tissue injury and repair processes, in which inflammation plays a central role in coordinating different pathways that regulate tissue regeneration and functional recovery. Indeed, after acute injury, a low level inflammation (acute inflammation) occurring after specific triggers, is crucial in stimulating wound healing and tissue repair, facilitating the resolution of inflammation and restoring tissue structure/function (inflammation drives regeneration). On the other hand, in the case of abnormal damage repair, chronic unregulated inflammation can lead to pathological processes, including hormonal metabolic changes, which culminate in the onset of specific diseases, including cancer and fibrosis[174,175]. Therefore, the regulation of both acute and chronic inflammation is essential for a proper restorative response and, in this scenario, MSCs can have a crucial physiopathological role. In fact, it has been shown that when MSCs coordinate damaged tissue for repair, they undergo local stimuli such as inflammatory cytokines, and hypoxia, which in turn boost and direct the reaction of MSCs to orchestrate tissue regeneration[85,176]. In Figure 3, we depict a hypothetical model that occurs during physiopathologic tissue injury and repair. In this model, MSCs are activated differently by various microenvironment stimuli to manage tissue functional recovery. One of the first factors that arises after tissue injury is the establishment of a hypoxic and weakly inflammatory microenvironment, which in turn activates local cells to protect/regenerate tissues[3,177]. Hypoxia rapidly up-regulates the level of intercellular adhesion molecule-1 in local-inflamed endothelium, promoting MSC migration to injured tissues[178,179]. Moreover, a mild inflammation may stimulate MSCs to release chemokines for attracting immune cells and amplifying immune responses[180]. Once MSCs reach the site of injury, the paracrine properties of MSCs to release chemotactic and angiogenic factors is significantly amplified under hypoxic conditions[181]. In this case, naïve MSC are activated to recruit neutrophils and stimulate the formation of new blood vessels. Neutrophil action is followed by monocyte/macrophage activity that ensures sustained release of pro-inflammatory cytokines and potentiation of the fibroproliferative response[182,183]. If these processes are not adequately regulated, a state of chronic inflammation occurs. Thus, cytokines such as IFN-γ, TNF-α, and IL-1 accumulate in the injured tissues, and the inflammatory environment becomes central in affecting the regulatory role of MSCs that exhibit immunosuppressive capacities[184]. The MSC phenotype is switched into a lower regenerative potential and a higher anti-inflammatory phenotype (Figure 3). Thus, high amounts of pro-inflammatory cytokine confer a dramatic immunomodulatory ability to MSCs[40,91,124,125,185,186] which, in turn, act as a homeostatic regulator to control the inflammatory response. Overall, this scenario describes what occurs when MSCs are exposed to low levels of both oxygen and inflammation, and their phenotype is potentially inclined to low immunomodulation and high stimulation of tissue regeneration. Otherwise, high levels of inflammation can imprint a MSC phenotype inclined toward high immunomodulation and weak stimulation of tissue regeneration (Figure 3). In this regard, Vigo *et al*[87] found that IFN-γ can orchestrate MSCs functions in a dose-manner, and this is reflected in the opportunity to modulate MSC properties before their use in clinical practice. In addition, considering the heterogeneous immune regulatory functions of MSCs due to intrinsic characteristics of individual clones, the priming of MSCs with pro-inflammatory factors can equally amplify immune therapeutic properties of MSCs, and eliminate the variances among different MSC clones[173].

Priming with inflammatory signals polarizes MSCs toward an anti-inflammatory and pro-trophic phenotype allowing, on the one hand, the regulation of inflammatory responses, and on the other the final remodelling and recovery of damaged tissue. Likewise, different priming strategies can be used to direct the therapeutic effects of naïve MSCs toward specific pathological processes. As also highlighted by the studies we have noted in this review, while hypoxic priming of MSCs could be used mainly to treat acute disease, to principally stimulate angiogenesis and tissue regeneration, inflammatory cytokines could be used mainly to prime MSCs for treating chronic immune-related disorders. The change of perspective from regeneration to inflammation implies in the MSCs the shift in the production of functional factors that stimulate regenerative or anti-inflammatory pathways (Figure 2). Interestingly, the 3D culture of MSCs as priming strategy appears to be an intermediate functional priming between the two mentioned above. The production of priming type-specific functional factors in MSCs could well pave the way for optimizing their therapeutic potential, aimed at a greater effectiveness as an

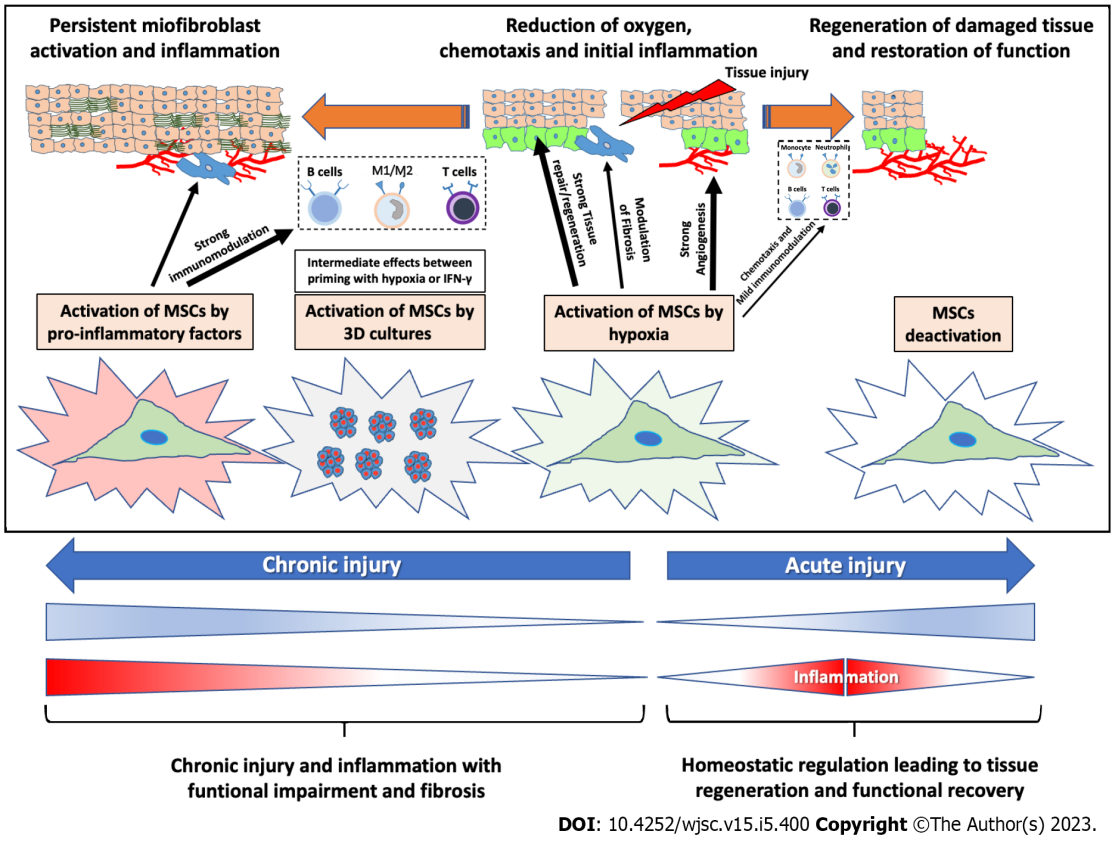

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Figure 3 Schematic illustration of the physiological role and biological action of mesenchymal stromal/stem cells primed *in vivo* in a model of tissue injury and repair. During tissue injury and repair, mesenchymal stromal/stem cells (MSCs) are differently activated by various microenvironment stimuli to orchestrate tissue repair and functional recovery. First, naïve MSC activation (hypoxic activation) leads to the release of both angiogenic factors and chemokines, which stimulate the formation of new blood vessels, the recruitment of neutrophils, and the expression of adhesion molecules. Neutrophil action is followed by macrophage activity, which ensures sustained release of pro-inflammatory cytokines, and potentiation of the fibroproliferative response. If this process is not adequately regulated, a state of chronic inflammation occurs; the MSC phenotype is switched into an anti-inflammatory phenotype. MSCs: Mesenchymal stromal/stem cells; 3D: Three-dimensional.

advanced therapy medicinal product.

FOOTNOTES

Author contributions: Miceli V collected the literature, prepared illustrative materials, and wrote the original draft; Miceli V, Zito G, Bulati M, Gallo A, Busà R, Iannolo G, and Conaldi PG wrote, reviewed, and edited the draft; Miceli V and Conaldi PG supervised the manuscript; and all authors have read and agreed to the published version of the manuscript.

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REVIEW

Communication between bone marrow mesenchymal stem cells and multiple myeloma cells: Impact on disease progression

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Abstract

Multiple myeloma (MM) is a hematological malignancy characterized by the accumulation of immunoglobulin-secreting clonal plasma cells at the bone marrow (BM). The interaction between MM cells and the BM microenvironment, and specifically BM mesenchymal stem cells (BM-MSCs), has a key role in the pathophysiology of this disease. Multiple data support the idea that BM-MSCs not only enhance the proliferation and survival of MM cells but are also involved in the resistance of MM cells to certain drugs, aiding the progression of this hematological tumor. The relation of MM cells with the resident BM-MSCs is a two-way interaction. MM modulate the behavior of BM-MSCs altering their expression profile, proliferation rate, osteogenic potential, and expression of senescence markers. In turn, modified BM-MSCs can produce a set of cytokines that would modulate the BM microenvironment to favor disease progression. The interaction between MM cells and BM-MSCs can be mediated by the secretion of a variety of soluble factors and extracellular vesicles carrying microRNAs, long non-coding RNAs or other molecules. However, the communication between these two types of cells could also involve a direct physical interaction through adhesion molecules or tunneling nanotubes. Thus, understanding the way this communication works and developing strategies to interfere in the process, would preclude the expansion of the MM cells and might offer alternative treatments for this incurable disease.

Key Words: Multiple myeloma; Mesenchymal stem cells; Bone marrow microenvironment; Soluble factors; Extra-cellular vesicles; Cells adhesion molecules; Tunnelling-

nanotubes

Core Tip: Mesenchymal stem cells (MSCs), the main cell population of the bone marrow (BM) stroma, can influence BM microenvironment through their paracrine activity, involving both soluble factors and extracellular vesicles, but also through direct communication. Being the BM the predominant localization of multiple myeloma cells (MM), finding the appropriate conditions at this niche, is key for the survival and expansion of tumour cells and thus, for the progression of the disease. Since the activity of BM-MSCs could determine the fate of MM cells at BM, these cells could be interesting targets for the design of new antitumor drugs.

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INTRODUCTION

Multiple myeloma (MM) is one of the most common hematological diseases, only second to non-Hodgkin lymphoma[1]. MM affects mainly older adults, with the median age of diagnosis being around 69 years. Only in 2020, 32270 new cases and 12830 deaths in the United States were estimated by the American Cancer Society Statistics Centre. In global terms, the cases would reach 160000, accounting for 0.9% of all cancer diagnosis. Importantly, incidence of MM has risen 126% globally, and hence, there is an increasing need to find new effective treatments for this incurable disease[2,3].

Besides the initial treatments for MM, consisting in alkylating agents often combined with corticosteroids, the last couple of decades have seen an important advance in the available treatments for this disease. We first saw the introduction of proteasome inhibitors (Bortezomib), histone deacetylase inhibitors (Panobinostat) and drugs such as Selinexor, with a nuclear export inhibition activity. In recent years monoclonal antibodies such as Daratumumab (anti-CD38) or Elotuzumab (anti-SLAMF7), and more recently the use of chimeric antibody receptor (CAR) T-cell products, has introduced immunotherapy as a viable approach to MM treatment[4]. According to data from the National Cancer Institute (Bethesa, MD, United States), all these treatments have had a deep impact on patients' survival, substantially raising the survival rate to 55% in the period between 2011 and 2017. More recently, the use of small molecules, with a molecular weight smaller than 1kDa, has also improved treatments, since it offers important advantages compared to the former therapies, as the easy cell entry, the simplicity of the molecules, and a much lower production cost than other drugs[5]. However, despite these advancements, there are still limitations to existing treatment options. Some patients may not respond to or may develop resistance to certain medications, many patients can become refractory to treatment and thus, there is a high risk of relapse. This promotes the search for new treatments to handle relapsed or refractory MM.

MM is caused by aberrant plasma cells (PC) proliferation in the bone marrow (BM). The premalignant states, known as monoclonal gammopathy of undetermined significance (MGUS) and smoldering MM, transition under specific conditions to the malignant state of PC leukemia or extramedullary myeloma [6]. A key characteristic of MM is the infiltration into and the colonization of the BM, one of the two primary lymphoid organs[7]. This colonization produces typical lytic bone lesions that would be present in approximately 80% of patients with newly diagnosed MM and are the major source of morbidity[8]. The bone lesions, resulting from the stimulation of bone resorption by B-cell plasmacytomas, are associated with hypercalcemia and often, severe bone pain and bone fractures[8,9].

While the initiation of a tumor mainly depends on the accumulation of genetic defects, the transition from a premalignant to a malignant state highly relies on the interaction of the tumor cell with a permissive microenvironment that would support the malignant transformation and the proliferation of the tumor cells, aiding them to evade apoptosis. The relevance of tumor microenvironment in disease progression was first discussed in the "seed and soil hypothesis" formulated by Stephen Paget in 1889, where the establishment of tumor metastatic sites is influenced by the cross-interaction between the seeds (cancer cells) and the soil (a particular microenvironment)[10]. This is not different in MM[11,12]. The progression to MM, which would occur in approximately 50% of patients diagnosed with MGUS [6], requires multiple genomic events, but also a permissive BM microenvironment[13]. MM cells proliferate almost exclusively within the BM niche, highlighting the role of this microenvironment in

supporting cancer growth. In fact, there is also mounting evidence indicating this BM microenvironment is not only key for PCs survival, but also has a crucial role in resistance to treatment and disease recurrence[14,15].

The MM cells infiltrating the BM will encounter a complex microenvironment formed by cellular and non-cellular components. Amongst the non-cellular components influencing the BM microenvironment, it is important to consider the extracellular matrix (ECM) proteins as well as a milieu of cytokines, chemokines, and growth factors. Many of these factors can have a positive effect on MM cells, boosting their proliferation and survival and the resistance to different types of drugs. A good example of these cytokines supporting MM progression are interleukin (IL)-6 and ligands of the B-cell maturation antigen, such as a proliferation-inducing ligand and B-cell activating factor (BAFF)[16,17]. Regarding the cellular components of the BM niche, many different types of bone cells (osteoblasts, or bone forming cells, and osteoclasts, or bone resorbing cells, and osteocytes) and cells from the immune system (macrophages, natural killer cells and regulatory T-cells) share this niche. Other cells present here are fat cells (adipocytes), fibroblasts, endothelial cells and two multipotent stem cells, BM mesenchymal stem cells (BM-MSCs), which differentiate into different mesodermal cell lineages, and hematopoietic stem cells (HSCs), that would differentiate into hematological lineages, including the myeloid lineage that would give rise to osteoclasts. MM cells are likely to interact with all the cells in the BM niche and elicit mutual influence[18]. In fact, it is known that communication between MM cells and BM-MSCs is essential in the progression of MM[19]. Once MM cells infiltrate the BM, their presence in the BM niche alters the activity of many of the cells found there, including those involved in bone homeostasis such as osteoclasts[20,21] and osteoblasts[22-24]. While in normal bone homeostasis, the activities of osteoblast and osteoclasts are carefully balanced to ensure a correct bone regeneration, the influence of MM cells disrupts this balance increasing both the resorptive activity of osteoclast and their numbers, and decreasing osteoblasts numbers as well as their osteogenic capacity[25], overall leading to an increase in bone destruction and the appearance of the aforementioned osteolytic lesions typical of this disease. Other cells at the BM niche which activity is highly influenced by MM cells are BM-MSCs. The presence of MM cells at the BM niche alters the MSCs behavior in different ways. In fact, changes in the expression of certain microRNAs (miRNAs) in BM-MSCs leading to important alterations of their secretory profile and osteogenic differentiation potential have been observed after co-cultivation of BM-MSCs and MM cells[26,27]. These changes at the BM niche upon MM invasion produce a microenvironment that would support disease progression. Indeed, there is strong evidence indicating that is precisely this interaction what leads to the formation of the lytic bone lesions[28]. One of the characteristics of this permissive microenvironment is the high presence of pro-inflammatory cytokines that would favor the progression of neoplasia[29]. The crosstalk between MM cells and the BM-MSCs at the BM niche is key to sustain this pro-inflammatory microenvironment and thus, to allow MM cell persistence and growth[30]. It is important to clarify, that this pro-inflammatory microenvironment would be the result of the action not only of the infiltrated MM cells but also of other cells residing at the BM niche, including BM-MSCs.

MSCs, have a key role in regulating the BM microenvironment through their paracrine activity, but also through direct cell-to-cell interaction. Regarding their paracrine activity, these cells produce a plethora of soluble biomolecules and vesicular components, known altogether as "secretome", that exert multiple actions on other cells at the BM microenvironment[31]. BM-MSCs role in MM disease development and progression has been reported as having both inhibitory[32] and supportive roles[33,34]. Sadly, the latter is the most frequent. Once at the BM niche, MM will exert their influence on resident MSCs, altering their signaling and gene expression pattern and thus, also their secretion pattern. After interaction with MM cells, MSCs will produce a secretome rich in pro-inflammatory cytokines. In fact, it has been previously described how MSCs react to IL-1 produced by the myeloma PCs by producing large quantities of IL-6, a cytokine that would in turn stimulate the survival of the MM cells[35,36]. Therefore, the soluble part of this secretome has a key role in the progression of tumor. Moreover, in the last few years, several molecules (miRNAs) that are present in the cargo in the extracellular vesicles (EVs) produced by BM-MSCs upon MM cells stimulation also seem to have a key role in the disease promotion. Although the soluble proteins and EVs produced by the BM-MSCs are the main actors in the communication between BM-MSCs and MM cells, other ways of communication have also been implicated. This will be discussed in the following sections.

Current available treatments for MM patients mainly target MM cells but have none or limited effect on other cells in the BM or de BM microenvironment. Knowledge of the different interactions between BM-MSCs and MM cells is key to understand how MM cells behave and grow within the BM and how osteolytic lesions are formed. In this work, we will address key aspects of the different ways of communication between MSCs and MM cells as well as the outcome of this crosstalk.

SOLUBLE FACTORS IN THE COMMUNICATION BETWEEN BM-MSCs AND MM CELLS

The multiple cellular interactions taking place in the BM, make this microenvironment a dynamic compartment with a myriad of soluble factors that would affect the behavior of the various cell types

concurring at that microenvironment. Although many of those cells have paracrine activity, BM-MSCs are the ones that have a stronger impact in the BM microenvironment due to the wide variety of soluble and non-soluble factors secreted by these cells. Various constituents of the, so called, BM-MSC secretome orchestrate the fate of the MM cells, from the first step encompassing the homing of those cells to the BM, onwards.

Role of soluble factors in the homing of MM cells to the BM

A key factor in the communication between BM cells and MM cells during the first stages of BM colonization, is the cytokine stromal cell derived factor 1α (SDF1α), also known as CXCL12. This factor, produced by BM-MSCs, works as a chemoattractant, being responsible of the homing of HSCs to the BM once they abandon the fetal liver during development[37]. SDF1α activity is mediated by the binding to a specific G-protein 7-span transmembrane receptor (CXCR4) at the target cells. CXCR4 is expressed at the surface of different cells in the BM microenvironment[38], and also at the surface of MM cells and other tumor cells[39]. Thus, SDF1α/CXCR4 interaction might have a relevant role in directing de metastasis of hematopoietic malignancies. Similar to its effect on HSCs, the interaction of SDF1α with its receptor at the MM cells, increases their migration, homing and adhesion towards the BM, in fact, knock down of CDCR4 in BM-MSCs or the use of the CXCR4 inhibitor AMD3100 (AnorMED), that blocks the binding of SDF1α to its receptor[40], seems to inhibit the migration of MM towards the BM[41]. The binding of SDF1α to its receptor at the MM cells, also triggers the activation of the phosphatidylinositol 3-kinase (PI3K) and the MAPK kinase (MEK)-extracellular signal regulated kinase (ERK, MEK/ERK) pathways, inducing a rearrangement in the cytoskeleton of MM cells that facilitates BM colonization [41]. SDF1α has also been described to act in a more indirect way, not mediated by the binding to CXCR4. SDF1α interacts with other molecules including matrix metalloproteinases (MMPs), integrins or growth factors such as hepatocyte growth factor (HGF), insulin like growth factor-1 (IGF-1) or molecules of the GTPases family. All of these effects elicited by SDF1α, in one way or another, lead to a promotion in MM cells migration, homing or adhesion into the BM[38].

Role of soluble factors in the promotion of proliferation and MM cell survival

Many of the factors secreted by BM-MSCs and by other cells of the BM microenvironment, activate key signaling pathways in the MM cells that would increase their chances to survive and proliferate in the BM microenvironment. A summary of these factors as well as the signaling pathways involved in this communication are shown in Figure 1. In fact, some mutations activating those pathways have also been found in patients with MM. We will address some of those key pathways in this section.

Once in the BM, for the tumor to progress further, MM cells would need a permissive microenvironment. This microenvironment would be created by multiple soluble factors secreted by the different cell types present at the BM. The soluble factors produced by the BM-MSCs seem to be the main, but not the only, effectors of the changes elicited in the MM cells. Besides SDF1α, BM-MSCs seem to secrete other important soluble factors such as IL-6, IL-17, vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), tumor necrosis factor-α (TNF-α), BAFF or leukemia inhibitory factor-1; osteoclasts mainly secrete IL-6 and VEGF; and vascular endothelial cells secrete cyclophilin-A[42,43]. These factors will activate specific signaling pathways in the MM cells such as PI3K/Akt, MEK/ERK, Janus kinase 2 (JAK2)-signal transducer and activator of transcription 3 (STAT3, JAK2/STAT3) pathways, related to cell survival, proliferation and drug resistance[43]. It is important to highlight that this communication is bi-directional, since MM cells would also produce cytokines such as IL-1β, VEGF, and transforming growth factor-beta (TGF-β) that would exert their effect on BM-MSCs, activating the nuclear factor kappa-B (NFκB) pathway and thus, inducing further secretion of cytokines by the BM-MSCs into the BM microenvironment, particularly IL-6[44,45].

IL-6 is the main activator of the JAK2/STAT3 pathway, known to be implicated in the pathogenicity of cancer. JAK2/STAT3 pathway activation promoted by IL-6 leads in MM cells to the expression not only of potent proto-oncogenes such as c-myc and cyclin D1, but also of anti-apoptotic genes like Mcl-1, Bcl-XL and Bcl-2. Moreover, STAT3 activation has also a immunosuppressive effect since it regulates T-cell mediated cytotoxic immune response[46], contributing to the establishment of a immunosuppressed microenvironment that would contribute to the survival and proliferation of the MM cells in the BM. On the other hand, IL-6 activation of JAK2/STAT3 pathways, also has an important role in bone destruction, a hallmark of MM. IL-6/JAK2/STAT3 axis induces the expression of the receptor activator of NFκB ligand (RANKL)[36,47] whose binding to its receptor at the surface of pre-osteoclasts, promotes their differentiation towards mature osteoclasts, activating bone resorption and thus, promoting the formation of osteolytic lesions.

It is important to highlight that the NFκB signaling pathway also has an important role in the survival of MM cells and in the maintenance of the tumorigenic microenvironment at the BM. Both canonical and non-canonical NF-kB pathways are activated by different factors present in the BM microenvironment, including IL-6, IGF-1, TNF-α or BAFF[48]. While IGF-1 is able to activate NFκB pathway, inducing the expression of anti-apoptotic, caspase-8 inhibitors FLIP and cIAP-2[49], TNF-α has a pro-survival effect through NFκB pathway mediators such as NFκB (NEMO) and IκB kinase subunit 2[44]. On the other hand, BAFF activates NFκB non-canonical pathway upregulating the expression of antiapoptotic proteins including Mcl-1, Bcl-XL, Bcl-w and Bcl-2[50]. There is also evidence indicating that IL-6 is

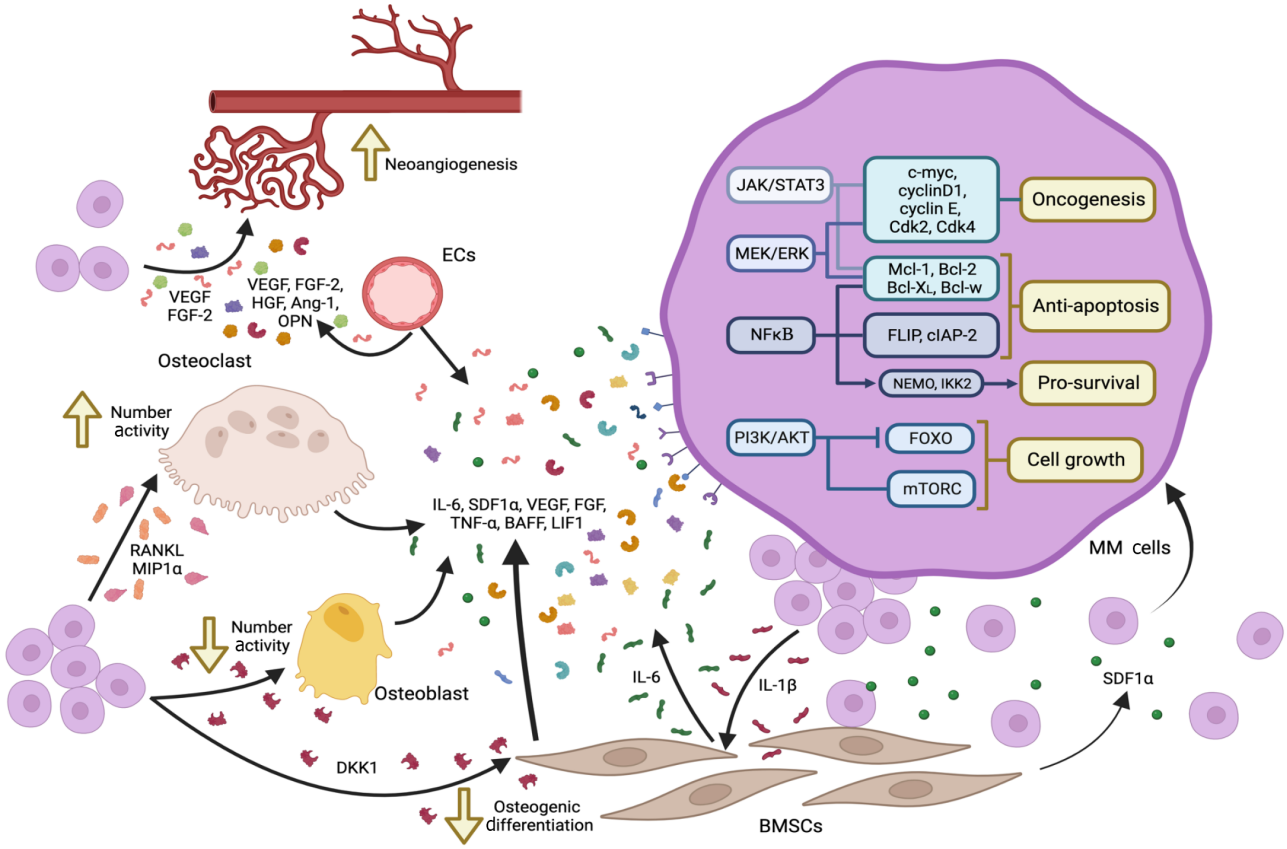

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Figure 1 Schematic representation of the main factors involved in the bidirectional communication between multiple myeloma cells and cells in the bone marrow microenviroment (bone marrow mesenchymal stem cells, osteoclasts, osteoblast, *etc.*). The main signaling patwthays activated by these factors are also depicted (Created with Biorender.com). VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factors; HGF: Hepatocyte growth factor; OPN: Osteopontin; ECs: Endothelial cells; IL: Interleukin; SDF1α: Stromal cell derived factor 1α; TNF-α: Tumor necrosis factor-α; BAFF: B-cell activating factor; DKK-1: Dickkopf-1; MM: Multiple myeloma; BM-MSC: Bone marrow mesenchymal stem cells; JAK: Janus kinase; STAT3: Signal transducer and activator of transcription 3; NFκB: Nuclear factor kappa-B; PI3K: Phosphatidylinositol 3-kinase; RANKL: Receptor activator of NFκB ligand; Ang-1: angiopoietin-1; MEK: MAPK kinase; ERK: Extracellular signal regulated kinase; LIF1: Leukemia inhibitory factor-1. Created with BioRender.com.

linked to the expression of VEGF in MM cells, being some of the VEFG isoform expression driven by the NFκB pathway[51,52].

The MEK/ERK pathway is the signaling pathway most found activated in MM patients, with a prevalence in between 43% and 53% of the patients[53]. Changes in MEK/ERK pathway have important effects in cell cycle, due to the alteration in the expression of molecules such as cyclin D1, cyclin E, Cdk2 and Cdk4 and in apoptosis prevention by the induction of the phosphorylation of the pro-apoptotic protein Bim. This phosphorylation results in the release of anti-apoptotic molecules such as Mcl-1, Bcl-XL and Bcl-2, also related to Akt pathway[54]. In the absence of mutations that activate this pathway, the stimulation of the MEK/ERK pathway in the MM cells might also occur by the action of different soluble factors present in the BM microenvironment such as BAFF, IL-6, SDF1α, VEGF or TNF-α among others[42]. As with other relevant signaling pathways that become activated in MM, the MEK/ERK is also studied as a potential therapeutic target.

PI3K/Akt signaling pathway also has a relevant role in cell proliferation, cell cycle and apoptosis. Alteration of the PI3K/Akt/mTOR pathway due to genetic modifications or its hyper-activation contributes to carcinogenesis, metastasis, invasion, proliferation and drug resistance of tumor cells. However, no activating mutations have been described in MM cells yet. Despite this fact, PI3K/Akt/mTOR pathway is important for MM cells survival[55,56].

Role of soluble factors in angiogenesis and bone homeostasis

Up to this point, we have mentioned some of the effects of the pro-tumorigenic microenvironment in the BM on the MM cell survival and growth. However, once modified by the BM microenvironment, MM cells will start to release different soluble factors that will not only perpetuate that tumorigenic microenvironment, but also will have a deep impact in angiogenesis and bone homeostasis.

Neovascularization in the bone is an essential feature for MM progression and the presence of high density of micro-vessels in the BM microenvironment is characteristic in MM. Cells residing at BM, such as BM-MSCs, osteoblasts, HSCs, or endothelial precursor cells, commonly express various angiogenic

factors, such as VEGF, FGF-2, TNF-α, HGF, IL-6, BAFF, SDF-1α, angiopoietin-1 or osteopontin (OPN). Also, MM cells are able to directly produce VEGF stablishing a VEGF autocrine loop where the produced VEGF would stimulate MM cells proliferation through the MEK-1/ERK pathway[57,58]. FGF-2 is another key pro-angiogenic molecule that would be produced by both MM cells and BM-MSCs[59]. However, contrary to VEGF, which is produced by all MM cells, FGF-2 production by MM does not seem to be a general feature in all MM cases[59]. Other molecules with pro-angiogenic activity such as MMPs[60,61] or OPN, also produced by MM cells, have also a relevant role in promoting micro-vessels formation in the BM microenvironment. The overall increase in the production of such angiogenic factors is elicited by the MM cells. The activation of angiogenesis linked to tumor progression is known as "angiogenic switch"[62] .

Bone homeostasis is a dynamic process driven by osteoclasts, osteoblast and osteocytes. Alterations in the balance between these cell types will lead to the remodeling of the bone. The characteristic bone lesions found in MM derive from the disruption of bone homeostasis initiated by the activation of JAK2/STAT3 pathway by IL-6 and the subsequent induction of RANKL expression by MM cells. Not only this but, as will be discussed later, cell-to-cell interaction of MM cells with BMSCs also induce the expression of the macrophage inflammatory protein (MIP)-1α[63]. Both RANKL and MIP-1α are mediators in the bone destruction driven by MM as they have an both in the number of osteoclasts and in their activity. MIP-1α is a chemoattractant for osteoclasts and stimulates osteoclast formation[64], while RANKL after being recognized by its receptor RANK, will induce the commitment of the macrophage/monocyte precursor cells to the osteoclast lineage[65].

Secreted by MM cell in response to the activation of the JNK pathway, Dickkopf-1 (DKK-1) is also a disruptor in bone homeostasis[66]. DKK-1 is an extracellular inhibitor of the Wnt pathway. DKK-1 interacts with membrane receptors as transmembrane proteins Kremen 1/2 and the human low-density lipoprotein receptor-related protein 5/6, thus competing with Wnt[67]. As one of the main regulatory pathways for osteogenic differentiation of BM-MSCs into osteoblasts[68], the inhibition of the Wnt/β-catenin pathway by DKK-1 will result in a reduced number of osteoblasts. By the action of these factors, RANKL, MIP-1α and DKK-1, the balance between bone formation and bone resorption driven by osteoblasts and osteoclasts is disrupted, resulting in the characteristic bone lesions present in MM patients.

A table summarizing the latest scientific evidence regarding key factors involved in MM/BM-MSCs communication and their effect is shown (Table 1).

EVs-MEDIATED COMMUNICATION BETWEEN BM-MSCs AND MM CELLS

Under non-pathological conditions, BM homeostasis is maintained by cell-to-cell contact, soluble molecules, and EVs. Whereas, over the years solid evidence has accumulated about the relevance of the first two, the involvement of EVs-mediated communication in the maintenance of BM homeostasis has started to be contemplated only in the last few decades[69]. Despite being a fairly new field, important advances have been made in the knowledge of EVs, such as their classification, in terms of their size and biogenesis, into three major categories (exosomes, micro-vesicles, and apoptotic bodies) and the fact that its content varies according to the state of their parental cells[31].

As we have previously discussed, MM cells have the capacity to alter the environment in which they reside[70] as well as the characteristics of cells present in that microenvironment. Thus, it is not surprising that the EVs produced by MM cells also play a key role in disease progression. In fact, it has recently been shown that, exosomes (a particular class of EVs) produced by both BM-MSCs and MM cells are largely responsible for MM pathogenesis[71]. This recent demonstration of the relevance of EVs in MM progression has resulted in several studies in the lats few years, however, the multitude of agents and interactions involved in the development and progression of this disease has made it difficult to fully understand the molecular mechanisms involved. In this section we aim to gather the available information so far.

Effect of MM-EVs on the BM-MSCs and bone homeostasis

As previously mentioned, osteolysis, one of the main hallmarks of MM disease, is linked to the negative effect of MM cells on cells responsible for bone homeostasis, such as MSCs, osteoblasts and osteoclasts [72]. In particular, myeloma bone disease (MBD) has a unique feature compared to other diseases that encompass bone destruction, since in MBD osteoblast activity is also severely impaired[24]. Several authors have suggested that an essential part of this bone damage is related to EVs directly produced by MM cells (MM-EVs). Zhang *et al*[73] demonstrated that the cargo of MM-EVs was enriched in various molecules which negatively regulate osteogenesis. They confirmed that MM-EVs induced high expression of miR-103a-3p in BM-MSCs, which led to impaired osteogenesis *in vitro*. Moreover, they showed that injection of MM-EVs in mouse tibia resulted in defective bone formation. Interestingly, *in vitro* assays also revealed that MM-EVs were also able to influence MM cells increasing viability and IL-6 production, known to regulate MM cell proliferation thus, establishing an autocrine feedback. MM-EVs also increased miR103a-3p expression in MM cells however, in those cells the increased prolif-

Table 1 Summary for the key soluble factors involved in multiple myeloma/bone marrow mesenchymal stem cells communication

Soluble factors	Origin	Function	Ref.
SDF1α	BMSCs	Chemoattractant of MM cell towards the BM microenvironment	[38]
IL-1β	MM cells	Act over BMSCs inducing the secretion of soluble factors, mainlyIL-6	[45]
IL-6	BMSCs	Closely related with cancer pathogenicity due to it proto-oncogenic and anti-apoptotic effect over MM cells	[46,47]
		Immunosuppressive effect over T cells	
		Also related with bone destruction by inducing the expression of RANKL by the MM cells	
VEGF	BM cells, MM cells	Promotes bone neovascularization, essential for tumour progression	[58]
RANKL	MM cells	Induce the commitment of the macrophage/monocyte precursor cells to the osteoclast lineage. Promoting indirectly bone destruction	[63]
DKK-1	MM cells	Disruptor of bone homeostasis by inhibiting BMSCs differentiation into osteoblasts	[66,67]

SDF1α: Stromal cell derived factor 1α; BM-MSC: Bone marrow mesenchymal stem cells; MM: Multiple myeloma; IL: Interleukin; RANKL: Receptor activator of NFκB ligand; VEGF: Vascular endothelial growth factor; DKK-1: Dickkopf-1.

eration of MM cells after exposures to MM-EVs does not seem to be related to miR103a-3p but to other miRNAs also present in the MM-EVs cargo, such as miR107 and miR181a-3p[24].

Among the different biomolecules found as part of the exosome cargo, long non-coding RNAs (lncRNAs) and miRNAs have been the focus of attention due to their key regulatory roles. Various miRNAs found in MM-EVs have been studied for their involvement in the disruption of osteogenesis. miR-129-5p was identified as a player in vesicle-mediated bone disease[74]. In particular, miR-129-5p seemed to inhibit the transcription factor specificity protein 1, leading to a reduction of ALPL, both at the mRNA and protein levels, during the early osteogenic differentiation of MSCs. On the other hand, the long non coding RNA Long Intergenic Non-Protein Coding RNA 461, found as part of the MM-exosomes cargo, has also been found to inhibit osteoblast differentiation by reducing the activity of Wnt/β-Catenin pathways, responsible for osteoblast proliferation, differentiation and activity[75]. Other molecules, such as soluble proteins present in the MM-EVs cargo also showed anti-osteogenic activity Faict *et al*[72] revealed that Wnt/β-Catenin inhibitor DKK-1 is present in MM-EVs and observed a lower expression of Osterix (OSX), Collagen 1A1 and alkaline phosphatase in differentiated MC3T3-E1 cells after MM-EVs treatment.

Runx2 is the master regulator of early osteogenic differentiation, and therefore a possible target for the anti-osteogenic effect of MM-EVs. In fact, lncRNA RUNX2-AS1 present in the MM-EVs cargo was identified as a bioactive molecule able to reach MSCs and form a transcriptionally repressed RNA duplex with RUNX2 premRNA, reducing the osteogenic activity[76]. In addition, a MM-EVs impact in osteoblastic differentiation through reduction of Runx2, together with OSX and OCN, has been described by Liu *et al*[77]. These authors also record increased levels of IL-6 secretion *via* APE1/NF-kB which, as aforementioned, is an important survival factor of MM cells.

Once the EVs produced by MM cells reach the BM-MSCs, their cargo modifies the BM-MSCs behaviour in the benefit of MM cells**.** A clear example of this is miR-146a which acts in a positive loop to favor disease progression[19]. Once this miRNA targets BM-MSCs, it produces an increase in the secretion of several cytokines and chemokines from those cells, including CXCL1, IL-6, IL-8, inducible protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), and CCL-5, which, in turn, once released into the BM microenvironment, would favor MM cell viability and migration. In addition, MM-EVs cargo miR-146a and miR-21, participate in proliferation and transformation of MSCs into cancer associated fibroblasts (CAFs). This is a type of cell which could contribute to a tumour-supportive microenvironment through secretion of cytokines, including IL-6 and TGF-β[78].

Interestingly, it has been shown that conventional chemotherapeutic agents including melphalan, and anti-proteases such as bortezomib and carfilzomib can stimulate a considerable MM-EVs release. The EVs produced under these circumstances are called "chemoexosomes". These chemoexosomes are characterized by the high presence of the heparanase enzyme in their surface. This heparanase is implied in several cellular changes leading to chemoresistance and the subsequent relapse of the patient. Heparanase EVs content is delivered in MM cells and activate ERK pathway as well as TNF-α production by macrophages, matrix degradation and migration promotion[71].

Effect of EVs produced by BM-MSCs on MM cells and MM progression

So far, we have analyzed the influence of MM-EVs on BM-MSCs, however, this communication, as previously mentioned, is bidirectional. In 2016, Wang *et al*[69] showed that BM-MSC-EVs from MM patients contained a lower level of the tumor suppressor miR-15a, and higher levels of oncogenic

proteins, cytokines, and adhesion molecules, when compared to EVs from healthy BM-MSCs. Cytokines such as IL-1ra, interferon-IP-10, MCP-1, MIP-1α, MIP-1β, and SDF1α were detected in murine BM-MSC-EVs. They confirmed that BM-MSC-EVs from MM patients act on MM cells activating proliferation, survival, and migration, as well as drug resistance to bortezomib, a widely used clinical drug for MM treatment.

In a similar study, a reduction of mir-15a levels in the cargo of BM-MSCs-EVs from MM patients was also detected. This change was shown to promote cell proliferation and dissemination or metastasis to other niches, which is a hallmark of MM. The same authors also revealed the importance of some of the proteins present in BM-MSCs-EVs cargo, as they detected higher content levels of IL-6, CCL2, γ-catenin and fibronectin, which are key to MM pathogenesis[70]. Other miRNAs cargo were also implicated in these processes. miR-483-5p was found packed in BM-MSCs-EVs and was responsible for promoting MM cell proliferation and reduced apoptosis *via* the miR-483-5p/TIMP2 axis[79]. Umezu *et al*[80] highlighted the role of miR-10a in MM disease since its transference *via* BM-MSC-EVs promoted cell proliferation in several MM cell lines (RPMI 8226, KMS-11, and U266) compared to BM-MSC-EVs with miR-10a blocked. Moreover, Gao *et al*[81] studied miR-155 present in BM-MSC-EVs cargo, which turned out to be involved in viability, stemness and drug resistance in MM cells. The role of miR-155 was underscored by the fact that incubation of the MM cells line mitochondrial pyruvate carrier 11 (MPC-11) with miR-155-mimics for 24 h resulted in a significantly reduced cell apoptosis *in vitro* and augmented expression of stemness maintenance markers OCT-4 and Nanog and drug resistance-associated proteins MRP1, ABCG2 and P-g.

As in the previous section, a table summarizing the main works referred to the relevance of communication between MM cells and BM-MSCs through EVs and the role of their cargo is shown (Table 2).

The resistance to treatment is precisely one of the major problems in MM at the clinical level, as this is directly responsible for the relapses. Some studies investigating the mechanisms behind this resistance have highlighted the implication of the activation of several signaling pathways, including p38, p53, c-Jun N-terminal kinases and Akt through the assessment of bortezomib treatment. The role of BM-MSC-EVs in interfering with the antitumor effect developed by bortezomib in MM was confirmed through different experiments. BM-MSC-EVs were able to alter apoptosis-related proteins Bcl-2, Bax, caspase-8, caspase-9, and caspase-3 promoting an antiapoptotic profile in both murine and human cells. These EVs blocked the significant reduction of Bcl-2 expression caused by bortezomib and reduced cleaved caspase-9, caspase-3, and PARP either in the absence or presence of bortezomib. Moreover, the use of GW4869, a neutral sphingomyelinase inhibitor of the formation of exosomes by the ceramide pathway, in combination with bortezomib treatment led to a significant effect on tumor load reduction[71,82].

In conclusion, the two-way communication between MM cells and BM-MSCs mediated by EVs is extremely intricate and plays a pivotal role in the progression of the disease. Since BM-MSCs-EVs have a key role in supporting MM development, this could become a key target to develop new therapies for the treatment of this hematological disease.

COMMUNICATION THROUGH CONTACT DEPENDENT MECHANISMS

As well as the already described interactions through paracrine secretion of different cytokines and EVs, MM cells also interact with BM-MSCs by direct cell-to-cell contact. These cell-to-cell interactions are not restricted to MM and BM-MSCs since MM cells also interact with other cells of the BM microenvironment such as osteoclasts and osteoblasts, endothelial cells, and lymphocytes. It is known that these contacts are also key to protect MM cells against chemotherapy, helping them to accumulate inside the BM[83], to adhere to endothelium, and to spread into the bloodstream[84], although the detailed mechanisms involved in those processes have not been completely elucidated[85].

Cell adhesion molecules in MM/BM-MSCs communication

Direct cell-to-cell adhesion and communication mechanisms have been known for more than 40 years [86,87]. These cell-to-cell communication is mediated by Cell Adhesion Molecules (CAMs), a subcategory of adhesion proteins located at the cell surface, involved in binding either to other cells, or in attaching cells to proteins of the ECM[88], suchas fibronectin, laminin or collagen (Figure 2). While it has been well documented that the ECM promotes the survival of different types of tumors, much less is known about the influence of the direct contact of BM-MSCs in their progression.

CAMs play a central role in cell communication and the maintenance of tissue homeostasis[89]. There are different superfamilies or groups of CAMs with different specificities and distributions. These families would include the Immunoglobulin superfamily CAMs (IgCAMs), integrins, cadherins and one superfamily of proteins that contain a C-type lectin-like domain (C-type lectin domain proteins or CTLDs)[89]. Following other criteria, CAMs can be classified into calcium-independent or calcium-dependent molecules[90], meaning that these molecules would need Ca^{2+} ions binding to different domains of the protein in order to rigidify their extracellular domains and enable interaction[91]. Integrins and IgCAMs belong to the calcium independent group whereas CLTDs and selectins belong to

Table 2 Summary of evidence about the relevance of different cargo molecules in the extracellular vesicles of multiple myeloma cells and bone marrow mesenchymal stem cells s to the progression of multiple myeloma

	Function	Ref.
MM-EVs cargo		
lncRNA RUNX2-AS1	Form a RNA duplex with RUNX2 premRNA, reducing the osteogenic activity in MSCs	[76]
miR-146a	Increase the secretion of several cytokines in BM-MSCs that favor MM cell viability and migration and induce CAF transformation	[78]
DKK-1	Lower expression of OSX, COL1A1 and ALP in osteoblast precusor cell line (MC3t3-E1)	[72]
MSC-EVs cargo		
mir-15a	Promote MM cell proliferation and dissemination to other niches	[70]
miR-483-5p	Induce MM cell proliferation and reduced apoptosis	[79]
miR-155	Reduce MM cell apoptosis and augment expression of stemness maintenance and drug resistance markers	[81]

EVs: Extracellular vesicles; MM: Multiple myeloma; BM-MSC: Bone marrow mesenchymal stem cells; CAF: Cancer associated fibroblast; DKK-1: Dickkopf-1; OSX: Osterix; COL1A1: Collagen 1A1; ALP: Alkaline phosphatase.

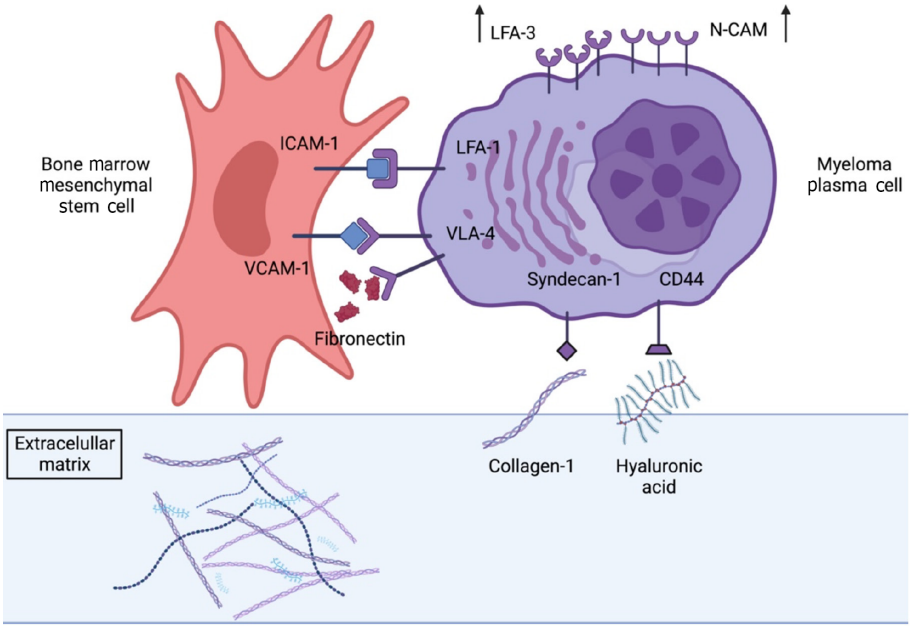

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Figure 2 Schematic representation of the main cell adhesion molecules in multiple myeloma cells and bone marrow mesenchymal stem cells. The main interactions between cell adhesion molecules (CAMs) of these two types of cells as well as the interactions of these CAMs with proteins of the extracellular matrix are displayed (Created with Biorender.com). ICAM-1: Intercellular adhesion molecule 1; VCAM-1: Vascular cell adhesion molecule-1; LFA-1: Leukocyte function-associated antigen 1; N-CAM: Neural cell adhesion molecule; VLA: Very late antigen. Created with BioRender.com.

calcium dependent group[92]. Cell adhesion molecules bind to different ligands. Cadherins, selectins and IgCAMs are associated with the cell-to-cell contact, while integrins are involved in the attachment of MM cells to the ECM[93]. All these molecules are integral in modeling cellular mechanisms such as growth, contact inhibition and apoptosis. In fact, changes in cell adhesion, involving these molecules, can be the defining event in a wide range of diseases, including cancer development[94], as lower intercellular adhesiveness allows malignant cells to scape from their site, thus, destroying the architecture of the original tissue, commonly the first step leading to cancer[94].

As well as the already described interactions through paracrine secretion of different cytokines and EVs, MM cells also interact with BM-MSCs by direct cell-to-cell contact. In fact, recent studies revealed that many of the changes undergone by BM-MSCs supporting the progression of MM, are acquirable by physical contact with MM cells[95]. In MM, this cell-to-cell interactions are not restricted to MM and BM-MSCs since MM cells also interact with other cells of the BM microenvironment such as osteoclasts and osteoblasts, endothelial cells, and lymphocytes. All these interactions are regulated by CAMs. It is

known that these contacts are key to protect MM cells against chemotherapy, helping them to accumulate inside the BM[83], to adhere to endothelium, and to spread into the bloodstream[84], although the detailed mechanisms involved in those processes have not been completely elucidated[85].

The most relevant role of CAMs in MM pathophysiology is related to the homing of malignant PCs to the BM. To complete the process of homing, mediated by CXCL12, MM cells need to adhere to either ECM proteins or BM-MSCs. This is mediated by CAMs such as very late antigen (VLA)4, VLA5, CD44, leukocyte function-associated antigen 1 (LFA-1), intercellular adhesion molecule 1 (ICAM-1), MPC-1 and syndecan (Figure 2).

One way of ensuring adhesion of MM to the ECM is the binding of its integrin VLA-4 to fibronectin, a common component of the ECM. VLA-4, which is in fact a heterodimer of two integrins CD49d(a4) and CD29(b1), also mediates the interaction of MM cells with BM-MSCs, through the vascular cell adhesion molecule-1 (VCAM-1), located at the BM-MSCs[96]. This interaction activates the secretion of MIP-1α and MIP-1β in MM cells, leading to an increase of osteoclastogenic activity[97]. Moreover, the direct contact of these two types of cells through VLA-4 also induces the production of DKK-1 by MM cells, which inhibits osteoblastic differentiation of BM-MSCs. Thus, these two actions, promotion of osteoclastogenesis and inhibition of osteogenesis, would have a detrimental effect on bone structure, contributing to the typical osteolytic lesions in MM. In addition, BM-MSCs unable to undergo osteoblastic differentiation would produce higher levels of IL-6, a cytokine that would stimulate the proliferation of DKK-1-secreting MM cells[25]. Moreover, it has been observed that VLA-4–fibronectin binding is an essential step that supports the IL-6-mediated induction of PCs in normal BM, since antibodies against VLA-4 were found to inhibit the secretion of IL-6 in co-cultures of MM cells and BM-MSCs cells[96,98,99].

The interaction between MM cells and BM-MSCs is also mediated by ICAM-1 (CD54) and LFA-1 (CD11α/CD18) expressed in BM-MSCs and MM respectively. The glycoprotein ICAM-1 is the main ligand for b2 integrins and its expression is induced in response to an inflammatory microenvironment [100], such as the one resulting in the BM following the colonization by MM cells. The ICAM-1/LFA-1 interaction seems to have a key role in the progression of MM since the blocking of LFA-1 through the use of monoclonal antibodies, inhibits the production of IL-6 by BM-MSCs. Thus, this interaction is focus of various studies aimed to the development of treatments for MM[101].

Syndecan (CD138) is the principal transmembrane proteoglycan expressed in the surface of MM cells and has in fact been used as a marker for the detection of this pathology. Syndecan has multiple functions in MM. This molecule mediates de adhesion of MM cells to the collagen in the ECM through direct interaction with collagen molecules but can also mediate myeloma cell-cell adhesion[102]. Syndecan-1 also plays a broad role in cells signaling since heparan sulfate chains on syndecan-1 can bind to and sequester growth factors and cytokines, regulating their availability to cells. Also, a recent study has shown that syndecan contributes to the survival of mature MM cells by enhancing IL-6 signaling[103]. Finally, the binding of syndecan to VEGF and other angiogenic factors, has been shown to promote angiogenesis in MM[104].

Finally, CD44, a transmembrane glycoprotein, interacts mainly although not exclusively with hyaluronic acid in the ECM[105]. CD44 signaling has been shown to activate various signaling pathways in different types of cancer including PI3k/AKT, MAPK/ERK and NF-kB[106], which, as we have seen, promote MM cell survival.

Although normal PC and MM cells express basically the same set of CAMS, some of these molecules were found to be more significantly overexpressed in MM cells when compared to healthy patients. In this group we can include, leukocyte adhesion molecule LFA-3 (CD58)[107] and neural cell adhesion molecule (CD56)[108]. MM cells can also express the lymphocyte function-associated antigen LFA-1 (CD11α/CD18) which was associated with tumor growth and homotypic tumor cell adhesion or aggregation[109]. It is also worth mentioning that some homing molecules could not be detected on MPCs: Selectin molecule L-selectin and collagen receptor VLA-2[89]. Although this study provides relevant information, for this information to be biologically relevant, ligands of these receptors had to be available within the tumor environment.

Overall, given the importance of some of these CAMs in the process of MM cells homing, these molecules could be important targets for designing antitumoral treatments. Several approaches have been explored, including antibodies specifically targeting these molecules on the cell surface, as well as small molecule inhibitors that interfere with the binding of the CAMs to their ligands. Moreover, receptor-blocking antibodies against most of these CAMs (VLA-4, CD56, MPC-1, CD21) were found to partially block MM cells adhesion to the BM stroma. This partial effect could be attributed to an additional adhesion mechanism yet to be discovered[110,111].

In MM a specific type of drug resistance seems to be mediated by CAMs, the so called, CAMs mediated drug resistance[112]. CAMs can activate intracellular pathways that promote cell survival, promote cancer cell adhesion to the ECM and regulate the expression of drug transporters that could pump chemotherapy drug out of cancer cells and reduce their efficacy. It is also important to highlight that MM spreading in the last stages of the diseases also involve important changes in cell adhesion. MM can abandon de BM microenvironment and become stroma independent because of different processes involving changes in the expression levels of CAM and certain cytokines. Once this happens, cells can be found to spread extramedullary at different sites such as lungs, liver, or pleural fluid[113, 114].

Role of tunneling nanotubes in MM/BM-MSCs communication

As we have seen, communication between MM and BM-MSCs cells can take place through mechanisms that can be classified as contact-dependent and/or contact-independent mechanisms[115]. While the previous section has been dedicated to direct communication mediated by cell adhesion molecules, in this last section we will briefly discuss transport *via* tunneling nanotubes (TNTs), another form of contact-dependent interaction.

TNTs are transient intercellular structures formed by the polymerization of F-actin which provide an important and general mechanism of cell-to-cell communication[116,117] and constitute a reliable infrastructure for vesicle and protein trafficking[118]. Numerous examples of communication between MSCs and malignant hematological cells such as B cells, MM and chronic lymphocytic leukemia, are already known, as well as the effects of this communication, such as increased drug resistance. This has already been demonstrated in acute myeloid leukemia (AML), B-cell precursor acute lymphocytic leukemia (ALL) or CML[119,120]. Therefore, TNTs are considered one of the key pharmacological targets in current research.

The role of TNTs is to deliver autophagosomes, mitochondria and other lipophiles to MSCs. This induces the secretion of specific cytokines, including interferon-γ-IP-10, CXCL10, IL-8, MCP-1 and CCL2 and other growth factors which, in turn, induce tumor cell survival, enhanced growth and even drug resistance[121]. This has been checked in AML, where increased survival of cells against chemotherapy treatments is observed by means of mitochondrial transfer from MSCs routed by TNT. In this case, the mitochondrial transfer translates into an increase of up to 14% in mitochondrial mass in co-cultures of tumor cells with MSCs and a 1.5-fold increase in mitochondrial adenosine triphosphate production (ATP), making them less prone to mitochondrial depolarization and thus resulting in increased survival against chemotherapy treatments[122].

Numerous lines of treatment are currently under development for various hematological diseases that reduce the formation of TNT by blocking actin polymerization. This inhibits the cellular communication that promotes disease progression. Those treatments include cytochalasin D, cytarabine, latrunculin A and B, daunorubicin, everolimus, metformin, nocodazole CK-666, ML-141 or 6-thio-GTP [123]. In addition, vinca alkaloids or taxanes are also being targeted because of their role in the polymerization of microtubules[124].

Although the fate of mitochondria transferred into tumor cells remains unclear, there is evidence indicating that MSCs play a key role in the progression of AML, ALL, MM and mitochondrial transfer chemoresistance. It is well known that the initiation of cancer requires metabolic adjustments, since rapid proliferation cancer cells have high metabolic requirements. This mitochondrial and/or mitochondrial DNA transfer to cancer cells increases mitochondrial content and enhances the mitochondrial process of oxidative phosphorylation (Oxphos), which generates a larger quantity of ATP than glycolysis, thus, promoting cell proliferation and invasion[125]. Therefore, targeting mitochondrial respiration and Oxphos is also a treatment option, FOXM1 is known to regulate myeloma cell metabolism by increasing glycolysis and Oxphos. NB73 is a FOXM1 inhibitor that promotes FOXM1 degradation and thus growth of MM cells, making it a potential drug targeting Oxphos[126].

Studies to date have elucidated that mitochondrial transfer dynamically induced resistance occurs between MM cells and other cells in the BM microenvironment *via* TNT, providing a starting point for the development of new targeted therapies[127]. An example of this line of treatment for MM is the use of anti-CD38 monoclonal antibodies[128]. This antibodies have different mechanisms of action, including cell apoptosis[129]. Moreover, their administration in mice has shown inhibition of mitochondrial transfer, a reduction in tumor volume and, in general, increased survival[1]. However, it should be noted that, although patients who have received this treatment show increased survival, it has been observed that resistance to these treatments can be acquired in the long term.

CONCLUSION

Conditions at the BM microenvironment are essential for the establishment and progression of MM. The complex BM microenvironment encompasses hematopoietic cells, immune cells, and cells involved in bone homeostasis such as osteoclasts, osteoblasts and BM-MSCs. Thus, it is understandable that the disruption of microenvironment homeostasis by MM cells results in angiogenesis, osteolysis, immune suppression and anemia[69].

As key regulators of this microenvironment, BM-MSCs play an important role in the progression of the disease. The crosstalk between MM cells and BM-MSCs takes place at different levels, through soluble cytokines, EVs, and direct cell-to-cell contact.

The interaction between these two cell types can have both positive and negative effects on the proliferation and survival of MM cells. The communication between MM cells and BM-MSCs can promote tumor growth. The survival and proliferation of MM cells once they reach the BM is associated with immune suppression, eliminating the possibility of an effective antitumor response. Although it is the interaction between all cells in the BM what produces this immunosuppressive microenvironment, BM-MSCs have a relevant role in the construction of this particular microenvironment due not only to their

important paracrine activity, but also to their ability to establish direct communication with other cells in that microenvironment. All these direct or indirect interactions activate a pleiotropic proliferative and antiapoptotic cascades favoring disease progression.

On the other hand, the communication between MM cells and BM-MSCs can also have a negative impact on cancer cell growth and survival. BM-MSCs can secrete factors that inhibit the growth and survival of MM cells.

Currently, therapeutic advances in the treatment of this disease are based on targeted therapies using monoclonal antibodies or CAR-T. These treatments have improved patient prognosis, although long-term resistance is still observed, and further research is needed into the specific mechanisms by which cells acquire this resistance. In the quest for new effective treatments for MM, the importance of communication between MM cells and BM-MSCs cannot be overstated. Understanding the molecular mechanisms involved in this two-way communication can provide valuable insights into MM pathogenesis and help identify key targets involved in the survival and proliferation of MM cells in the BM microenvironment and thus, opening new opportunities for the design of targeted therapies to avoid disease progression.

FOOTNOTES

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MINIREVIEWS

Molecular signaling in cancer stem cells of tongue squamous cell carcinoma: Therapeutic implications and challenges

Priyanka Joshi, Sanjeev Waghmare

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Abstract

Head and neck squamous cell carcinoma is the seventh most common cancer worldwide with high mortality rates. Amongst oral cavity cancers, tongue carcinoma is a very common and aggressive oral cavity carcinoma. Despite the implementation of a multimodality treatment regime including surgical intervention, chemo-radiation as well as targeted therapy, tongue carcinoma shows a poor overall 5-year survival pattern, which is attributed to therapy resistance and recurrence of the disease. The presence of a rare population, *i.e.*, cancer stem cells (CSCs) within the tumor, are involved in therapy resistance, recurrence, and distant metastasis that results in poor survival patterns. Therapeutic agents targeting CSCs have been in clinical trials, although they are unable to reach into therapy stage which is due to their failure in trials. A more detailed understanding of the CSCs is essential for identifying efficient targets. Molecular signaling pathways, which are differentially regulated in the CSCs, are one of the promising targets to manipulate the CSCs that would provide an improved outcome. In this review, we summarize the current understanding of molecular signaling associated with the maintenance and regulation of CSCs in tongue squamous cell carcinoma in order to emphasize the need of the hour to get a deeper understanding to unravel novel targets.

Key Words: Head and neck squamous cell carcinoma; Cancer stem cells; Signaling; Tongue squamous cell carcinoma

Core Tip: Tongue squamous cell carcinoma is one of the most common and aggressive oral cavity carcinomas, particularly among the Indian population. Despite various treatment strategies employed, the survival rates of the patients remain poor. A rare population *i.e.*, cancer stem cells (CSCs), plays an important role in resistance, recurrence as well as metastasis which are factors responsible for the poor survival outcome. In this review, we discuss the recent findings regarding cell signaling pathways and markers associated with the CSCs and the need to gain a deeper understanding on the properties of the CSCs.

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INTRODUCTION

Global cancer statistics by GLOBOCAN in 2020 showed that 2.0% of new cancer cases reported worldwide were lip and oral cavity cancers, while 1.8% of the total cancer-related deaths were of lip and oral cavity cancers. Lip and oral cavity cancers are the most commonly diagnosed cancers that are responsible for most cancer-related deaths in India[1]. Most cases of oral squamous cell carcinomas (OSCCs) are presented at advanced stages, *i.e.*, stages III or IV [tumor-node-metastasis (TNM) staging], where the 5-year survival of the patients is less than 50%. Further, 40% of the oral carcinomas are presented as tongue carcinomas[2].

Head and neck squamous cell carcinomas (HNSCCs) are carcinomas of the oral cavity, nasopharynx, oropharynx, larynx, and hypopharynx[3]. The oral cavity carcinomas comprise the anterior 2/3rd of the tongue, buccal mucosa, retromolar trigone, lower and upper alveolar ridge, hard palate, and floor of the mouth[3].

The poor survival observed in HNSCCs is primarily attributed to loco-regional/ distant metastasis and therapy resistance. Therefore, understanding the molecular mechanism underlying these properties of tumors has become very crucial. Current treatments for HNSCCs include surgery, chemo-radiotherapy, and targeted therapy.

The first evidence of cancer stem cells (CSCs) was observed in acute myeloid leukemia[4], where it was reported that only 10000 cells expressing $CD34^+/CD38^-$ could give rise to leukemia in non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice. These cells possess high tumorigenic potential, which are termed as CSCs. CSCs exhibit stem cell-like properties such as self-renewal, slow cycling, and the ability to divide and differentiate into various sub-populations[5]. Further, CSCs were isolated from solid tumors such as breast cancer, HNSCCs, colorectal cancer, ovarian cancer, lung cancer, *etc*[5].

Owing to their unique properties, these CSCs escape the current treatment regimes, thereby adversely affecting patient survival. Therefore, to design an effective treatment regime in order to achieve better efficiency and treatment outcome, it is crucial to understand the molecular mechanism involved in maintaining these CSCs within the tumor. In this review, we focus on the tongue squamous cell carcinoma (TSCC) and have summarized the known molecular markers of CSCs, molecular signaling involved in the regulation of CSCs, the inhibitors used in clinics for treatment, and the ones that are in clinical trials.

CSC MARKERS FOR TSCC

CD44 and variants

CD44 is a single-chain proteoglycan transmembrane glycoprotein expressed on human embryonic stem cells at the developmental stages of cell types such as bone marrow and connective tissue. CD44 interacts with molecules such as hyaluronic acid (HA), collagen, osteopontin, fibronectin, chondroitin and serglycin/sulfated proteoglycan. CD44 has variant isoforms such as CD44, CD44s, CD44v3, CD44v6, CD44v8-10[6] with HA as the most specific ligand for CD44 and its isoforms[6].

The first report of isolation and characterization of CSCs from HNSCC showed that 5000 $CD44^+/Lin^-$ cells gave rise to *in vivo* tumors in NOD-SCID mice[7]. CD44 expression is co-related with the expression of known stem cell marker BMI-1 in HNSCC cells.

Recently, CD44v3 is reported to be overexpressed in HNSCC tumors as compared to the cut margin. Transfection of CD44v3 in the HNSCC cells increases cell migration[8]. Tumor cell growth, migration, matrix metalloproteinase activity, and lymph node metastasis in patients are associated with CD44v3

overexpression in HNSCC cells[9]. Decreased expression of CD44v9 co-relates with poor overall survival (OS) in TSCC[10]. Overexpression of CD44 co-relates with tumor invasiveness and epithelial-mesenchymal transition (EMT). Expression of CD44 in invasive margins of OSCC was associated with samples showing poor histopathological differentiation, high tumor budding activity, and single-cell invasion[11]. Further, increased expression of CD44, together with increased expression of NANOG was associated with poor survival in HNSCC patients as compared to those showing low expression of CD44 and NANOG[12].

Cells overexpressing CD44 ($CD44^+$) showed self-renewal property with high tumorigenic potential, metastasis, and chemo-resistance. Therefore, $CD44^+$ cells in HNSCC tumors are considered as CSC-rich population. CD44, paired with the overexpression of other stem cell markers, such as aldehyde dehydrogenase (ALDH) and CD133, are being used for the isolation of CSCs from HNSCC tumors[13]. In TSCC, $CD44^+/CD133^+$ cells showed stem cell-like properties such as high proliferation, invasion, and migration with high tumorigenicity[13].

There have been recent reports linking $CD44^+$ cells in HNSCC tumors to early angiogenesis[14], lymph node metastasis[15], and occult metastasis[16]. Moreover, overexpression of CD44 in adjacent normal epithelia of TSCC co-related with clinical stage and nodal metastasis in patients[17]. CD44 mRNA expression did not show any co-relation with age, sex, smoking history, size of the tumor, or 5-year survival rate[18].

ALDH

ALDH is an enzyme superfamily which converts aldehydes to carboxylic acids that are involved in drug resistance and detoxification. The human ALDH1A subfamily is involved in the retinoic acid pathway, which regulates gene expression and cell development in both normal and cancer cells. The enzymes belonging to ALDH1A subfamily viz., ALDH1A1, ALDH1A2, and ALDH1A3 are located in the cytosol that catalyze the irreversible conversion of retinaldehyde to retinoic acid.

Amongst the ALDH1A subfamily, ALDH1A1 is overexpressed in the CSCs of HNSCC. Overexpression of ALDH has co-related with overexpression of other stem cell markers such as OCT-3/4, NANOG, STELLA, SNAIL, and BMI-1 in HNSCC. $ALDH^{high}$ cells have also been shown to have increased *in vitro* sphere formation ability and *in vivo* tumorigenesis ability[19,20]. Higher expression of ALDH also co-related with poor patient survival. Importantly, 500 $ALDH^{high}$ cells isolated from HNSCC tumors showed a higher tumorigenic potential upon *in vivo* serial transplantations as compared to $ALDH^{low}$ cells[21]. High expression of ALDH1A1 in oropharyngeal carcinoma co-related with poor differentiation in tumors and poor OS patterns in patients[22]. $ALDH^{high}$ TSCC cells showed serum independency and a higher ability to form tumorospheres than $ALDH^{low}$ cells. $ALDH^{high}$ cells also exhibited overexpression of stem cell-related genes such as NOTCH2[23]. ALDH1A1 expression was directly co-related with OS and lymph node metastasis in HNSCC[24]. The study showed a co-relation of ALDH1A1 expression with TWIST1 expression in primary tumor tissues and lymph node metastases. Recent reports have demonstrated the involvement of ALDH isoforms in cisplatin resistance in HNSCC. Treating cells with ALDH inhibitors showed decreased cell viability and reduced tumor burden *in vivo* when given in combination with cisplatin as compared to only cisplatin treatment. This study also showed that treating cells with the ALDH3A1 activator along with cisplatin increased cell survival[25]. Overexpression of ALDH1A1 in HNSCC tissues co-relates with poor survival as compared to low ALDH1A1 expression[26]. Additionally, the expression of ALDH1 increased from epithelial dysplasia to oral carcinoma, that co-related with poor survival rates in OSCC patients[27]. In addition, low ALDH1A1 in the HNSCC patients showed significantly better OS as compared to high ALDH1A1 expression[25].

CD133

CD133/AC133/prominin-1 is a 97 kDa pentaspan transmembrane glycoprotein encoded by the prominin 1 (*PROM1*) gene. CD133 protein has an intracellular C-terminal domain, an extracellular N-terminal domain, and five transmembrane segments[28].

Spheroids obtained from HNSCC patient tumor cells showed higher CD133 expression than normal epithelial cells[29]. High expression of CD133 with high expression of CD44 and CD117 was observed as marker of CSCs in OSCC cells[30]. TSCC cells overexpressing CD133 showed a higher *in vitro* and *in vivo* tumorigenicity as compared to cells with low expression of CD133[31]. Recent reports showed that $CD133^+$ OSCC cells exhibit properties such as self-renewal, drug resistance, higher tumorigenic potential, and higher growth rate as compared to $CD133^-$ cells. Further, increased expression of stem cell markers such as NANOG, OCT4, SOX2, and ALDH1A1 in $CD133^+$ cells suggested that CD133 is a potential CSC marker for OSCC CSCs[32]. TSCC-derived spheroids were reported to overexpress CD133[33].

Other markers

Stemness markers such as OCT-3/4, NANOG, SOX2, KLF-4, and BMI-1 have been associated with characteristics such as self-renewal, pluripotency, and development of embryonic stem cells are overexpressed in CSCs in TSCCs.

Invasive TSCC cells overexpressing CD44 and SOX9 showed a higher expression of SOX2 and OCT-4 [34]. SOX2 overexpression in TSCC tissues co-related with poor OS in patients[35]. In addition, SOX2 overexpression co-related with tumor size, cell differentiation, nodal metastasis, and clinical TNM stage. In TSCC cells, the knockdown of SOX2 showed a decrease in cell proliferation, cell migration and invasion, and colony forming, which was reversed with overexpression of SOX2[36]. Moreover, an increased SOX2 expression was associated with poor OS, disease-specific survival, and disease-free survival (DFS) in TSCC[37]. Downregulation of SOX2 by MTA-3 was reported to repress CSC properties and tumor growth in TSCCs, and patients exhibiting $MTA3^{low}/SOX2^{high}$ showed the worst prognosis [38]. Additionally, SOX2 regulated HEY1, which in turn regulates NOTCH4 expression, followed by increased EMT in HNSCC cells[39].

The expression of both OCT-3/4 and NANOG was high in side population cells that co-related with distant metastasis[40]. Also, high OCT-4 expression in TSCC samples and NANOG in adjacent cut margin tissues have been reported as indicators of lymph node metastasis and worse prognosis[12]. Furthermore, TSCC showed an association between BMI-1 overexpression and increased proliferation, nodal metastasis, and decreased OS in patients. Further, knocking down BMI-1 in TSCC cells showed a reduction in cell proliferation and migration, increased cell apoptosis, senescence, and cisplatin sensitivity[41]. Ectopic overexpression of BMI-1 increased susceptibility of tongue carcinogenesis after exposure to 4-nitroquinoline-1-oxide in mice. The ectopic expression of BMI-1 was shown to regulate the pathways such as mTOR signaling, EIF2 signaling, and p70S6K signaling[42]. Additionally, high expression of OCT4 and BMI-1, along with ALDH1, co-related with poor survival in OSCC patients[27].

The *TRIM* (tripartite motif) gene family have ubiquitin ligase function that plays an important role in various human diseases such as muscular dystrophies and atrophies and HIV infections *etc*[43]. A recent report showed that overexpression of TRIM14 induces CSC-like properties with an increased sphere formation ability and cisplatin resistance in TSCC cells. Further, on inhibition of TRIM14 by miR-15b, these characteristics were reversed, implying that TRIM14 might play an important role in the maintenance of these properties[44].

MOLECULAR SIGNALING

The Wnt pathway

The Wnt signaling consists of the Canonical (involving β-catenin) and non-canonical pathways. Canonical Wnt signaling initiates when Wnt ligand binds to FRZ receptor and low-density LRP5/6 co-receptors. In the absence of the Wnt ligand, a complex of Axin, APC, GSK3, and CK1 phosphorylates β-catenin, leading to ubiquitination and subsequent proteasomal degradation of β-catenin. When present, Wnt ligand binds to FRZ receptor leading to FRZ-induced LRP5/6 phosphorylation followed by activation of the scaffold protein DVL. Activated DVL recruits Axin to receptors, which then inhibits the phosphorylation of β-catenin. Subsequently, β-catenin translocates into the nucleus promoting the transcription of Wnt target genes by interacting with T cell-specific factor/lymphoid enhancer-binding factor. Proper functioning of the Wnt signaling pathway is important for embryonic development and self-renewal of normal stem cells[45]. Deregulated Wnt signaling is involved in the development of various cancers such as colorectal cancer, epidermal cancer[46], hepatocellular carcinoma, breast cancer, glioma, *etc*[47].

Recent studies showed that the suppression of the Wnt signaling inhibits the progression of OSCC. Micro RNAs such as miR-29a[48], miR-638[49], miR-106a*[50], *etc.*, have been shown to suppress tumor progression by regulating the Wnt signaling. The miR-638 and miR-106a* regulate Wnt through downregulating oncogenes PLD1 and MeCP2, while miR-29a caused reduction in β catenin levels. In addition, chemical compounds such as quercetin (bioactive flavonoid) and niclosamide (anthelminthic) were reported to inhibit tumor progression by affecting the Wnt signaling in OSCC. Quercetin induced miR-22 expression, thereby inhibiting the Wnt1/β catenin axis[51], while Niclosamide downregulated the expression of β-catenin, DVL2, phosphorylated GSK3β, and Cyclin D1[52]. SOX8 was shown to regulate chemo-resistance and EMT in TSCC cells by activating the Wnt pathway suggesting that it might play a crucial role in the maintenance of tongue CSCs[53]. Furthermore, the spheroid forming ability and expression of CSC markers (CD44 and ALDH) was negatively impacted in the presence of the Wnt antagonist sFRP4 in tongue carcinoma cells. In presence of the Wnt ligand, WNT3a, these properties were reverted[54]. The Wnt on and off pathway and its role in TSCC is shown in (Figure 1).

The Hedgehog pathway

The Hedgehog pathway has three different types of ligand proteins in mammals such as Sonic-Hedgehog (SHH), Indian-Hedgehog, and Desert-Hedgehog. The ligand binds to the receptor PTCH1 and removes the inhibition on the transmembrane protein Smoothened (SMO). This further leads to SMO accumulation in the cytoplasm. Subsequently, the translocation of glioma-associated oncogene (Gli) proteins into the nucleus initiates the transcription of target genes that are involved in intercellular communication, organogenesis, regeneration, and homeostasis[55].

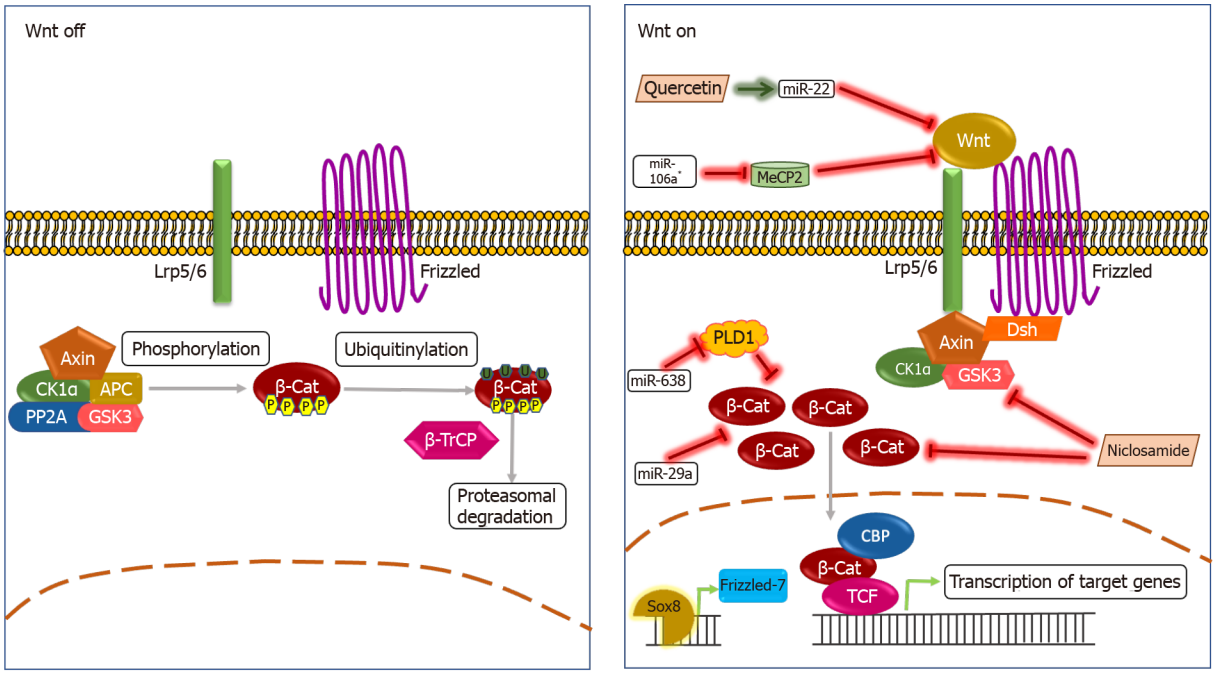

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Figure 1 The Wnt signaling pathway and cancer stemness. In the absence of a Wnt ligand, in the canonical pathway,, a complex of Axin, CK1α, APC, PP2A, and GSK3 (termed destruction complex) phosphorylates β-catenin targeting it for ubiquitinylation, that leads to its proteasomal degradation. When the Wnt ligand binds to the Frizzled receptor and LRP5/6 co-receptor, the destruction complex gets localized to the receptor, preventing the degradation of β-catenin that localizes to the nucleus that further activates transcription of the target genes. It has been reported that miRNAs such as miR-29a, miR-638, and miR-106a* reduce levels of β-catenin and Wnt ligand. The miR-638 targets PLD1, a generally accepted oncogene, leading to the reduction β-catenin levels[49]. The miR-29a also directly causes a reduction in β-catenin levels in TSCC cell lines[48]. The miRNA miR-106a* causes a reduction in MeCP2 (a gene expression regulator and oncogene) levels that inhibit the binding of Wnt ligand to the receptor that in turn causes downregulation of the Wnt pathway[50]. Chemical compounds such as Quercetin and Niclosamide downregulate the Wnt signaling pathway. Quercetin causes an increase in levels of miR-22 that in turn inhibits the Wnt1/β-catenin axis [51], while Niclosamide directly binds to DVL2, phosphorylated GSK3β, and Cyclin D1 that reduces levels of β-catenin[52]. Sox8 is shown to activate the Wnt pathway by inducing the expression of Frizzled-7[53].

Hedgehog pathway activation promotes angiogenesis in OSCC. Overexpression of SHH ligand in human TSCC and expression of PTCH1, Gli1, and Gli2 proteins in microvascular cells have been observed in the tumor invasive front[56]. The involvement of Hedgehog pathway has been shown in angiogenesis by macrophages and endothelial cells[57]. Hedgehog and TGFβ signaling are involved in bone invasion and destruction. Gli2 expression is associated with bone invasion. Silencing of Gli2 showed a reduction in invasiveness in orthotopic mice models[58]. Gli3 knockdown in TSCC cells have resulted in the downregulation of the CSC markers such as CD44, OCT-4, and BMI-1 genes and a reduction in CSCs[59]. Further, increased expression of Gli1 has been shown in spheroid forming cells in TSCC cell line[60]. The Hedgehog pathway and its role in TSCC have been shown along with inhibitors in clinical trials for various cancers except for HNSCC (Figure 2).

The Notch pathway

The Notch pathway has four receptors such as NOTCH 1, NOTCH 2, NOTCH 3, and NOTCH 4. The ligands are of two types, viz. Delta-like ligands (DLL1, DLL3 and DLL4) and Jagged ligands (JAG1 and JAG2). Notch pathway also involves proteolysis by metalloprotease, tumor necrosis factor-α-converting enzyme (TACE), and γ-secretase. The binding of the ligand to the receptor releases the extracellular domain by TACE activity, which then binds to the receptor on an adjacent cell, while the intracellular domain is cleaved by γ-secretase activity that further gets translocated into the nucleus, which acts like a transcriptional factor for the activation of the target genes (HES family, HEY, NF-κB, VEGF, and c-MYC) [61].

Notch signaling has been shown to induce EMT in OSCC cells. Activation of Notch signaling is directly co-related with the expression of markers such as E-cadherin and Vimentin and increased invasiveness of OSCC cells[62,63]. Decreasing NOTCH1 in the TSCC cells showed a reduction in the invasiveness of the cells and decreased expression of MMP-2 and MMP-9 (associated with metastasis and invasion) in TSCC[64]. Additionally, activation of the notch intracellular domain in TSCC cell line co-related with stemness characteristics such as spheroid formation and expression of stemness markers viz. OCT4, SOX2 and CD44. The knockdown of NOTCH1 co-related with chemo-sensitization and loss of spheroid-forming ability. Further, high expression of NOTCH1 showed a significantly poor OS as well as DFS in HNSCC patients[65]. Further, NOTCH4 expression promoted cell-cycle dysregulation,

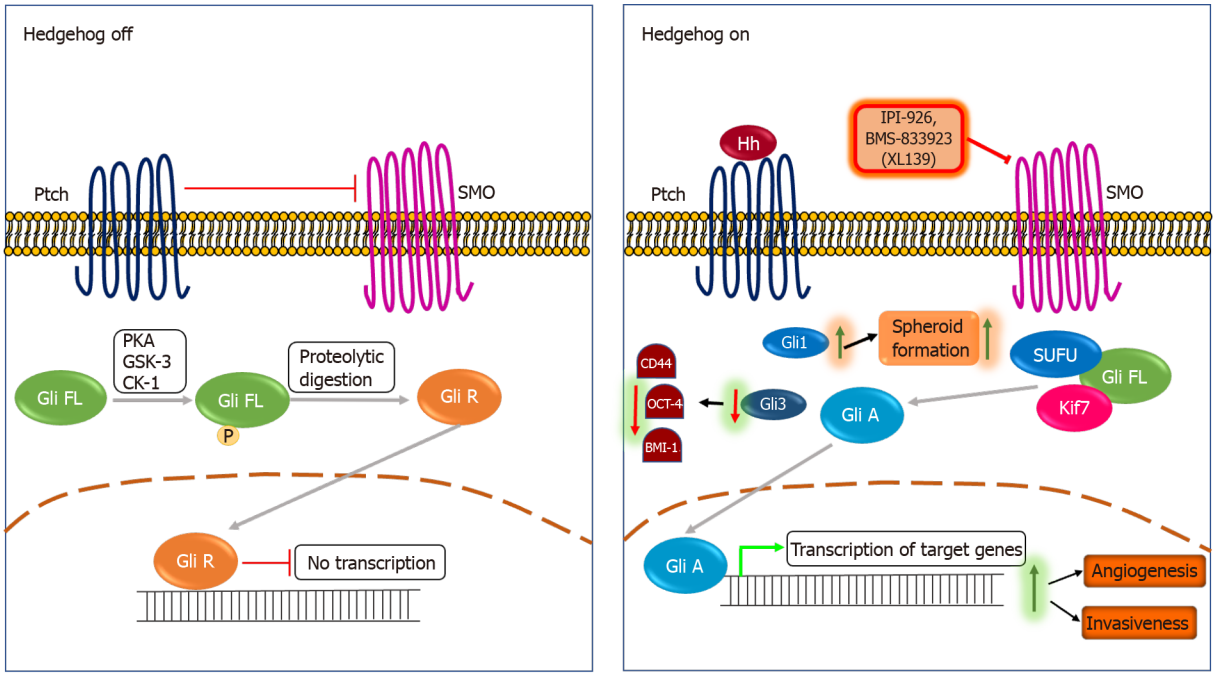

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Figure 2 The Hedgehog signaling pathway and cancer stemness. In the absence of Hedgehog ligand, Patched inhibits smoothened (SMO), leading to the full-length Gli protein that gets phosphorylated by PKA, GSK-3 and CK-1 and converted into Gli repressor through proteolytic digestion. The Gli repressor further inhibits the Hedgehog pathway. When the Hedgehog ligand binds to the Patched receptor, the inhibition on SMO is released, leading to the dissociation of Gli from SUFU and Kif7 that lead to the activation of the Gli protein (Gli A). Further, Gli A translocates to the nucleus that further activates transcription of target genes. Activation of the Hedgehog pathway promotes angiogenesis and invasiveness *in vivo*[57,58]. Downregulation of Gli3 reduces expression of stemness markers such as CD44, OCT-4, and BMI-1 in tongue carcinoma (TSCC) cells[59], while upregulation of Gli1 increases the spheroid formation ability of TSCC cells[60]. IPI-926 and BMS-833923 (XL139) are SMO inhibitors used to therapeutically target the Hedgehog pathway. IPI-926 is a semi-synthetic derivative of cyclopamine, while BMS-833923 (XL139) is a small molecule inhibitor of SMO. Ptch: Patched; SMO: Smoothened; Hh: Hedgehog ligand; Gli FL: Full length glioma associated oncogene; Gli A: Gli activator; Gli R: Gli repressor; IPI-926, BMS-833923 (XL139): Smoothened inhibitors.

cell proliferation, drug resistance, and inhibition of apoptosis. Elevated expression of NOTCH4 along with HEY1 co-related with OCT4, SOX2, and CD44 overexpression that showed increased migration and invasion in TSCC cells[66]. Recently, it was reported that a STAT3-activated long non-coding RNA, hepatocyte nuclear factor 1 homeobox A antisense RNA 1, promoted tumorigenesis by activating the Notch pathway in OSCC cells[67]. Further, high expression of NOTCH1 and JAG1 have been shown to be predictors of poor OS as well as DFS in oral carcinoma patients[68]. The notch signaling pathway and its role in TSCC is shown in (Figure 3).

The HGF/c-MET pathway

The HGF/c-MET pathway is involved in tumorigenesis in various cancers such as HNSCC, non-small cell lung cancer, hepatocellular carcinoma, ovarian cancer, bladder cancer, cervical cancer, *etc.*[69]. The binding of ligand HGF to the kinase receptor c-MET leads to the dimerization of two subunits. The dimerization results in the auto-phosphorylation of tyrosine residues in the cytoplasmic domain of the receptor, which then creates a docking site for various adaptor proteins that regulate pathways such as PI3K/AKT pathway and Wnt pathway[69].

HGF treatment has been shown to increase the expression of CSC markers and sphere forming ability of HNSCC cells, which were decreased upon c-MET knockdown. The transcriptional level of c-MET was higher in cells with high ALDH activity (one of the HNSCC CSC markers). Moreover, c-MET knockdown in the HNSCC stem-like cells resulted in better survival in *in vivo* orthotopic tongue xenograft models[70].

Significant co-relation has been observed in TSCC between the c-MET expression and tumor stage, nodal status, clinical stage, locoregional recurrence, and distant metastasis. In addition, high expression of c-MET and autocrine motility factor receptor (AMFR) was associated with worse DFS. The study suggested that c-MET and AMFR expression can be potent prognosis marker that targets to decrease metastasis in OSCC[71]. Immunostaining for the c-MET showed a significant co-relation with lymph node metastasis, recurrence, and pathological stage of TSCC[72]. High c-MET expression was co-related with lymph node metastasis, greater depth of invasion, decreased patient survival, increased invasion & migration in *in vitro* and subcutaneous *in vivo* mice model injected with TSCC cells[73]. Further, the knockdown of c-MET has shown to reduce cervical lymph node metastasis and improve survival patterns in *in vivo* models[74]. Overexpression of c-MET was shown to co-relate with occult metastasis

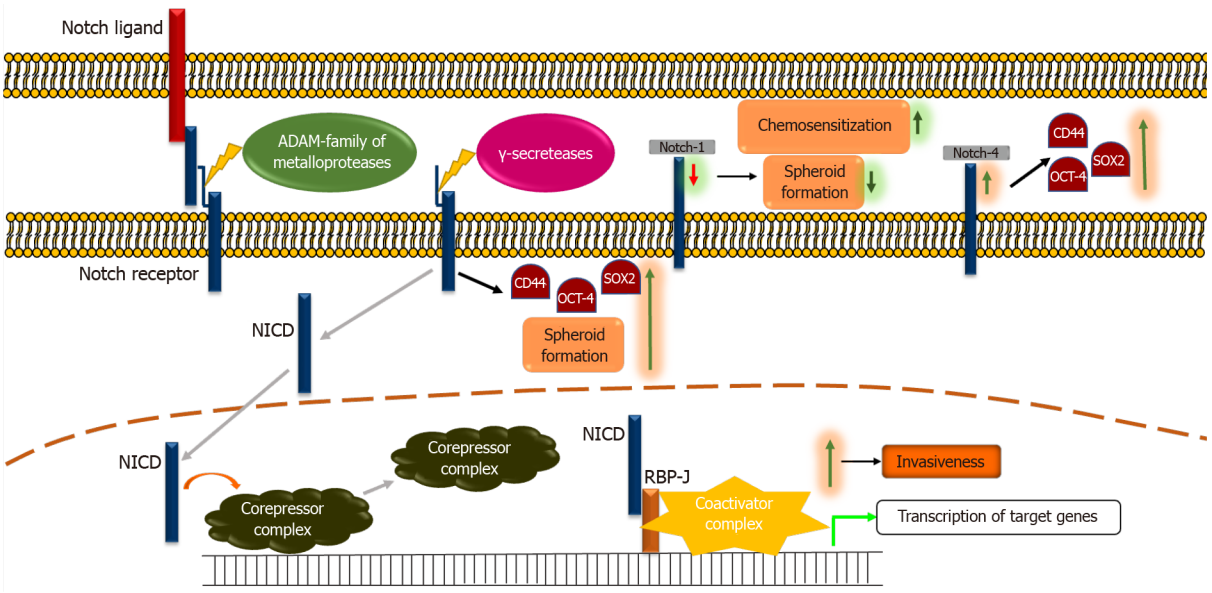

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Figure 3 The Notch signaling pathway and cancer stemness. In absence of the Notch ligand, the Notch pathway is in the inactivate state. The binding of ligand to the Notch receptor leads to cleavage by ADAM-family metalloproteases releasing the extracellular domain of the receptor. Further, the receptor is cleaved by γ-secretase leading to the formation of the Notch intracellular domain (NICD), thereby activating it. The NICD then translocates to the nucleus and releases inhibition on the target genes by dissociating the corepressor complex and forms a complex with RBP-J and co-activator complex thereby activating the transcription of target genes. Further, knockdown of Notch-1 expression has been shown to increase chemo-sensitization and decrease the spheroid formation ability of tongue squamous cell carcinoma (TSCC) cells[64]. An increase in Notch-4 levels increases stemness markers such as CD44, SOX2, and OCT-4 in TSCC cells[66]. NICD activation has been shown to increase levels of CD44, OCT-4, and SOX2 in TSCC cells[65]. Activation of the Notch pathway increases the invasiveness in TSCC cells[62,63,66,67]. NICD: Notch intracellular domain.

in TSCC[75]. The HGF/c-MET pathway and its role in TSCC is shown along with inhibitor in clinical trials for various cancers except for HNSCC (Figure 4).

Other pathways

The transcription factor Nrf2 has been shown to induce the expression of genes involved in cellular antioxidant and anti-inflammatory responses. Normally, Nrf2 is located in the cytoplasm. Upon activation, it translocates to the nucleus, forming heterodimers with proteins such as c-JUN and small musculoaponeurotic fibrosarcoma protein that bind to antioxidant response element, which regulate the expression of around 200 genes that regulate anti-inflammatory and antioxidant response. Nrf2 is involved in the regulation of mitochondrial biogenesis pathways[76]. A compound named Plumbagin is shown to suppress EMT and stemness characteristics by regulating redox homeostasis and inducing reactive oxygen species (ROS) generation within the cell by suppressing the Nrf2-regulated pathways [77].

The Hippo/transcriptional coactivator with PDZ-binding (TAZ) signaling pathway is also involved in the regulation of properties such as cell proliferation, apoptosis, invasion, migration *etc.* in TSCC. When the pathway is off, the Yes-associated protein (YAP) and TAZ motif translocate to the nucleus, thereby inducing the transcription of various genes by binding to the TEA domain family proteins and other transcription factors. LATS1 is activated by MST1 with Salvador through phosphorylation, which then phosphorylates the YAP/TAZ, retaining it in the cytoplasm, which then binds to 14-3-3 and gets degraded[78]. Factors such as HIF-1α[79] and epigallocatechin-3-gallate[80] affect the Hippo pathway to modulate proliferation, apoptosis, invasion, and migration in TSCC cells.

Approximately 90% HNSCCs overexpress the EGFR pathway[81]. The EGFR is a receptor tyrosine kinase of the ErbB family. The ligands of the EGFR are EGF, heparin-binding EGF, and TGFα. Upon receptor-ligand binding, the inactive monomer of the receptor dimerizes, either with another monomer of EGFR forming a homodimer or with another ErbB family receptor forming a heterodimer. This active dimer then auto-phosphorylates the C-terminal domain of the receptor providing a docking site for the phospho-tyrosine binding domain and Src homology 2 domain resulting in the activation of several signaling pathways such as MAPK, PI3K/Akt pathway, and phospholipase Cγ pathways[82]. Stimulation of OSCC cells by EGF showed an induction of EMT in the cells, which revealed morphological changes with the downregulation of E-cadherin and the upregulation of vimentin in the cells. In addition, stimulated cells showed enrichment of stem-like population ($CD44^+/CD24^-$) with an increase in CSC markers such as ALDH1 and BMI-1, suggesting that EGF may be responsible to induce CSC properties in OSCCs[83].

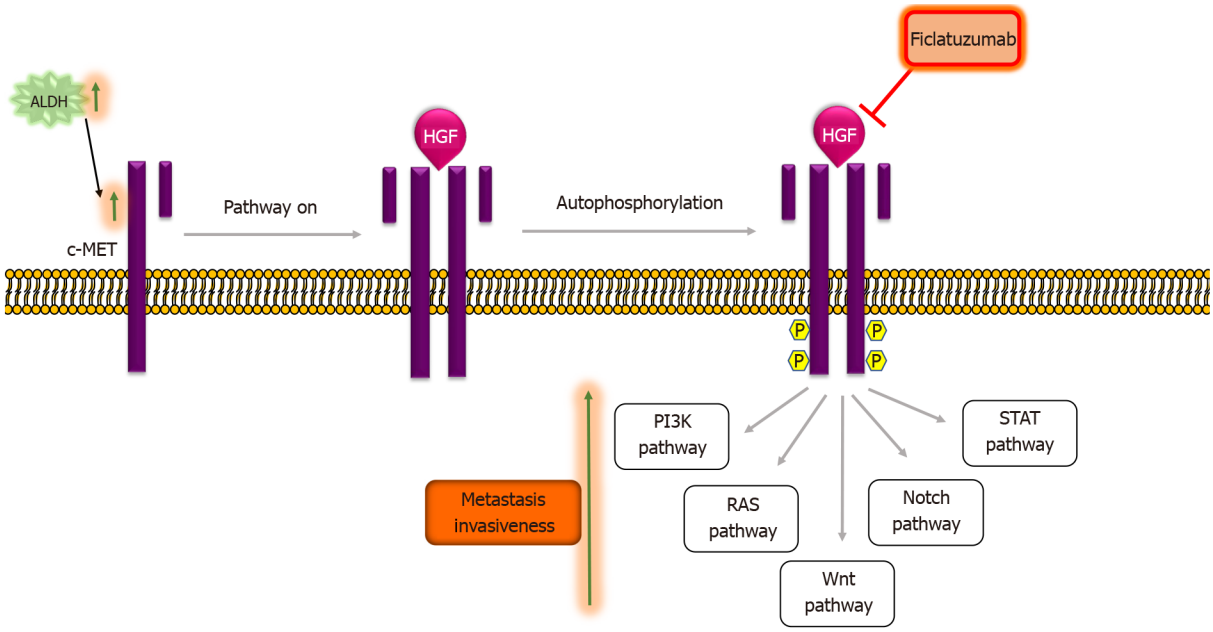

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Figure 4 The HGF/c-MET signaling pathway and cancer stemness. In the absence of the HGF ligand, the HGF/c-MET pathway remains inactivated. The binding of the HGF ligand to the c-MET kinase receptor results in the dimerization of two subunits that further leads to auto-phosphorylation of the tyrosine residues in the cytoplasmic domain of the receptor creating a docking site for adaptor proteins, which regulate pathways, such as PI3K, RAS, Wnt, Notch and STAT pathway. Transcriptional levels of c-MET were high in ALDH^{high} cells[70]. Activation of c-MET results in an increase in invasiveness and metastasis *in vitro* as well as *in vivo*[73, 74]. Ficlatuzumab is a humanized IgG1 monoclonal antibody targeting HGF used to therapeutically target the HGF/c-met pathway. ALDH: Aldehyde dehydrogenase; Ficlatuzumab: HGF/c-MET pathway inhibitor.

Ephrin (EPH) receptors and their ligands play important roles in controlling the actin cytoskeleton and cellular responses, including attraction/repulsion, migration, and cell positioning during developmental stages[84]. Recent report has shown regulation of the expression of stemness markers by EPHA10. EPHA10 and its ligand EFNA4 increased cell migration, sphere formation, and expression of markers such as SNAIL, NANOG, and OCT4 in OSCC cells. It was also reported that high mRNA levels of EFNA4 with NANOG or OCT4 co-related with poor survival patterns in OSCC patients[85].

CURRENT TREATMENT REGIME

The current treatment regime for HNSCCs is dependent on the TNM staging of the carcinoma based on T (tumor size considering the depth of invasion), N (nodal metastasis considering extranodal extension or ENE), and M (presence of distant metastasis)[86]. For early stage of cancer, single modality treatment, in which mostly surgery, is preferred. With the progression of disease manifested either through larger tumor dimensions or nodal metastasis, multimodality in treatment is employed, wherein surgery is followed by adjuvant chemotherapy or radiotherapy. In case of distant metastasis, where surgical intervention is difficult, chemotherapy is the preferred choice. Further, administration of adjuvant chemo or radiation therapy following surgery is shown to exhibit better patient survival[87]. Chemotherapeutic agents commonly administered are cisplatin, carboplatin, docetaxel, 5-fluorouracil, methotrexate, and paclitaxel. Radiotherapy is also employed in cycles of 1.8-2 Gy/day with a total dose of 66-72 Gy[88]. Targeted therapies, specifically acting on certain upregulated pathways, such as EGFR, are administered. For example, cetuximab targets the EGFR pathway in HNSCC patients or anti-PD1 agents for immunotherapy in HNSCC. Agents such as pembrolizumab and nivolumab, which target PD-1, have shown promising results in clinical trials[87]. Although, recent reports highlighted that owing to heterogeneity in PD-L1 expression throughout tumors and utilization of different methods and antibodies, there might arise errors in immuno-histochemical assessment of PD-L1 prior to therapy decisions[89-92]. In the course of currently existing assessment methods across various cancers, Marletta *et al*[91] observed that in HNSCC, the registration trial utilized the 22C3 clone (Dako) on Agilent autostainer link 48 while the European Medicines Agency granted administration of immunotherapy regardless of antibody, and the instrument used. The reports emphasized on the establishment of a standardized uniform protocol considering the heterogeneity of expression as well as the antibodies and platforms used for the assessment of PD-L1 before deciding whether immunotherapy should be administered to the patients[89-92].

Table 1 Clinical trials currently active for head and neck squamous cell carcinoma

Region	Drug name	Target	Phase	NCT number
Worldwide	Everolimus	Inhibitor of mTOR	I	NCT01637194
	Cetuximab	Monoclonal anti-EGFR antibody		
	Bevacizumab (with fluorouracil and hydroxyurea)	Anti VEGF-A antibody	I	NCT00023959
	Trastuzumab (with IL-12)	Monoclonal anti-EGFR antibody	I	NCT00004074
	Erlotinib	Tyrosine kinase receptor (EGFR)	I, II	NCT00101348
	Cetuximab	Monoclonal anti-EGFR antibody		
	With or without bevacizumab	Anti VEGF-A antibody		
	Erlotinib hydrochloride	Tyrosine kinase receptor (EGFR)	I, II	NCT00101348
	Cetuximab	Monoclonal anti-EGFR antibody		
	Erlotinib hydrochloride	Tyrosine kinase receptor (EGFR)	I	NCT00397384
	Cetuximab	Monoclonal anti-EGFR antibody		
	Zalutumumab (after radiotherapy)	Monoclonal anti-EGFR antibody	III	NCT00496652
	Temsirolimus	mTORC1 inhibitor	II	NCT01256385
	With or without cetuximab	Monoclonal anti-EGFR antibody		
	Cetuximab	Monoclonal anti-EGFR antibody	II	NCT00939627
	Sorafenib tosylate	Tyrosine kinase inhibitor		
	Cetuximab	Monoclonal anti-EGFR antibody	II	NCT01316757
	Erlotinib hydrochloride	Tyrosine kinase receptor (EGFR)		
	Varlilumab	Monoclonal anti-CD27 antibody	I, II	NCT02335918
	Nivolumab	Monoclonal anti-PD-1 antibody		
	MEDI7247	Monoclonal anti-ASCT2 antibody conjugated with pyrrolobenzodiazepine dimer	I	NCT03811652
	Cetuximab with lenalidomide	Monoclonal anti-EGFR antibody	I	NCT01254617
	Durvalumab	Monoclonal antibody that blocks PD-1/PD-L1 interaction	I	NCT03144778
	With or without tremelimumab	Monoclonal antibody against CTLA-4		
	Sitravitinib	Inhibitor of receptor tyrosine kinases	Early phase I	NCT03575598
	Nivolumab	Monoclonal anti-PD-1 antibody		
	Nivolumab	Monoclonal anti-PD-1 antibody	I	NCT03229278
	Pembrolizumab with trigriluzole	Monoclonal anti-PD-1 antibody		
	BKM120	PI3K inhibitor	I, II	NCT01816984
	Cetuximab	Monoclonal anti-EGFR antibody		
	FATE-NK100		I	NCT03319459
	Cetuximab	Monoclonal anti-EGFR antibody		
	Trastuzumab	Monoclonal anti-EGFR antibody		
	Nivolumab with SBRT	Monoclonal anti-PD-1 antibody	II	NCT02684253
	BYL719	PI3K inhibitor	II	NCT03292250
	Poziotinib	EGFR inhibitor		
	Nintedanib	Angiokinase inhibitor		
	Abemaciclib	CDK4 and CD6 inhibitor		
	Durvalumab	Monoclonal antibody that blocks PD-1/PD-L1 interaction		

	Tremelimumab	Monoclonal anti-CTLA-4 antibody		
	Nivolumab and tadalafil	Monoclonal anti-PD-1 antibody	Early phase I	NCT03238365
Involving Indian institutes	Gefitinib (Iressa) with cisplatin and radiotherapy	EGFR inhibitor (tyrosine kinase inhibitor)	II	NCT00229723
	Lapatinib	EGFR inhibitor (tyrosine kinase inhibitor)	II	NCT00371566
	Gefitinib with methotrexate	EGFR inhibitor (tyrosine kinase inhibitor)	III	NCT00206219
	P276-00 with EBRT	CDK inhibitor	I, II	NCT00899054
	P276-00	CDK inhibitor	II	NCT00824343
	Lapatinib with chemoradiation	EGFR inhibitor (tyrosine kinase inhibitor)	III	NCT00424255
	Lapatinib with chemoradiation	EGFR inhibitor (tyrosine kinase inhibitor)	II	NCT00387127
	MEDI4736	Monoclonal antibody blocking interaction between PD-L1 with its receptors	III	NCT02551159
	Tremelimumab with chemotherapy	Monoclonal anti-CTLA-4 antibody		

SBRT: Stereotactic radiation therapy.

Recently, a clinical trial consisting of 13 TSCC patients showed that immunotherapy of cytokine-induced killer cells after chemotherapy helped in the reversion of immunosuppression caused during chemotherapy and surgery. Twelve out of thirteen patients survived for more than 90 mo post-therapy. No immune system related toxicities were reported in the surviving patients. No other side effects of the treatment were observed except complaints of aphthous ulcers by two patients[93]. This showed that multiple modality treatments might improve the survival and quality of life of the patients. However, for further improvement in treatment, more targets specific to CSCs need to be explored.

There have been few clinical trials worldwide as well as those involving Indian institutes for newer molecules targeting EGFR pathway, immune checkpoints, PD-L1, cyclin-dependent kinases, *etc.*, as single treatment agent or in combination with other chemotherapeutic agents/radiation in HNSCC (Table 1). However, these trials are still in the early phases and do not particularly target CSCs. Therefore, further detailed study is essential in finding newer targets specific to CSCs in HNSCC.

CONCLUSION

The CSC population plays an important role in therapy resistance, recurrence, and metastasis. These factors adversely affect patients' survival. In spite of several years of research, most of the treatment regimes employed currently target the tumor bulk. CSCs possess self-renewing property, slow cycling, aberrant cell signaling, dynamic ABC transporter system, DNA repair mechanism, epigenetic modifications, and metabolic regulation, *etc.*, which enable CSCs to escape this therapy and thereby play an important role in recurrence, therapy resistance, and loco-regional/distant metastasis.

A better understanding of the aberrant signaling pathways involved in poor prognosis and maintenance of the CSC population would be more effective in improving treatment outcome. Such an understanding would also be important in the prognosis, prediction, and designing of treatment regime that not only reduce the bulk of the tumor but also effectively eliminate the CSC population thereby improving patient survival.

Molecules specifically targeting signaling pathways that regulate the CSC population administered in combination with conventional therapy or as a single treatment modality need to be studied in TSCC. Uncovering the signaling pathways for CSCs, and targeting them would enable better clinical outcomes.

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MINIREVIEWS

Human pluripotent stem cell-derived extracellular vesicles: From now to the future

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Abstract

Extracellular vesicles (EVs) are nanometric particles that enclose cell-derived bioactive molecules in a lipid bilayer and serve as intercellular communication tools. Accordingly, in various biological contexts, EVs are reported to engage in immune modulation, senescence, and cell proliferation and differentiation. Therefore, EVs could be key elements for potential off-the-shelf cell-free therapy. Little has been studied regarding EVs derived from human pluripotent stem cells (hPSC-EVs), even though hPSCs offer good opportunities for induction of tissue regeneration and unlimited proliferative ability. In this review article, we provide an overview of studies using hPSC-EVs, focusing on identifying the conditions in which the cells are cultivated for the isolation of EVs, how they are characterized, and applications already demonstrated. The topics reported in this article highlight the incipient status of the studies in the field and the significance of hPSC-EVs' prospective applications as PSC-derived cell-free therapy products.

Key Words: Pluripotent stem cells; Extracellular vesicles; Exosome; Cell-free therapy

Core Tip: The research on extracellular vesicles (EVs) derived from different cell types, such as adult stem cells, has shown potential in the treatment of various pathologies. However, little has been explored regarding EVs derived from human pluripotent stem cells (hPSC-EVs). In this review, we provide an overview of studies carried out on these EVs, highlighting methodologies used for the culture of hPSCs for isolating EVs, their characteristics, and potential applications. We note the potential of hPSC-EVs as future acellular therapies. However, studies are in the infancy, and more research is needed to confirm their benefits.

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INTRODUCTION

Extracellular vesicles (EVs) are nanometric particles that are enclosed by a lipid bilayer and released by all cell types. They lack a functional nucleus and are therefore unable to replicate[1]. EVs are composed of bioactive factors such as lipids, proteins, and nucleic acids, including mRNAs and non-coding RNAs [2]. EV is an umbrella term that encompasses a heterogeneous population of membrane vesicles generated through a variety of mechanisms. The two major EV subpopulations include microvesicles (MVs) and exosomes (EXOs). EXOs are intraluminal vesicles of endosomal origin released when multivesicular bodies fuse with the plasma membrane, whereas MVs or ectosomes are generated from the outer budding of the plasma membrane[3]. Due to their distinct biogenesis, MVs are generally larger (up to 1000 nm in diameter) than EXOs (less than 200 nm). However, these vesicle populations overlap not only in terms of size but also in composition[4]. Recently, other nomenclatures were described in the "Minimal information for studies of extracellular vesicles 2018" guidelines (MISEV2018) based on the physical characteristics of EVs, for example, size (< 200 nm, small EVs; > 200 nm, medium or large EVs) or density (low, middle, or high)[1].

Potential uses of EVs, such as for the diagnosis and treatment of pathologies or as potential drug carriers, have been investigated. In the field of regenerative medicine, the secretomes of adult stem cells, primarily mesenchymal stem/stromal cells (MSCs), including their EVs, are of great interest as they have been shown to act mainly in a paracrine manner rather than their potential for differentiation[5]. An interesting list of advantages and disadvantages of the use of EVs instead of stem cells has been presented by Öztürk *et al*[6]. Among the advantages of using EVs cited by them and others are low immunogenicity and toxicity; minimal risk of malign transformation; minimal risk of getting trapped in the lung or causing vasculature obstruction; avoidance of contamination with undesired cell types; avoidance of uncontrolled cell division; the ability to manipulate EVs in order to obtain potential improvements; optimization of MSC culture to obtain a higher amount of EVs; and their ability to cross the blood-brain barrier, among others[4,6]. In addition, EVs mimic the beneficial effects of MSCs in cell therapies in a wide range of animal models for different diseases[7-9].

MSC-derived EV (MSC-EV) has been extensively studied and has demonstrated several promising effects, as reviewed by Gowen *et al*[10], Tieu *et al*[11], Fuloria *et al*[12], Kou *et al*[13], and Yudintceva *et al* [14]. However, despite the high potential of MSC-EVs, several factors limit their use. Recently some reviews highlighted the difficulty of establishing criteria to define the specific characteristics of MSC-EV and discussed the great variation in the MSC-EV preparations[15,16]. Disadvantages of MSCs as a source for EVs include the variability between cells derived from different tissues, the variability between different donors, their limited ability to proliferate, the fact that they enter senescence, and genomic instability after a few passages[17]. This raises the question of whether pluripotent stem cell (PSC) derived EVs have a similar to or better therapeutic potential than adult stem cell-derived EVs.

In this context, our objective is to show, using a non-systematic search, studies that use or characterize EVs derived from human PSC (hPSC-EVs) to understand the advances in the area. We also aim to identify the conditions in which the cells are cultivated for the isolation of EVs, how these are characterized, and any demonstrated applications (*in vivo* or *in vitro*).

HPSC-EVS

Overview of hPSCs

hPSCs are characterized by unlimited proliferation and the potential to generate specialized cell lineages [18]. Human embryonic stem cells (hESC) were first isolated from human blastocysts in 1998 by Thomson *et al*[19], and to date, hundreds of hESC lineages have been established worldwide. hESC-based therapeutic technologies have applications in many diseases and conditions, such as spinal cord injuries, age-related tissue degeneration, and diabetes[20]. However, ethical issues related to using cells from embryos have hindered the application of hESCs in research and treatment, leading to the development of the induced PSC (iPSC) technology by Takahashi and Yamanaka[21] and Takahashi *et al* [22]. Since the generation of the first iPSC, many research groups have developed human iPSC (hiPSC) lineages reprogrammed from different adult cells, and obtained lineages very similar to hESC in terms of morphology and differentiation potential[23]. For more information about hPSCs, see Karagiannis *et al*[24], Liu *et al*[25], and Yamanaka[26].

Especially after the discovery of hiPSCs, pluripotent cells represented a promising alternative for regenerative medicine, transplants, disease modeling, and many other research applications[27-29]. The possibility of generating pluripotent cells from patients and, from them, differentiated cells for tissue repair may mitigate common transplant issues, such as immunologic rejection. Nevertheless, the immunogenicity of pluripotent cells remains controversial[30], and the potential for tumorigenesis hinders the wide application of these cells in clinics. The risks of contaminating the differentiated cell populations with remaining pluripotent or proliferative cells, as well as the transmission of active pluripotency transcription factors or the acquisition of mutations by the pluripotent cells during *in vitro* culture[26,31], limit the acceptance of hPSC-based therapies. Therefore, cell-free therapeutic approaches, including EVs, offer promising possibilities for applying hPSC-derived products[32].

It seems that the role of the secretomes of these cells has only recently begun to be investigated, possibly due to the difficulties still encountered in using hPSCs in the clinic. Some interesting studies show that EVs from ESCs could help with embryo implantation[33] and maintaining ESC stemness[34], while others have investigated the biogenesis of ESC-EVs[35,36], although they used murine PSCs. We will focus this review on studies with hPSCs due to their potential clinical applications.

HPSC-EVs: Isolation and characterization methodologies

The first investigation on the isolation of EVs from hPSC dates from 2015. In this initial approach, EVs were isolated from hiPSC cultured in Essential 8™ medium using differential centrifugation (DF)/ ultracentrifugation (UC). It was shown that the hiPSC-derived EVs (hiPSC-EV) contain a variety of microRNAs (miRNAs) (such as miR-382, miR-611, and others) related to pathways such as focal adhesion, Wnt, PI3K-Akt, and MAPK signaling, as well as proteins related to processes involved in signal transduction, receptor binding, and others. In addition, the EVs positively affected the metabolism, proliferation, apoptosis rate, and differentiation capacity of cardiac MSCs. Better results were obtained when cells were exposed for only 22 h to EVs[37]. This initial attempt demonstrated how hPSC-EVs could be beneficial and of interest for future acellular therapy applications.

Despite the potential of hPSC-EVs, we observed that the number of publications in this area is still low, and most of the existing publications date from the last five years (Figure 1A, Table 1). Some studies evaluate EVs that were isolated during the differentiation process or from cells that differentiated from PSCs, such as hiPSC-derived keratinocytes[38]; hPSC-derived cardiac progenitors or cardiomyocytes[39-41]; hPSC-derived MSCs[42-44]; hiPSC-derived neurons[45-47]; and hESC-derived chondroprogenitor cells[48]. However, our review explores studies that isolated EVs from undifferentiated hPSCs.

Using a non-systematic search, we found 36 studies that isolate hPSC-EVs mainly from the hiPSC lineages (Figure 1B). Table 1 summarizes these studies, highlighting the cell culture medium used to culture the PSC, time of conditioned medium collection, EV isolation method, and EV mean size. The most common culture media were commercial, with defined components (Figure 1D). The two most common media used were mTeSR™1 (StemCell Technologies) and Essential 8™ medium (Thermo Fisher) (Table 1). A study published by Luo *et al*[49] aimed to optimize culture conditions for isolation of hiPSC-EVs. Using DMEM with different concentrations of EV-depleted KnockOut™ Serum Replacement (ED-KSR), they observed that cells remained viable at a 0.5% ED-KSR concentration and were able to isolate EVs from PSCs cultured in this condition efficiently. However, after 5 d of culture, there was a reduction in the expression of some pluripotency markers. Thus, although it may be cheaper than commercial media, it is necessary to consider the additional step of centrifugation of the KSR to remove particles, as well as the effects of the change in pluripotency-related parameters on the composition and potential of the EVs.

The biggest variations in EV isolation methods relate to the collection time of the conditioned medium: Many studies do not state the conditioning time. In most studies, however, the EVs were isolated after 24 h of cell culture or every 24 h for 3-5 consecutive days (Table 1), avoiding exceeding the 80%-90% cell confluence in the cell cultures. This collection time is possibly related to the nature of PSCs, as the culture medium must be changed daily, and cells must not reach 100% confluence to guarantee their viability and pluripotency.

Other relevant aspects of EVs are their size, morphology, and estimated particle concentrations. Most studies presented the information listed in MISEV2018, including positive and negative protein markers in EVs, usually using the western blot technique (31/36 articles) and performing a single EV analysis mainly using transmission electron microscopy (31/36 articles) to verify EV morphology and nanoparticle tracking analysis (20/36 articles) to verify their mean size and concentration (Figure 1C). The greatest number of studies used small EVs/EXOs, with sizes up to 200 nm (small EVs) (Table 1).

The most common method for hPSC-EV isolation is DF (here defined as the initial centrifugations to remove cellular debris and apoptotic bodies) followed by UC (Table 1). Although this is the most common method used, it is unsuitable for isolating EVs from large-scale experiments and clinical trials. Using a large-scale 2D culture, Andrade *et al*[69] isolated hPSC-EVs using tangential flow filtration (TFF) with or without subsequent UC (TFF + UC). The isolated EVs presented a size of approximately 100 nm, regardless of whether UC had been performed, with similar particle concentration, although TFF + UC resulted in a smaller number of proteins. The effect of different culture conditions (hypoxia - 1% O_2, physiological hypoxia - 5% O_2, and normoxia) on the therapeutic potential of hPSC-EVs was also

Table 1 Human pluripotent stem cell-derived extracellular vesicles: Methods of isolation and vesicle size

Ref.	Culture medium	EV collection time	EV isolation method	EV mean size (nm)
Bobis-Wozowicz *et al*[37], 2015	Essential 8™ medium	NI (cells in 70%-90% confluency)	DC + UC	146
Ju *et al*[50], 2017	PSCeasy medium (Cellapy)	24 h	DC + UC	122, 132
Zhou *et al*[51], 2017	mTeSR™-1 medium	NI (cells in 60%-90% confluency)	DC + 0.22 μm filter + UC	101
Ding *et al*[52], 2018	mTeSR™-1 medium	48 h	DC + UC	103.1
Kaur *et al*[53], 2018	Essential 8™ Flex medium	48 h	DC + UC or miR-CURY™ Exosome Isolation Kit (Exiqon A/S)	100-200
Kobayashi *et al*[54], 2018	DMEM-F12 + NEAA, 200 mM L-gln, KSR, 0.1 M BME	2-3 d before passage	MagCapture Exosome Isolation Kit PS (Wako)	100
Oh *et al*[55], 2018	Essential 8 medium	Daily, from day 2 to day 5	0.45 μm filter + ExoQuick-TC kit	85.8
Peng *et al*[56], 2018	mTeSR™-1 medium	24 h (cells in about 80% confluency)	MV: DC + 16500 g, 1 h; EXO: DC + 120000 g, 2 h	MV = 200-600; EXO = 40-80
Saito *et al*[57], 2018	mTeSR™-1 medium	NI	DC + concentration in 100-KDa filter + MagCapture™ Exosome Isolation Kit PS	179
Chen *et al*[58], 2019	mTeSR™-1 medium	NI	DC + UC	50-150
Liu *et al*[59], 2019	Essential 8™ medium	Daily, for 3-5 d	DC + concentration in 100-kDa filter + SEC	150
Marzano *et al*[60], 2019	mTeSR™-1 medium	Daily, for 4 d	0.22 μm filter + concentration in 100-kDa filter + Total Exosome Isolation Reagent (Thermo Fisher) or DC + UC	about 240
Povero *et al*[61], 2019	NI	24-48 h	DC + UC	300-400
Sun *et al*[62], 2019	mTeSR™-1 medium	48 h	DC + concentration in 100-kDa filter + Exosome Isolation Kit (PureExo) + UC + 0.22 μm filter	70-100 (cell-dependent)
Zhu *et al*[63], 2019	mTeSR™-1 medium	48h (cells 80%-90% confluency)	DC + 0.22 μm filter + UC	70.2
Collino *et al*[64], 2020	mTeSR™-1 medium	24 h	DC + UC	119
Hu *et al*[65], 2021	ncEpic hPSC medium	NI	DC + 0.22 μm filter + UC	72.4 ± 21.3
Kurtzwald-Josefson *et al*[66], 2020	DMEM/F12 Ham 1:1 + 20% KSR, 1% NEAA, 1% L-gln, 0.2% BME, 4 ng/mL rhFGF basic	24 h (cells in about 80% confluency)	Total exosome isolation reagent (Thermo Fisher Scientific)	115 ± 7
Liu *et al*[67], 2020	mTeSR™-1 medium	NI	DC + UC	50-75
Wang *et al*[68], 2020	PGM1 medium	NI	DC + 0.22 μm filter + UC	30-120
Andrade *et al*[69], 2021	mTeSR™-1 mediuma	Daily, for 4-5 days	TFF with or without subsequent UC	103-109
Ashok *et al*[39], 2021	StemMACS medium with 10 μM ROCK inhibitor and 0.2% Pluronic F68	Days 3, 4, and 5 prior to differentiation	DC + 0.22 μm filter + UC + SG	50
Hu *et al*[65], 2021	ncEpic hPSC medium	NI	DC + 0.22 μm filter + UC	-100
Karnas *et al*[70], 2021	Essential 8™ medium	NI	DC + UC	215.7
Ke *et al*[71], 2021	Exo-depleted FBS	48 h	MV: DC + 16500 g, 60 min; Exo: DC + 120000 g, 120 min	MV = 200-600; Exo = 40-80
Luo *et al*[49], 2021	DMEM/F12 + KSR (0.5%, 2.5%, 5%, or 20%)	Daily, for 5 d	DC + 0.45 μm filter + concentration in 10-kDa filter + 0.22 μm filter + UC or ExoQuick-TC kit (SystemBioscience)	187.8, 168.2
Saito *et al*[46], 2021	StemFit AK-03N medium (Ajinomoto)	NI	15000 × g, 30 min + 0.22 μm filter + UC	70

Wang *et al*[72], 2021	mTeSR™-1 medium	NI	DC + UC	120-140
Xia *et al*[73], 2021	Nuwacell hiPSC/hESCs medium	24 h	DC + 0.22 μm filter + UC	50-150
Bi *et al*[74], 2022	ncTarget medium (Nuwacell. Ltd, China)	24 h (cells in about 80% confluency)	DC + UC + 0.22 μm filter + UC	hESC = 133.1; hIPSC = 157.7
Gu *et al*[75], 2022	mTeSR™-1 medium	NI	0.45 μm filter + concentration in 100-kDa filter + GC + 0.22 μm filter + UC	143.5
Gupta *et al*[76], 2022	StemFlex™ medium	48 h	DC + one-step sucrose cushion UC	123.6 ± 60
Hsueh *et al*[77], 2023	StemFlex™ medium	48 h	DC + UC	136.8
Li *et al*[78], 2023	ncEpic hPSC medium	NI	DC + UC	74.70 ± 20.77
Li *et al*[79], 2022	mTeSR™-1 medium	NI	DC + 0.22 μm filter + UC	50.75-105.7
Pan *et al*[80], 2022	mTeSR™-1 medium	24 h (cells in about 80% confluency)	DC + UC	142.2 ± 64.1

DC: Differential centrifugation; EXO: Exosome; FBS: Fetal bovine serum; MV: Microvesicle; NI: Not informed; SEC: Size exclusion chromatography; SG: Sucrose gradient; TFF: Tangential flow filtration; UC: Ultracentrifugation; hPSC: Human pluripotent stem cell; hESC: Human embryonic stem cells; hIPSC: Human induced pluripotent stem cell; KSR: KnockOut™ Serum Replacement.

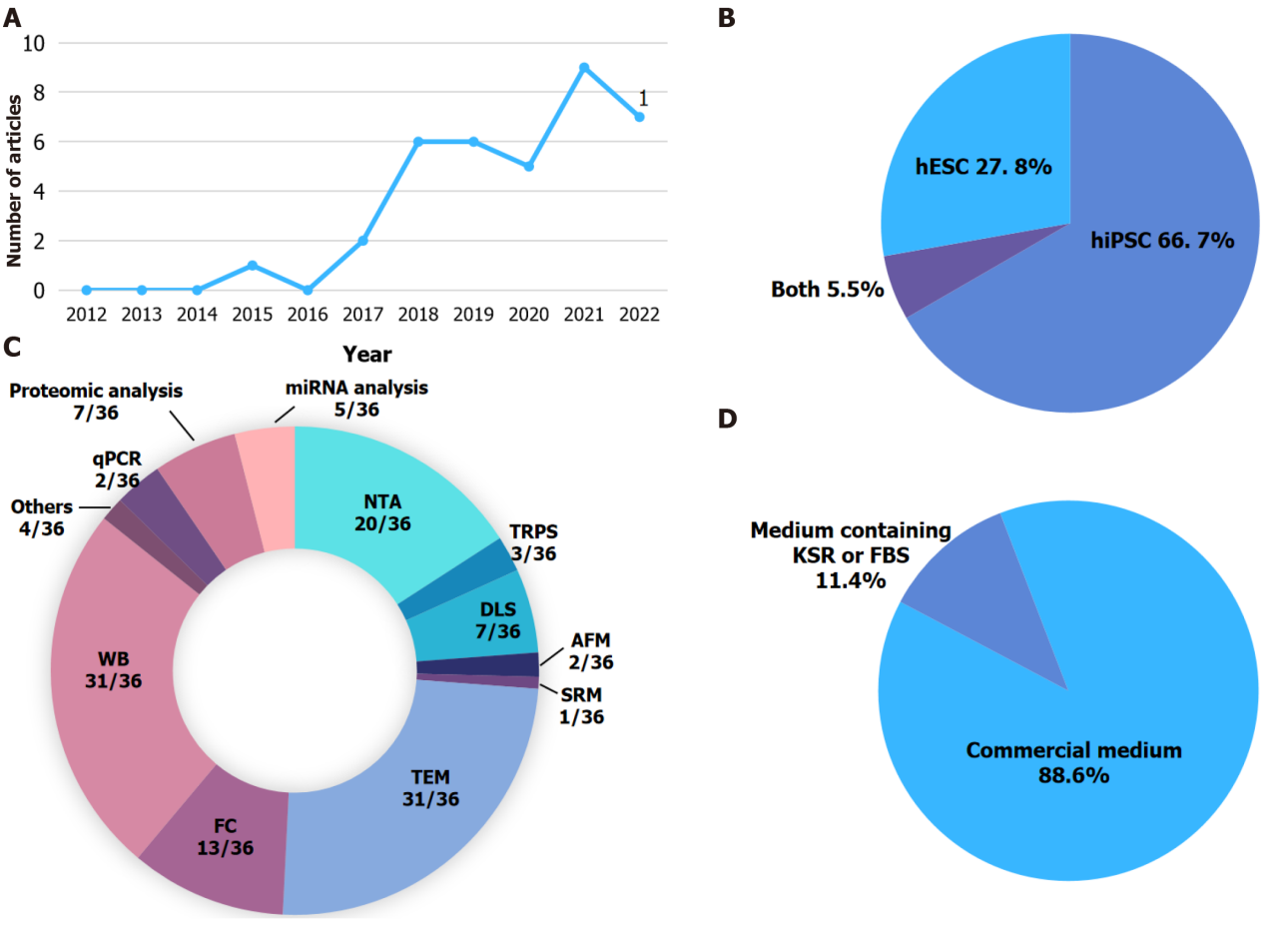

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Figure 1 Overview of studies on human pluripotent stem cell-derived extracellular vesicles published between 2012 and 2022. A: Timeline of published articles on human pluripotent stem cell-derived extracellular vesicles (hPSC-EVs). [1]Two articles were published online in 2022 but published in print in 2023; B: Analysis of the percentage of articles that use human embryonic stem cells, human induced pluripotent stem cell, or both cell types to isolate EVs; C: Methods used in the studies to characterize hPSC-EVs. The graphic depicts the number of articles using certain techniques/total number of articles included in the analysis; D: Analysis of media used to culture hPSC to isolate the EVs. AFM: Atomic force microscopy; DLS: Dynamic light scattering; FC: Flow cytometry; NTA: Nanoparticle tracking analysis; qPCR: Quantitative polymerase chain reaction; SRM: Super-resolution microscopy; TEM: Transmission electron microscopy; TRPS: Tunable resistive pulse sensing; WB: Western blot; hESC: Human embryonic stem cells; hIPSC: Human induced pluripotent stem cell.

investigated. The results showed that EVs derived from hPSC cultured in 1% O_2 (hypoxia) had greater angiogenic potential than those derived under other conditions and that better results were achieved when obtaining EVs using TFF[69].

Another highly discussed topic about PSCs is the possible formation of teratomas, as well as the biodistribution of these cells when applied in *in vivo* models. These concerns also extend to PSC-EVs. To clarify these points, Gu *et al*[75] evaluated the safety and biodistribution of hiPSC-EVs. They used several approaches to show that PSC-EVs are safe, have no adverse effects on cells (*e.g.*, do not cause hemolysis), are not genotoxic, and can be administered by different routes (nasal, intramuscular, or intravenous) without generating adverse effects (*e.g.*, inflammation at the site or pathological changes in the organs of rats).

Potential therapeutic applications of hPSC-EVs

Although few investigations have been carried out with hPSC-EVs, we notice that almost all of them have already applied hPSC-EVs to different disease models, both *in vitro* and *in vivo*. PSC-EVs have been described as having: Protective effects in *in vitro* and *in vivo* models of ischemia-reperfusion kidney injury[64]; neural protective abilities[60]; the capacity to modulate neuroinflammation and protect against ischemic stroke through Treg cell expansion[73]; antifibrotic effects *in vivo* and in *in vivo* models of liver injury[61,72]; and reduced cartilage degradation in an osteoarthritis model[77]. They have shown improvements in wound closure, angiogenesis, and increased nerve fiber density in a wound-healing diabetic mouse model[54,79]; and improved recovery of ovarian function in a premature ovarian failure mouse model[67]. EVs were also associated with acellular nerve grafts, demonstrating their potential to repair peripheral nerve defects[80].

It was also demonstrated that MVs, but not EXOs, dedifferentiated Müller cells into retinal progenitor cells *in vitro*[71]. Other studies showed the ability of PSC-EVs to promote regeneration of diseased or damaged retinas[56] and to accelerate corneal epithelium defect healing *in vivo*[68]. Other potential uses cited for PSC-EVs are: In antitumoral activity[51,63]; in angiogenesis stimulation[69]; as a gene delivery vector[50]; to increase the functional properties of cord blood-derived hematopoietic stem and progenitor cells[70]; and to improve the number of beating EBs depending on the hiPSC origin[66].

One noteworthy effect shown in some studies is the capacity of PSC-EVs to "rejuvenate" different cell types, such as senescent endothelial cells[52,58], senescent human dermal fibroblasts[55], senescent chondrocytes[77], and others. Considering this potential, the hPSC-EVs, hESC-EVs, and hiPSC-EVs were investigated as therapeutic tools for age-related diseases. Regarding neurological diseases, the hPSC-EVs showed potential in recovery of senescent hippocampal neural stem cells in rats with vascular dementia - partially through the transfer of miRNAs that inhibit mTORC1 activation - resulting in an improvement in disease status (*e.g.*, reverse cognitive impairment)[81]. Furthermore, using mice of varying ages, hPSC-EVs were found to rejuvenate hippocampal neural stem cells partly through the transfer of SMAD proteins that activate myelin transcription factor 1 (MYT1), which is reduced in senescent cells, and activates a signaling cascade in the MYT1-Egln3-Sirt1 axis[81].

In an ischemic stroke model, hPSC-EVs reduced the expression of inflammatory cytokines and leukocyte infiltration, and increased the number of regulatory T cells and other immunomodulatory effects that alleviate neurological deficits[73]. They also reduced blood-brain barrier damage in aged stroke mice through blood-brain barrier rejuvenation, partially through the transfer of AKT1 and CALM from EVs to endothelial cells leading to activation of the endothelial nitric oxide synthase-Sirt1 axis[78]. Therefore, hPSC-EVs could be a promising cell-free therapy to treat age-related diseases associated with cellular senescence.

In order to evaluate the benefit of hPSC-EVs compared to other EVs, one interesting study demonstrated that both hiPSC-EVs and hMSC-EVs, isolated through size exclusion chromatography (Table 1), could improve the proliferation of senescent MSCs and alleviate cellular aging in a replicative aging model, possibly modulating reactive oxygen species production with peroxiredoxins presented in EVs. However, despite the similar effects, EVs derived from iPSCs enter target cells more efficiently, and the production of hiPSC-EVs was about 16-fold higher than that of MSC-EVs (using the same culture medium)[59].

hPSC-EV composition

Even though many articles described the effects of hPSC-EVs, few made deeper characterizations of, for example, the protein and miRNA content of these EVs. Some performed proteomic analysis to help explain some of the effects[59] or as a control (time 0) to study the differentiation process[39,46]. In one interesting approach using high-density lectin microarray, Saito *et al*[57] demonstrated that rBC2LCN, a specific lectin for hPSCs, bound to hiPSC-derived EVs but not to adipose-derived stem cell-, hemodiafiltration-, or chondrocyte-derived EVs, which suggests a particular glycan-signature for hiPSC-EVs, resembling the glycome signature of the cell surface.

One recent study that provided a detailed description of the contents of hPSC-EVs was conducted by Bi *et al*[74]. The proteomics of hESC-, hiPSC-, and hMSC-EXOs showed that the main enriched proteins were related to distinct pathways between vesicles of pluripotent and multipotent cells. In hPSCs, EXO content was more focused on development, metabolism, and anti-aging properties, and in hMSCs, it was related to immune regulation. Another study in 2022 also indicated that hMSC-EV content is

strongly related to immune regulation while hPSC-EV content does not present many of the proteins related to this function[76]. Actually, 79 proteins were found to be shared between hMSC- and hPSC-EVs, yet the main biological processes related to them were DNA regulation, signal transduction and cell communication[76]. Liu *et al*[59] also compared the protein content of hiPSC-EVs and hMSC-EVs and described more than 1100 proteins shared between the different EVs, allowing to identify proteins that could be responsible for the anti-senescent effect observed in the study.

Considering the protein content of hESCs and hiPSC-EXOs, Bi *et al*[74] suggested that hESC-EXOs are more prone to regulate development and pluripotency pathways, and hiPSC-EXOs have a stronger correlation with metabolism. Regarding the most enriched miRNAs for both hPSC-EVs, it was shown that they were related to cell cycle and metabolism regulation. Interestingly, miRNAs found in both hESC-EXOs and hiPSC-EXOs were involved in cell differentiation, development, and cell cycle, even though the hiPSC-EXO set of miRNAs seemed to play a less significant role in these functions than the hESC-EXO set[74].

In order to explore whether apoptosis-linked gene 2-interacting protein X (ALIX), a protein present in the endosomal sorting complex required for the transport and biogenesis of EXOs, could regulate the protein content of EV, Sun *et al*[62] isolated EVs from hiPSCs in which ALIX was overexpressed (using lentiviral transduction) or were knocked out (using CRISP-Cas9 system). EVs isolated from these cell lineages were of similar size, although EVs generated from knockout cells were slightly larger. The evaluation of protein content in EVs showed that those derived from knockout cells had fewer proteins, while EVs from overexpressing cells presented a higher number of proteins. These differences could be related to the differences demonstrated in functional assays, *e.g.*, cell viability, apoptosis inhibition, and formation of capillary-like structures, where EVs from overexpressing cells had better effects. So, EVs with different protein profiles could have different therapeutic applications.

CONCLUSION

Although hPSC cultivation has been carried out for some time, the requirements for *in vitro* culture of these cells are very specific, as many factors are necessary to maintain them in their undifferentiated state. This, together with the cost, could be one of the reasons why secretomes and isolation of hPSC-EVs have not been extensively studied so far. Commercial media are now defined with a few components that are no longer as expensive as before, which may have contributed to the increase in publications in recent years.

An overview of the hPSC-EV studies is shown in Figure 2, which illustrates the potential use of these EVs for regenerative medicine. Regarding EV characterization, we observed in the publications that hPSC-EVs follow the basic requirements described in MISEV2018. However, despite the recent increase in research in this area, further characterization of the content of these EVs needs to be carried out. In addition, studies with modified cells aimed to enrich the content of EVs with some specific protein or miRNA may be of great interest. One interesting approach requiring more extensive discussion is the possible use of hPSC-EVs in reprogramming adult cells into PSCs. A recent study used EVs derived from ESCs undergoing cardiac differentiation to transdifferentiate fibroblasts to cardiomyocyte-like cells with relatively high efficiency[82].

Our review shows that hPSC-EVs have therapeutic potential, although no publications demonstrate that they are effectively better than other EVs, such as hMSC-EVs. hPSC can be obtained from different sources (embryonic or reprogrammed from adult cells) and, despite showing some heterogeneity between lineages, they are highly similar in their main characteristics: They are pluripotent and with a high proliferative capacity. The latter makes it possible to obtain a large number of EVs. It should be noted that PSC-EV derived from different hPSC lineages may show some variability in their content. But considering the fact that we can isolate EVs from a single source (a homogenous culture), this can possibly bring less variability between batches compared to other common EV sources. However, studies in this area are still needed as current results are highly variable. Alternatives to EVs include the use of cell-engineered nanovesicles generated by serial extrusion of hiPSCs, as described by Lee *et al*[83], which presented similar results to PSC-EVs, but with higher production yield. However, more studies are needed to verify the viability of this method for future applications. Thus, challenges that remain are the large-scale production of EVs, which in the case of hPSC cultivation can be expensive, and the investment in efficient methodologies for EV isolation that could be used in good manufacturing practices for future acellular therapies.

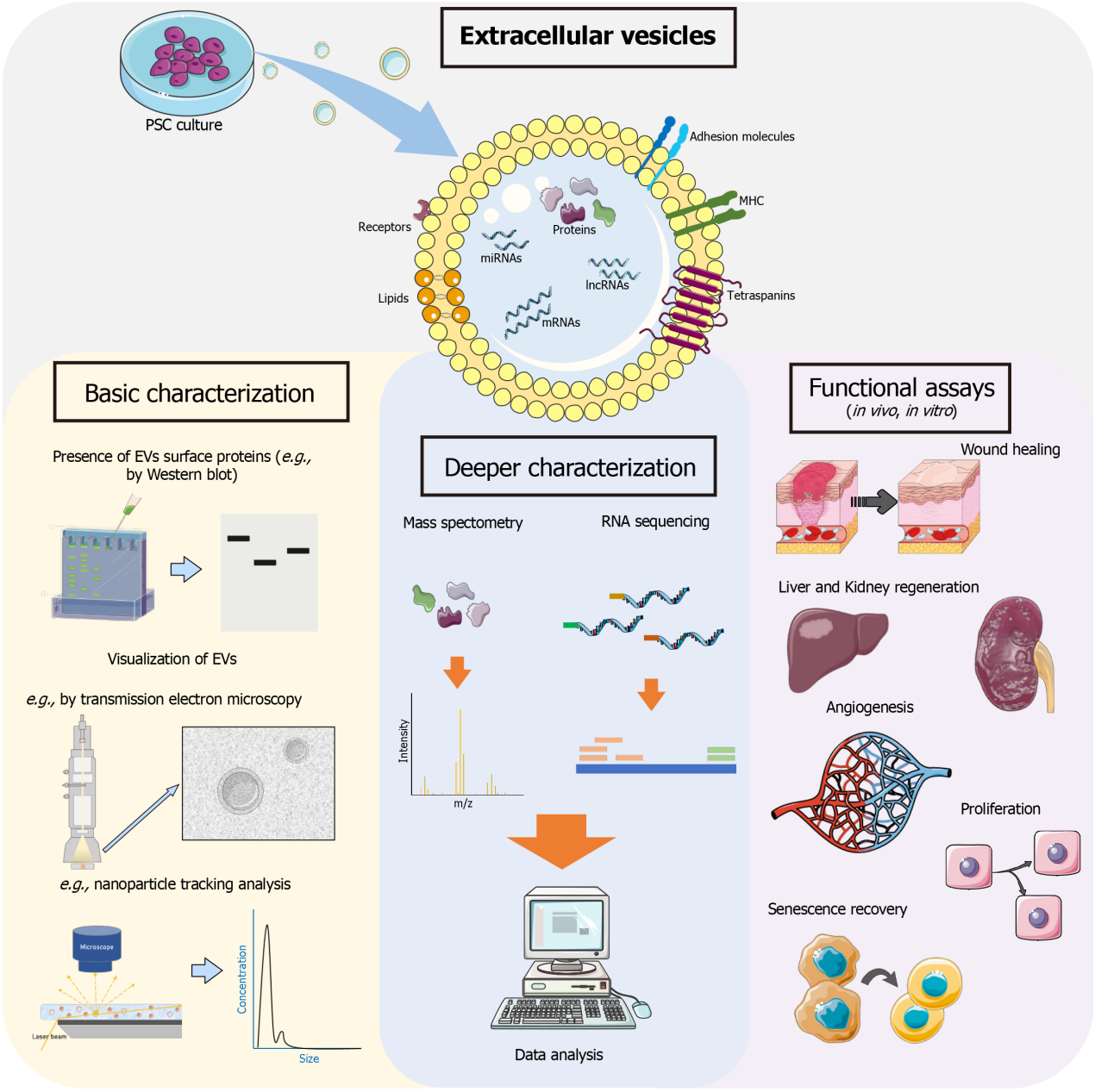

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Figure 2 Diagram of pluripotent stem cell-derived extracellular vesicle isolation, its most common characterizations, and the applications described or indicated for these extracellular vesicles. EV: Extracellular vesicle; miRNA: MicroRNA; lncRNA: Long noncoding RNA; PSC: Pluripotent stem cell. The images were obtained from Servier Medical Art (http://smart.servier.com), licensed under a Creative Commons Attribution 3.0 Unported License.

FOOTNOTES

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MINIREVIEWS

Single-cell RNA sequencing in cornea research: Insights into limbal stem cells and their niche regulation

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Abstract

The corneal epithelium is composed of stratified squamous epithelial cells on the outer surface of the eye, which acts as a protective barrier and is critical for clear and stable vision. Its continuous renewal or wound healing depends on the proliferation and differentiation of limbal stem cells (LSCs), a cell population that resides at the limbus in a highly regulated niche. Dysfunction of LSCs or their niche can cause limbal stem cell deficiency, a disease that is manifested by failed epithelial wound healing or even blindness. Nevertheless, compared to stem cells in other tissues, little is known about the LSCs and their niche. With the advent of single-cell RNA sequencing, our understanding of LSC characteristics and their microenvironment has grown considerably. In this review, we summarized the current findings from single-cell studies in the field of cornea research and focused on important advancements driven by this technology, including the heterogeneity of the LSC population, novel LSC markers and regulation of the LSC niche, which will provide a reference for clinical issues such as corneal epithelial wound healing, ocular surface reconstruction and interventions for related diseases.

Key Words: Cornea; Limbal stem cells; Single cell RNA sequencing; Heterogeneity; Novel markers; Niche regulation

Core Tip: Limbal stem cells (LSCs), a cell population that resides at the limbus in a highly regulated niche. With the advent of single-cell RNA sequencing, our understanding of LSC characteristics and their microenvironment has grown considerably. This review focuses on the current research on single cell sequencing in LSCs. We highlight the heterogeneity, novel specific markers and niche regulation of LSCs.

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INTRODUCTION

The cornea is a unique transparent tissue in the human body exposed to the external environment and is the window for sight[1,2]. Specifically, the corneal epithelium acts as a protective barrier on the ocular surface and is constantly regenerating. This unique property of the corneal epithelium is dependent on self-renewing epithelial stem cells located at the limbus, known as limbal stem cells (LSCs)[3-5]. LSCs reside in the "palisades of Vogt" (also known as limbal epithelial crypts) and are critical for corneal epithelial regeneration and wound healing. LSCs respond to corneal epithelial cell renewal or wound healing by differentiating to produce limbal progenitor cells (LPCs) and transient amplifying cells (TACs), which then migrate to the central cornea to replenish the corneal epithelium[6-9]. This process was summarized as the XYZ hypothesis[10] and explained the balance of cell numbers and homeostasis in the corneal epithelium maintained by LSCs.

Like the stem cells in other tissues, the surrounding microenvironment or limbal niche strictly supports and regulates the functional behaviors of LSCs[11,12]. The limbal niche has unique characteristics and components, including mesenchymal cells, immune cells, melanocytes, vascular cells, extracellular matrix and signaling molecules (*e.g.*, growth factors and cytokines)[13-16]. Significant pathology involving any component of the limbal niche can lead to the dysfunction of LSCs or even result in limbal stem cell deficiency (LSCD), a disease that is characterized by impaired wound healing or blindness[17,18].

Various studies have identified numerous markers of LSCs but identifying definitive molecular signatures to distinguish LSCs and other corneal epithelial cells is still challenging. The unclear internal heterogeneity of the LSC population can increase the difficulty in efficiently isolating pure LSCs for clinical transplantation. In addition, emerging evidence supports that reconstruction of the limbal niche may be introduced to treat LSCD. Therefore, understanding the function and niche regulation of LSCs is needed to discover novel therapies for ocular surface disease.

With the development and maturity of sequencing technology, more and more genomic, transcriptomic, epigenetic and proteomic sequencing technologies have been applied to studying eye tissues [19-22]. In recent years, single-cell RNA sequencing (scRNA-Seq) technology has provided a powerful tool for discovery of new cell types and for dissecting their potential heterogeneity in unprecedented resolution[23-25]. For multicellular organisms, cell heterogeneity is defined by differences in genetic background, transcriptomic and proteomic profiles[26]. Compared to other traditional techniques for detecting the average expression of genes in multiple cells, single-cell sequencing can detect differential signals between individual cells, improve the resolution of research from the tissue to the cellular level [27-29]. A single-cell atlas has been compiled for several ocular tissues, such as the uvea[30], retina[31-34], iris[35,36], sclera[37,38] and human cornea[39,40]. In this review, we summarize the current advances on LSCs derived from single-cell studies to better understand the features and functions of LSCs and the precise cellular and molecular mechanisms of niche regulation. Overall, this review presents key points from recent discoveries to enrich our knowledge on LSC biology and ocular surface homeostasis reconstruction or other clinical problems.

HETEROGENEITY OF THE LSC POPULATION

LSCs are located in the basal layer of the corneal epithelium. As previously mentioned, they are characterized by a high proliferative potential, small size, high nucleoplasmic ratio and slow cell cycle[41,42]. LSCs are scarce, and finding bona-fide markers to distinguish them from other basal epithelial cells is challenging. In addition, few studies have investigated the heterogeneity and hierarchy of LSCs. Understanding the heterogeneity of LSCs is important for comprehending the function to effectively

isolate them for clinical transplantation.

Dou *et al*[43] performed scRNA-Seq on human limbal tissues and identified four subclusters of stem/progenitor cells after single-cell transcriptome analysis. In this study, the authors annotated eight cell types, including prominent limbal epithelial cells, stromal cells and other rare cell populations. The authors then subclustered limbal epithelial cells and resolved their heterogeneity, including limbal stem/progenitor cells (LSPCs), limbal suprabasal cells and limbal superficial cells. To further explore the LSC population, the authors then subclustered LSPCs and obtained four subpopulations (Figure 1) including: (1) A subpopulation with the classical LSC marker *TP63*[44]; (2) A subpopulation with high expression of *CCL20*, which is a chemokine that can induce cell migration and proliferation[45]; (3) A subpopulation with specific expression of *GPHA2*, a marker recently identified in quiescent LSCs (qLSCs) from humans and mice[46,47]; and (4) A subpopulation with high expression of *KRT6B*, which is associated with rapid keratinocyte division and contributes to inhibiting the migration of mitotic cell populations from the basal layer[48]. The authors then investigated the differences in stemness and differentiation status and observed that *TP63*$^+$ and *CCL20*$^+$ cells presented a high stemness state, whereas *GPHA2*$^+$ and *KRT6B*$^+$ showed a high differentiation state.

Another study by Li *et al*[49] annotated five subtypes from the limbal basal epithelium of the human cornea. They characterized terminally differentiated cells (TDCs), post-mitotic cells, TACs, LPCs and LSCs. Furthermore, the authors discovered that these five subtypes represented the major stages and trajectories of human LSC proliferation and differentiation (from LSCs, LPCs, TACs and post-mitotic cells to TDCs), and they were spatially situated in different regions from the limbus to the central cornea. In TDCs, corneal epithelium-specific differentiation markers and keratinocyte keratinization markers were expressed at the highest levels, while the LSC differentiation markers had the lowest expression.

LSCs in mice are also heterogeneous and behave differently than human LSCs. Altshuler *et al*[46] combined scRNA-Seq and quantitative lineage tracing for in-depth analysis of mouse limbal epithelium. The authors revealed the presence of two distinct subpopulations of mouse LSCs that were in separate and well-defined spatial locations called the "inner" and "outer" limbus (Figure 2). The inner limbus contains active LSCs, which maintain the homeostasis of the corneal epithelium. The outer limbus contains qLSCs that have a significantly lower rate of division and are involved in wound healing and border formation. Spectral tracking experiments displayed that qLSCs can quickly exit the dormant state and enter the cell cycle in response to injury, suggesting that qLSCs are a reservoir for tissue regeneration. In addition, their circumferentially extended clonal growth model and continuous localization on the border highly indicates that these cells play a crucial role in border maintenance. Notably, this finding was also confirmed by a study utilizing the two-photon live imaging approach [50]. Collectively, LSCs are highly heterogeneous in both humans and mice, unlike stem cells in other tissues. Further studies are needed to investigate the self-renewal and differentiation mechanisms of LSCs.

NOVEL MARKERS FOR LSCS

Since 1989, when LSCs were discovered[4], a series of markers have been found to identify these cells, such as *TP63, KRT3, KRT12*. However, the marker pattern typically labels the broad limbal basal cell population. Accurately distinguishing LSCs from other epithelial cells is still challenging and is still an active area of research. Altshuler *et al*[46] discovered a novel set of markers to accurately identify LSCs. They applied *in situ* hybridization probes for *Krt4* and *Krt12* to label mouse conjunctival and corneal basal and suprabasal cells, respectively. *Gpha2* staining could obviously demarcate the outer LSCs (also known as qLSCs), while the inner LSCs (also known as active LSCs) were labeled as *Atf3*$^+$. Then, they used immunofluorescence staining to confirm that the outer limbal epithelial basal cells were *Krt15*$^+$/*Ifitm3*$^+$/*Cd63*$^+$, and the inner limbal epithelial basal cells were *Atf3*$^+$/*Mt1-2*$^+$. Next, the authors explored the correlation between mouse and human LSC markers. Immunofluorescence images revealed that *KRT15*, *IFITM3* and *GPHA2* were expressed in human limbus epithelial basal cells. *Ifitm3* was found to be restricted to cellular vesicles in the cytoplasm of undifferentiated limbal cells, which was consistent with a previous study's findings[51]. *Ifitm3* knockdown led to a differentiation phenotype and a reduced colony-forming capacity. These experiments suggest that *Ifitm3* and *Gpha2* can be used to identify LSCs, and *Ifitm3* mediates the undifferentiated state.

Gpha2 has been frequently studied in human LSCs. Dou *et al*[43] explored the four subclusters of LSPCs, which were identified by *TP63*, *CCL20*, *GPHA2* and *KRT6B*. Collin *et al*[47] identified several novel genes, one of which was *GPHA2*, using an unbiased approach to recognize marker genes that were highly expressed in human LSCs relative to other corneal epithelial cells. High and specific expression of *GPHA2* was observed in the limbus crypts, which was consistent with the findings of Altshuler *et al*[46]. Moreover, the authors used RNA interference (RNAi) to downregulate *GPHA2* and observed a significant reduction in cell proliferation and differentiation efficiency, indicating an important role of GPHA2 in maintaining the undifferentiated state of human LSCs. The authors also performed flow activated cell sorting analysis with colony forming efficiency assays to confirm the

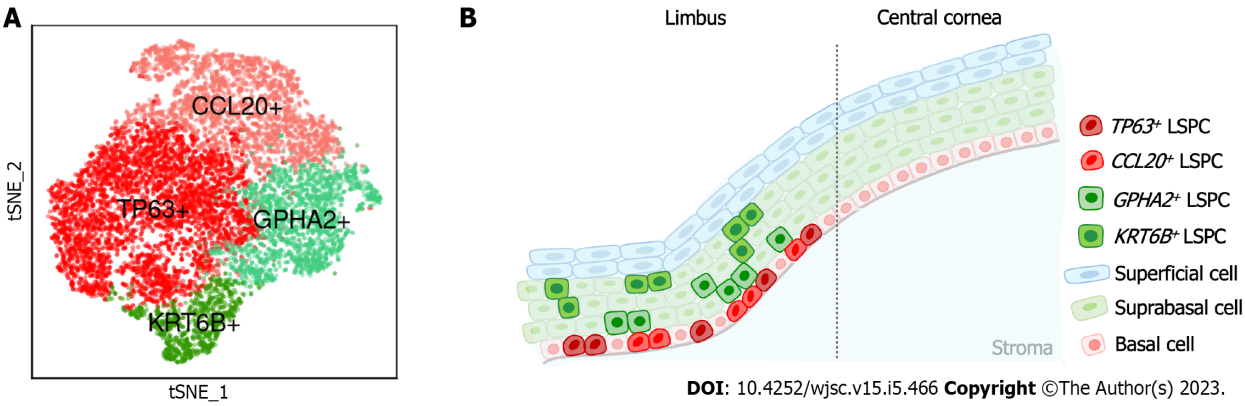

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Figure 1 Heterogeneity of limbal stem cells in humans. A: The *t*-distributed stochastic neighbor embedding plot of four subpopulations of limbal stem cells; B: Schematic diagram of the heterogeneous limbal stem cells in the human limbus. LSPC: Limbal stem/progenitor cell.

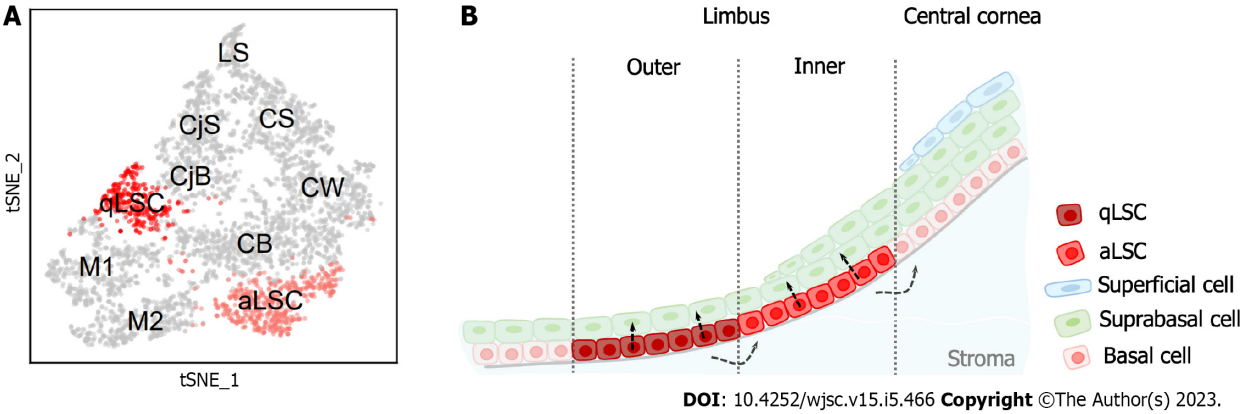

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Figure 2 Heterogeneity of limbal stem cells in mice. A: The *t*-distributed stochastic neighbor embedding plot of the corneal epithelial cell subpopulations in the mouse limbus. The limbal stem cells are highlighted in red; B: Schematic diagram of the heterogenous limbal stem cells in the mouse limbus. aLSC: Active limbal stem cell; CB: Corneal basal cell; CjB: Conjunctival basal cell; CjS: Conjunctival suprabasal cell; CS: Corneal superficial cell; CW: Corneal wing cell; LS: Limbal superficial cell; M1/M2: Cells in mitosis; qLSC: Quiescent limbal stem cell.

RNAi data.

Other LSC markers have also been identified. Li *et al*[49] identified *TSPAN7*$^+$ and *SOX17*$^+$ cells distributed in a scattered pattern in human limbus epithelium basal cells. The authors established an *in vitro* model of epithelial cells and discovered *TSPAN7* and *SOX17* were not strongly expressed in human limbal epithelial cells. However, mRNA and protein expression levels were significantly activated after injury, especially during cell migration and growth. The authors also utilized RNAi to downregulate *TSPAN7* and *SOX17* and observed inhibited cell proliferation and significantly delayed epithelial regeneration during wound healing. Overall, the discovery of novel markers of LSCs (Table 1) can help us to better distinguish LSCs from other cells to further understand the function and state of LSCs and provide a more effective strategy for the isolation, culture and clinical application of LSCs.

NICHE REGULATION OF THE LSCS AT THE LIMBUS

LSC proliferation, migration and differentiation are inseparable from the regulation of the limbal niche microenvironment. The stem cell niche is the local microenvironment directly promoting or protecting stem cell populations[52-54]. The LSC niche provides a sheltered environment that protects LSCs from stimulation[55-58]. If the LSC niche is involved in pathological damage, then LSC dysfunction can occur. Therefore, the study of the LSC niche is essential.

Collin *et al*[47] investigated the interaction between LSCs and the limbal niche by single-cell analysis. The authors combined scRNA-Seq and ATAC-Seq and performed CellPhoneDB analysis[59]. They identified multiple significant interactions between human LSCs and regulatory factors of immune cells such as proinflammatory cytokines [tumor necrosis factor, interleukin (IL)-1β, IL-6, IL-17A, interferon γ, and oncostatin M], proinflammatory cell surface receptor (triggering receptor expressed on myeloid cells 1), proinflammatory cytokine expression (adaptor complexes 1) and regulators of inflammatory

Table 1 Novel limbal stem cell markers identified by single-cell RNA sequencing

LSC subtype	Marker	Species	Ref.
LSPC with high stemness	*TP63, CCL20*	Human	[43]
LSPC with high differentiation	*GPHA2, KRT6B*	Human	
LSC	*TSPAN7, SOX17, SELE, ECSCR, RAMP3, RNASE1, NPCD1, NNMT, SLC2A3, KLF2, PDK4*	Human	[49]
Limbal progenitor cell	*DCN, PLIN2, DEGS1, MMP10, IFITM3, SLC6A6, LTB4R, SLP1*	Human	
qLSC	*Gpha2, Cd63, Ifitm3*	Mouse	[46]
aLSC	*Atf3, Socs3, Mt1, Prdm1*	Mouse	

aLSC: Active limbal stem cell; LSC: Limbal stem cell; LSPC: Limbal stem/progenitor cell; qLSC: Quiescent limbal stem cell.

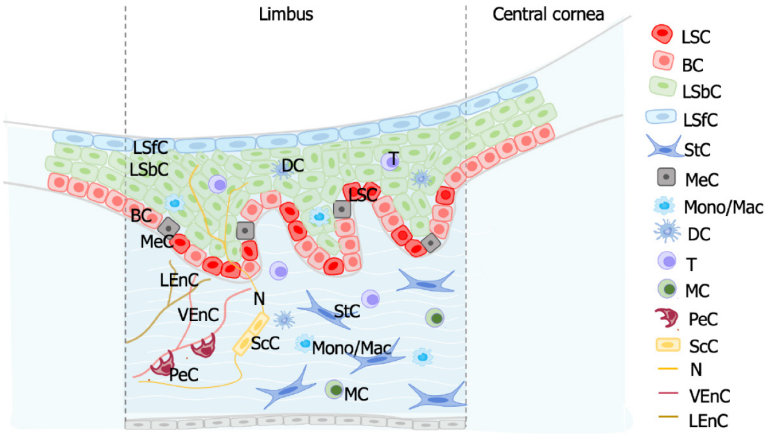
responses (nuclear factor kappa B, RELA, colony-stimulating factor 2, phosphoinositide 3-kinase, extracellular signal-regulated kinase 1/2, and F2). The authors verified that limbal epithelial cells were significantly reduced in cell culture medium containing tumor necrosis factor-α and IL-1β. This suggested that proinflammatory cytokines produced by immune cells were involved in the apoptosis of limbal epithelial cells[60], thus mimicking the central corneal defect and stimulating the proliferation of LSCs[61]. This was also consistent with other reports showing that the addition of proinflammatory factors to limbal epithelial cell cultures can directly affect the expression of LSC markers and their colony forming efficiency capacity[60,62-64].

Dou *et al*[43] systematically explored intercellular communication between LSPCs and other cell populations based on ligand-receptor analysis. By correlating the corresponding receptor-ligands in human LSPCs and their niche cells, the authors observed that LSPCs were regulated by the limbal niche as well as by other cells in the limbal niche. The Notch signaling pathway was also involved in cell-cell interaction between LSPCs and their niche cells. NOTCH1-4 receptors were expressed in LSPCs, and their relevant ligands were primarily identified in niche cells, such as Schwann cells, stromal cells, pericytes and LSPCs. Likewise, the WNT7A, WNT7B and WNT5A ligands, which participate in the Wnt/β-catenin signaling pathway, were detected on LSPCs. Their corresponding receptors were primarily detected on limbal epithelial cells, stromal cells, immune cells, Schwann cells and LSPCs. The presence of multiple chemokines, such as CCL4, CCL4L2, IL-1β and IL-24, on LSPCs and their paired receptors indicated that immune cell interactions may potentially regulate LSPCs.

Altshuler *et al*[46] revealed that T cells acted as niche cells and served its function in the maintenance of quiescence, epithelial thickness control and wound healing. By studying the limbus of the severe combined immunodeficiency (SCID) and non-obese diabetic SCID mice, which are unable to make mature T and B lymphocytes, it was observed that the GPHA2 protein was substantially decreased to almost undetectable levels. In contrast, the expression of *Ifitm3* did not rely on the existence of immune cells, implying that it was regulated by other niche cells. When T cells were inhibited by topical application of the corticosteroid dexamethasone, LSCs showed a dramatic reduction in *Cd63* and *Gpha2* expression levels and increased cell proliferation, demonstrating that T cells played a crucial role in regulating qLSCs. Finally, corneal epithelial debridement followed by epithelial closure by fluorescein dye infiltration revealed delayed epithelial wound healing in mice lacking T cells.

In addition, other niche cells were determined to be important for the microenvironment regulation of LSCs. Oxidative stress can lead to a variety of eye diseases, such as keratitis, cataracts and retinal diseases, which are subject to varying degrees of oxidative damage[65,66]. Recently, studies found that melanocytes in the limbal niche (as antioxidant systems) protected LSPCs from UV-induced oxidative damage and reduced oxidative stress through the transfer of melanosomes[67,68]. Moreover, by ligand analysis, Dou *et al*[43] identified the intercellular communications between melanocytes and LSCs. NAMPT, as a ligand, was highly expressed in melanocytes and had been reported to act as a critical switch in melanoma cells. CD44 acted as a receptor and was also highly enriched in melanocytes.

Vascular endothelial cells are also one of the important niche cells of LSCs. It has been reported that vascular endothelial cells were highly correlated with the classic Wnt signaling pathway involved in the regulation of the corneal limbal niche[69,70]. Furthermore, Dou *et al*[43] performed a differential expression analysis with the integration of the scRNA-Seq datasets from the limbus and the skin and observed that the vascular endothelial cells from the limbus highly expressed anti-vascular factors compared to that from the skin, consistent with characteristics of corneal angiogenic privilege. Above all, these studies have shown that the regulation of the LSC niche (Figure 3) occupies a key role in the growth, development, proliferation and differentiation of LSCs.



DOI: 10.4252/wjsc.v15.i5.466

Figure 3 Structure and cellular compositions in the limbal stem cell environment. Niche cells regulate limbal stem cells. LSCs: Limbal stem cells; BC: Basal cell; DC: Dendritic cell; LEnC, Lymphatic endothelial cell; LSbC: Limbal suprabasal cell; LSfC: Limbal superficial cell; MC: Mast cell; MeC: Melanocyte; Mono/Mac: Monocyte/macrophage; N: Nerve; PeC: Peripheral cell; ScC: Schwann cell; StC: Stromal cell; T: T cell; VEnC: Vascular endothelial cell.

CONCLUSION

The first Drop-Seq experiments were performed on mouse retina in 2015[23]. Since this revolutionary experiment, single-cell sequencing technology has been widely used in many fields, including ophthalmology, and gene expression has been studied at an unprecedented resolution in multiple ocular tissues. Corneal transparency is essential for normal vision; thus, comprehension of the mechanisms related to corneal wound healing and regeneration is crucial for the treatment of patients suffering from corneal disease. Currently, corneal epithelial regeneration is a relatively satisfactory approach and has the potential to treat corneal superficial scars. However, for multiple corneal basal scars or endothelial disease, corneal transplantation remains the only option to restore clear vision[71-73]. Unfortunately, corneal clouding remains one of the leading causes of blindness worldwide due to the lack of corneal donor tissue or the limited availability of corneal surgery[74,75]. Although most studies support corneal regeneration through LSC therapies[76,77], the study of LSCs is particularly important.

This review focused on the current research on single-cell sequencing in LSCs. We highlighted the heterogeneity of LSCs and presented several novel specific markers of LSCs and the role of niche regulation of LSCs. LSCs can be identified in both humans and mice, and several markers, such as *GHPA2* and *IFITM3*, can be highly and specifically expressed on LSCs. Moreover, both T cell regulation in mice studied by Altshuler *et al*[46] and immune cell regulation in humans studied by Collin *et al*[47] and Dou *et al*[43] suggest that niche regulation is of vital importance for LSCs.

Future research can still benefit from RNA-Seq technology as it can aid in acquisition of further knowledge on the functions and characteristics of LSCs, including in the discovery of more novel highly specific expression markers and more niche regulated components that can promote or inhibit the proliferation and differentiation of LSCs. These discoveries should be translated into better prevention and treatment strategies to treat blindness and improve the clinical prognosis of patients with LSCD and other LSC-related diseases.

FOOTNOTES

Author contributions: Dou SQ and Shi WY designed the report; Sun D collected the data and wrote the paper; Dou SQ and Shi WY reviewed and edited the manuscript; and all authors discussed the study's results and provided important intellectual comments on the manuscript.

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ORIGINAL ARTICLE

Basic Study

Exosomes from circ-Astn1-modified adipose-derived mesenchymal stem cells enhance wound healing through miR-138-5p/SIRT1/FOXO1 axis regulation

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Abstract

BACKGROUND

Wound healing impairment is a dysfunction induced by hyperglycemia and its effect on endothelial precursor cells (EPCs) in type 2 diabetes mellitus. There is increasing evidence showing that exosomes (Exos) derived from adipose-derived mesenchymal stem cells (ADSCs) exhibit the potential to improve endothelial cell function along with wound healing. However, the potential therapeutic mechanism by which ADSC Exos contribute to wound healing in diabetic mice remains unclear.

AIM

To reveal the potential therapeutic mechanism of ADSC Exos in wound healing in diabetic mice.

METHODS

Exos from ADSCs and fibroblasts were used for high-throughput RNA sequencing (RNA-Seq). ADSC-Exo-mediated healing of full-thickness skin wounds in a diabetic mouse model was investigated. We employed EPCs to investigate the therapeutic function of Exos in cell damage and dysfunction caused by high glucose (HG). We utilized a luciferase reporter (LR) assay to analyze interactions among circular RNA astrotactin 1 (circ-Astn1), sirtuin (SIRT) and miR-138-5p. A diabetic mouse model was used to verify the therapeutic effect of circ-Astn1 on Exo-mediated wound healing.

RESULTS

High-throughput RNA-Seq analysis showed that circ-Astn1 expression was increased in ADSC Exos compared with Exos from fibroblasts. Exos containing high concentrations of circ-Astn1 had enhanced therapeutic effects in restoring

EPC function under HG conditions by promoting SIRT1 expression. Circ-Astn1 expression enhanced SIRT1 expression through miR-138-5p adsorption, which was validated by the LR assay along with bioinformatics analyses. Exos containing high concentrations of circ-Astn1 had better therapeutic effects on wound healing *in vivo* compared to wild-type ADSC Exos. Immunofluorescence and immunohistochemical investigations suggested that circ-Astn1 enhanced angiopoiesis through Exo treatment of wounded skin as well as by suppressing apoptosis through promotion of SIRT1 and decreased forkhead box O1 expression.

CONCLUSION

Circ-Astn1 promotes the therapeutic effect of ADSC-Exos and thus improves wound healing in diabetes *via* miR-138-5p absorption and SIRT1 upregulation. Based on our data, we advocate targeting the circ-Astn1/miR-138-5p/SIRT1 axis as a potential therapeutic option for the treatment of diabetic ulcers.

Key Words: Adipose-derived mesenchymal stem cells; Circular RNA astrotactin 1; Diabetic; Exosomes; Angiogenesis

Core Tip: Circular RNA astrotactin 1 (circ-Astn1) promoted the therapeutic effect of adipose-derived mesenchymal stem cells-exosomes and thus improved wound healing in diabetes *via* miR-138-5p absorption and SIRT1 upregulation. Based on our data, we advocate targeting the circ-Astn1/miR-138-5p/SIRT1 axis as a potential therapeutic alternative for diabetic ulcers.

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INTRODUCTION

Diabetes affects 30 million children as well as adults in the United States, *i.e.* one out of every eleven people in the United States, which leads to $327 billion costs each year. Consequently, it is important to develop a new method of diabetes treatment. Interventions that improve healing rates and decrease diabetic ulcer size could lower the infection incidence, amputation rate, and care cost[1]. Diabetic foot (DF) is a severe complication of type 2 diabetes mellitus (T2D). DF infection is the main reason for DF development and deterioration, and controlling infection plays an important role in disease treatment. Previous studies have found that diabetes is associated with hyperglycemia, one of the most important causes of oxidative stress. Endogenous antioxidants are able to destroy the reactive species and create a balance between antioxidants and free radicals[2,3]. The impaired function and senescence of endothelial progenitor cells (EPCs) and high glucose (HG)-induced reactive oxygen species likely exacerbate DFs[4].

Accumulated evidence shows that mesenchymal stem cell (MSC)transplantation promotes angiogenesis and accelerates diabetic wound healing[5,6]. Adipose-derived mesenchymal stem cells (ADSCs) therapy provides potentially new therapeutic options to improve diabetic wound healing[7], and autologous stem cell transplantation reduces the cost of drug development, which in turn reduces financial costs. However, the mechanism is not clear.

Stem cells live in niches, which are complicated microenvironments that exert important functions in directing the division, differentiation, and activity of stem cells. However the direction of differentiation is affected by hypoxia, cytokines, trophic factors, chemical and pharmacological agents, and physical factors[8]. Considering the safety of *in vivo* transplantation, some investigations have suggested that exosomes (Exos) from ADSCs play a similar functional role to ADSCs in promoting diabetic wound healing. Exos are tiny endosomal membrane-bound vesicles, 50–200 nm in length, that have a variety of contents including protein and nucleic acids which vary with cell or tissue origin. They play their full role by fusing with selected cells and releasing their cargo that could contain bioactive molecules including lipids, proteins, non-coding-RNA (ncRNA)[9-11] and mRNAs. Previous studies have found that Exos can regulate the epithelial–mesenchymal transition and disease progression in different cancers[12,13]. Exos secreted from ADSCs attenuate diabetic nephropathy by promoting autophagy flux

and by inhibiting apoptosis in podocytes[14]. Exos from nuclear factor erythroid 2–related factor 2-overexpressing ADSCs accelerate cutaneous wound healing by promoting vascularization in a DF ulcer [4]. Exos from linc00511-overexpressing ADSCs accelerate angiogenesis in healing DF ulcers by suppressing progestin and adipoQ receptor family member 3-induced Twist1 degradation[15]. However, it remains largely unknown if Exos from ncRNA-modified ADSCs can improve wound healing.

The ncRNAs include circular RNA (circRNA), long non-coding RNA (lncRNA), and microRNA (miRNA). circRNA activity is indispensable during the regulation of gene expression, demonstrating that circRNAs function not only as candidate therapeutic agents but also as diagnostic markers. circRNA 5′ and 3′ extremities are linked to form an integrated circular structure, which makes circRNAs more resistant to RNA exonuclease degradation. as well as more stable 8than linear RNAs[16,17]. A previous study found that circRNAs possess activity and potential clinical benefits in skin wound healing[18].

To identify relevant circRNAs as therapeutic targets, we used high-throughput sequencing detection to identify the function of mmu_circ_0000101 (circ-Astn1), which acts as the key factor in delivery by ADSC Exos. Exos from circ-Astn1-modified ADSCs improve wound repair in diabetic rats through miR-138-5p/SIRT1 pathway regulation. The present study verified the effect of treatment with Exos from circ-Astn1-overexpressing ADSCs on HG-induced EPC dysfunction. The abundance and simple methods of sampling of ADSC-Exos make it safer in terms of trauma and other adverse reactions.

MATERIALS AND METHODS

Ethics statement

The Animal Care and Use Committee of Peking Union Medical College Hospital approved the investigation protocol (No: XHDW-2020-01; Beijing, China). We carried out all postoperative animal care along with surgical interventions following the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All surgeries and euthanasia were performed under sodium pentobarbital anesthesia (30 mg/kg) by intraperitoneal injection, and all efforts were made to minimize suffering.

High-throughput and strand-specific RNA sequencing library construction

Total RNA from ADSCs and fibroblast Exos was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). Our team prepared about 3 μg total RNA *per* sample using the VAHTS Total RNA-seq (H/M/R) Library Prep Kit from Illumina (Vazyme Biotech Co., Ltd., Nanjing, China) to isolate the ribosomal RNA and remove other RNAs such as ncRNA and mRNA. We then performed RNA purification using RNase R (Epicenter, 40 U, 37 °C for 3 h) followed by TRIzol. An RNA sequencing RNA-Seq) library was prepared using the KAPA Stranded RNA-Seq Library Prep Kit (Roche, Basel, Switzerland) and they were exposed in order following extensive codifying with Illumina HiSeq 4000 from Aksomics, Inc. (Shanghai, China).

Cell treatment

To investigate endothelial precursor cell (EPC) dysfunction as well as apoptosis, we cultivated EPCs at 37 °C with 5% carbon dioxide in EPC medium (Gibco, Carlsbad, CA, United States) and processed them after 1 d using 5.5 or 30 mmol/L glucose. We harvested EPCs for detection of apoptosis as well as to test their response to Exo therapy. In order to study the protective function of Exos on EPCs, we added 100 μg/mL Exos to cultures following 80% EPC fusion to evaluate the protective function against damage caused by prior HG treatment with various glucose concentrations.

ADSC isolation and identification

We isolated ADSCs from adipose tissue following the method used in a previous study[4]. We observed no uninduced differentiation in cultural expansion. We induced osteogenic differentiation *via* a 3-wk culture of ADSCs in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, and 10 mmol/L β-glycerophosphate. We induced adipogenic differentiation through culturing ADSCs for 2 weeks in DMEM supplemented with 10% FBS, 10 μM insulin, 0.5 mmol/L isobutylmethylxanthine, 200 μM indomethacin, and 1 μM dexamethasone. We also investigated the osteogenic or adipogenic differentiation of ADSCs through Oil-Red O and alkaline phosphatase staining.

Identification and isolation of ADSC-derived Exos

We isolated ADSC-derived Exos when cells reached 80%-90% confluence. Our team rinsed ADSCs from various groups with phosphate-buffered saline (PBS), and then cultured them in FBS-free endothelial cell growth medium (EGM)-2MV, which was supplemented with 1 × serum replacement solution (PeproTech, Rocky Hill, NJ, United States) for another 2 d. Then, we collected conditioned culture medium and centrifuged it at 300 × *g* for 10 min to remove cells and at 2000 × *g* for another 10 min to

remove apoptotic cells and cellular debris. Following centrifugation at 10000 × *g* for 30 min, we filtered the supernatant through a 0.22 μm filter (Millipore, Billerica, MA, United States), then transferred 15 mL supernatant to the Amicon Ultra-15 Centrifugal Filter Unit (100 kDa) and centrifuged it at 4000 × *g* to concentrate to approximately 1 mL. The ultrafiltration unit was washed twice with PBS centrifuged it again at 100000 × *g*, and the supernatant was aspirated. All processes were conducted at 4 °C. We resuspended the Exo pellets obtained in 500 μL PBS. Finally, the Exo protein content was evaluated using the Pierce bicinchoninic acid assay (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States). We stored Exos at -80 °C until subsequent use for experiments and identified Exos by western blotting and transmission electron microscopy.

Diabetic wound induction

We utilized Balb/c mice and induced diabetes through a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ) dissolved in 0.1 M citrate buffer (pH 4.5). Three days after STZ administration, we confirmed diabetes development by measuring fasting blood glucose levels in blood samples obtained from the tail vein. We considered a mouse with fasting blood glucose levels > 250 mg/dL diabetic, which we maintained for 1 mo and employed for subsequent analyses of posterior blood glucose stabilization. Following diabetes validation, we anesthetized mice through intramuscular injection with ketamine hydrochloride and xylazine cocktail at 80 and 10 mg/kg, respectively. Once anesthesia was established, hair was shaved from the dorsal leg area and the region was sterilized using povidone iodine solution. A sterile biopsy punch was used to generate a full-thickness 4 mm excisional wound. Then we allocated mice randomly to subcutaneous injection with 100 μL PBS containing 200 μg ADSC Exos or equivalent amount of PBS without Exos at four sites near the wound (25 μL/site). We euthanized mice and harvested skin specimens for histopathological validation.

RNA overexpression or interference

RNA overexpression or interference was induced by transfection of miR-138-5p mimics or inhibitor, circ-Astn1 and SIRT1 overexpression vector, and siRNA against circ-Astn1 (si-circ-Astn1) obtained from RiboBio (Guangzhou, China). Our team performed transfection using Lipofectamine 2000 (Thermo Fisher Scientific) following a method previously described[19].

Quantitative polymerase chain reaction

We isolated total RNA from skin tissue or cells from wounds using a TRIzol reagent kit. Our team synthesized cDNA to amplify with TaqMan miRNA Reverse Transcription Kit. Our team then performed quantitative polymerase chain reaction (qPCR) using a TaqMan Human miRNA Assay Kit, using the $2^{-\Delta\Delta CT}$ approach to detect fold changes with respect to expression. We used *U6* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as internal references. Primers utilized were: Circ-Astn1, F: 5′-CTGGACCCTTGTGAACACCAATG-3′, R, 5′-GGATCATCACCAGGCACAAGATG-3′; FOXO1, F: 5′-AAGGCCATCGAGAGCTCAGC-3′, R, 5′-GATTTTCCGCTCTTGCCTCC-3′; miR-138-5p, F: 5′-GCTGGTGTTGTGAATCAG-3′, R: 5′-GAACATGTCTGCGTATCTC-3; U6, F: 5′-AGTAAGCCCTTGCTGTCAGTG-3′, R: 5′-CCTGGGTCTGATAATGCTGGG-3′; GAPDH: F: 5′-GTCTCCTCTGACTTCAACAGCG-3′, R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′, and were designed by Gene Pharma (Shanghai, China).

Apoptosis detection

To assess apoptosis, we collected cells into centrifuge tubes and centrifuged them at 1000 rpm for 5 min. We resuspended cells in PBS at 4 °C and removed the supernatant following centrifugation. We resuspended the cell pellet at 1–5 × 10^6/mL in 1 × binding buffer, then 100 μL cell suspension was mixed with 5 μL Annexin V/fluorescein isothiocyanate in the dark at room temperature for 5 min. Lastly, we added 10 μL propidium iodide (PI) and 400 μL PBS to stain the cells. We analyzed data using the FlowJo package.

Immunofluorescence and immunohistochemical assays

We fixed skin tissue samples in 10% formalin solution, embedded them in paraffin, and sectioned them at 5 μm. Our team stained tissue sections with hematoxylin and eosin (HE) for histological detection, and cluster of differentiation 31 (CD31) immunofluorescence staining was used to detect histopathological changes associated with angiogenesis. We performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to identify apoptotic cells. Our team visualized sections using fluorescence (Nikon, Tokyo, Japan) or light microscope (Zeiss, Oberkochen, Germany), and photographed them using a digital camera.

Western blot analysis

Skin tissues were lysed, and lysates were centrifuged at 12000 rpm at 4 °C following addition of a protease inhibitor. The protein concentration was determined using the Pierce BCA kit (Thermo Fisher). Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to PVDF membranes. The primary antibodies used to assay protein expression were SIRT1

(1:600), forkhead box O1 (FOXO1) (1:600) (all from Santa Cruz Biotechnology, Santa Cruz, CA, United States), and anti-GAPDH (1:1000; Sigma-Aldrich, St. Louis, MO, United States), followed by a horseradish peroxidase-conjugated secondary antibody (1:1000; Abcam, Cambridge, MA, United States). An enhanced chemiluminescence kit (Millipore, Burlington, MA, United States) was used to read the bands.

Luciferase reporter assay

We created and cloned wild-type (WT) and 3'-UTR mutant (MUT) *SIRT1*, as well as WT and MUT *circ-Astn1* into pMIR firefly luciferase-expressing vectors. We co-transfected the vectors into HEK293T cells once they reached 70% confluence, using 500 ng pMIR-SIRT1-wt/pMIR-SIRT1-Mut or pMIR-*circ-Astn1*-wt/pMIR-*circ-Astn1*-Mut combined with 50 nM miR-138-5p mimics using a Lipofectamine 2000 Transfection Kit for the luciferase assay. We assayed luciferase activity using a Dual-Luciferase Reporter System (Promega, Madison, WI, United States). We performed five independent assays.

Tube formation assay

We performed an EPC tube formation assay using Matrigel (BD Biosciences, Franklin Lakes, NJ, United States). Matrigel solution was mixed with ECMatrix diluent buffer then spread on μ-Slide plates and incubated at 37 °C for 1 h for the matrix solution to solidify. Next, we added various treatments to the EPCs (2×10^4 cells/well) to wells containing solid matrix and cultured them with EGM-2 medium at 37 °C for a period of 12 h. Our team detected tube formation under an inverted light microscope (100 ×) and evaluated three independent representative fields from each well to determine mean tube number.

Cell Counting Kit (CCK)-8 assay

EPC proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) (BD Biosciences). Our team cultivated transfected cells in 96-well plates with Exos in HG conditions for 1 d in wells to which 10 μL CCK-8 reagent and 90 μL fresh culture medium was previously added. Absorbance was detected at 450 nm using a microplate reader following incubation at 37 °C for 2 h.

Statistical analyses

We denoted continuous parameters by the mean ± SD and employed one-way variance of analysis (ANOVA) to compare data using GraphPad Prism (GraphPad, La Jolla, CA, United States). $P \leq 0.05$ indicated a statistically significant difference.

RESULTS

ADSC and Exo characterization

Isolated ADSCs have classical cobblestone-like morphology (Figure 1A). Immunofluorescence staining showed that ADSCs from mouse adipose tissue samples were positive for expression of the mesenchymal cell surface markers CD29 (Figure 1B), CD44 (Figure 1C), CD90 (Figure 1D), and CD105 (Figure 1E), but negative for expression of the endothelial cell marker CD31 (Figure 1F) as well as von Willebrand Factor (Figure 1G). The results of Oil Red O staining (Figure 1H) together with alkaline phosphatase staining (Figure 1I) verified that isolated ADSCs possessed both osteoblastic and adipocytic differentiation capacity. We concluded that ADSCs have the potential for multidirectional differentiation[20].

Exos were isolated by ultra-high-speed centrifugation. Transmission electron microscopy revealed that ADSC Exos had spherical or cup-shaped morphology with a diameter ranging from 50 to 120 nm (Figure 1J) as previously reported[21]. Western blotting suggested that ADSC Exos were positive for the Exo markers CD81 and CD63, which are cellular components (Figure 1K).

Exos derived from circ-Astn1-modified ADSCs play important roles in the restoration of EPC function by decreasing apoptosis under HG conditions

To determine the role of circRNAs in ADSC Exo-mediated restoration of EPC function under HG conditions, circRNA expression in ADSCs and fibroblast Exos was explored by RNA-Seq. The results verified that the contents of mmu_circ_0000101, mmu_circ_0008040, mmu_circ_0008061, and mmu_circ_0008099 were all significantly upregulated in ADSC Exos compared with fibroblast Exos (Figure 2A). RT-qPCR analysis confirmed that mmu_circ_0000101, mmu_circ_0008040, mmu_circ_0008061 and mmu_circ_0008099 expression in EPCs decreased after exposure to HG conditions (Figure 2B), with expression of mmu_circ_0000101 in particular decreasing most significantly. Consequently, mmu_circ_0000101 was selected for subsequent study. Mmu_circ_0000101 originated from *Astn1* gene exon 5, so mmu_circ_0000101 was also known as circ-Astn1. The entire mature spliced sequence length was 967 bp. The gene is on chromosome 1: 160432178-160441253 (Figure 2C).

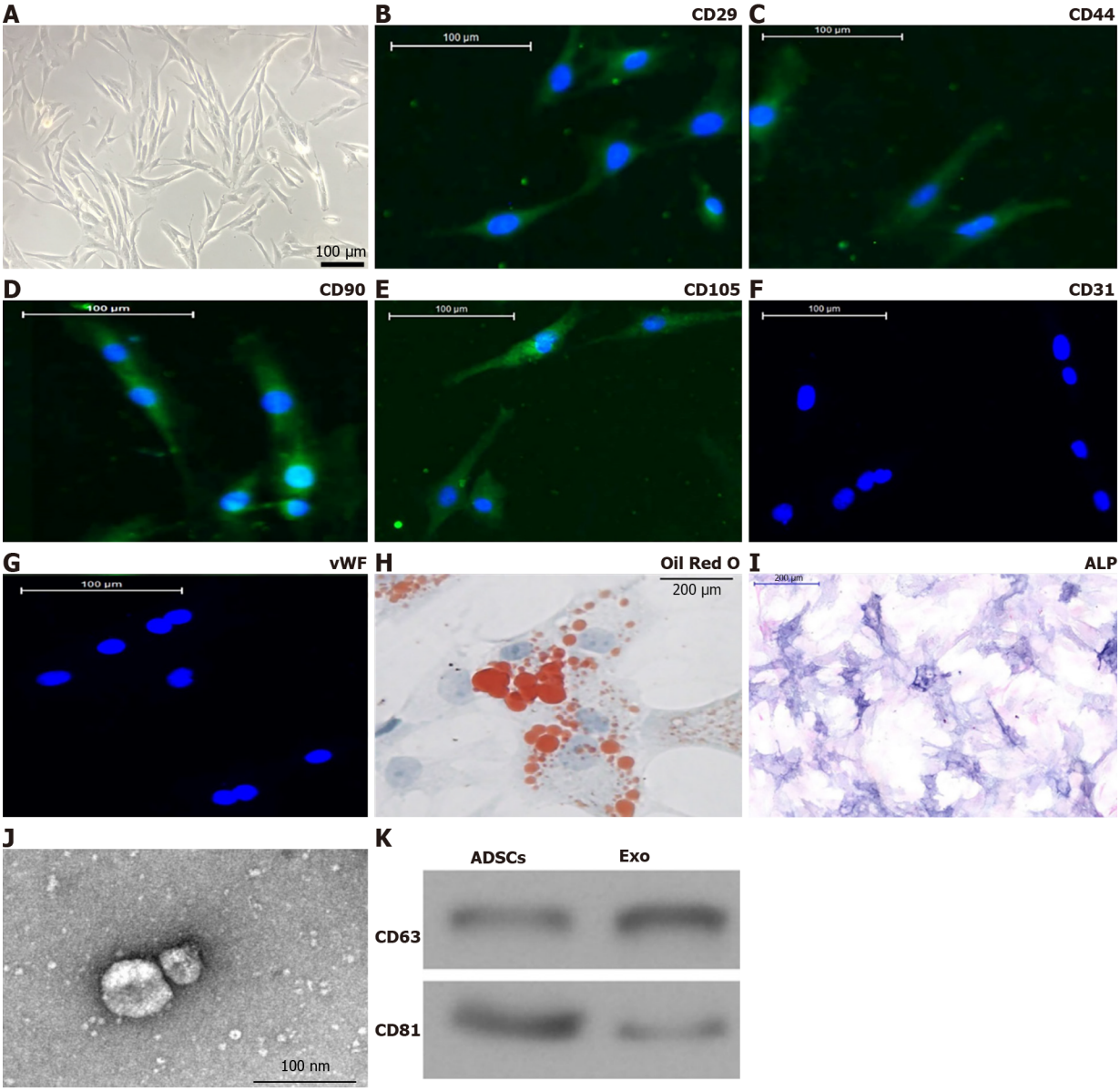

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Figure 1 Characterization of adipose-derived mesenchymal stem cells and exosomes. A: Adipose-derived mesenchymal stem cells (ADSCs) showed a typical cobblestone-like morphology. Scale bar: 100 μm; B–G: Immunofluorescence staining of cell surface markers. ADSCs exhibited positive expression of cluster of differentiation 90 (CD90), CD29, CD44, and CD105, but not von Willebrand factor or CD34. Scale bar: 100 μm; H and I: The differentiation potential of ADSCs assessed by Oil Red O (H) and alkaline phosphatase (I) staining. Scale bar: 200 μm; J: Transmission electron micrographs demonstrated ADSC exosome morphology. Scale bar: 100 nm; K: Western blotting detection of CD81 and CD63 expression in exosomes and ADSCs.

Flow cytometry investigations have shown that HG (30 mmol/L glucose) treatment promotes EPC apoptosis. Treatment with Exos from WT ADSCs suppressed HG-induced EPC apoptosis, and treatment with Exos from ADSCs overexpressing circ-Astn1 had a more significant effect in suppressing HG-induced apoptosis of EPCs than Exos from WT ADSCs (Figure 2D and E), suggesting that circ-Astn1 played an important role in ADSC-Exo-mediated EPC protection under HG conditions. CCK8 detection confirmed that treatment with Exos containing high levels of circ-Astn1 had a greater effect in restoring the proliferative ability of EPCs under HG conditions (Figure 2F). We used tubule formation by EPCs in Matrigel-coated culture wells as an *in vitro* angiogenesis model, and evaluated their potential by counting the branch numbers formed. HG conditions suppressed angiogenesis, and treatment with Exos containing high levels of circ-Astn1 was more effective in promoting angiogenesis of EPCs under HG conditions (Figure 2G-J).

The circ-Astn1-mediated miR-138-5p/SIRT1/FOXO1 signaling pathway protects EPCs under HG conditions by promoting angiogenesis

Bioinformatics data showed that circ-Astn1 regulates SIRT1 expression *via* inhibition of miR-138-5p. SIRT1 functions critically in promoting angiogenesis by activating the FOXO1 signaling pathway[22]. To

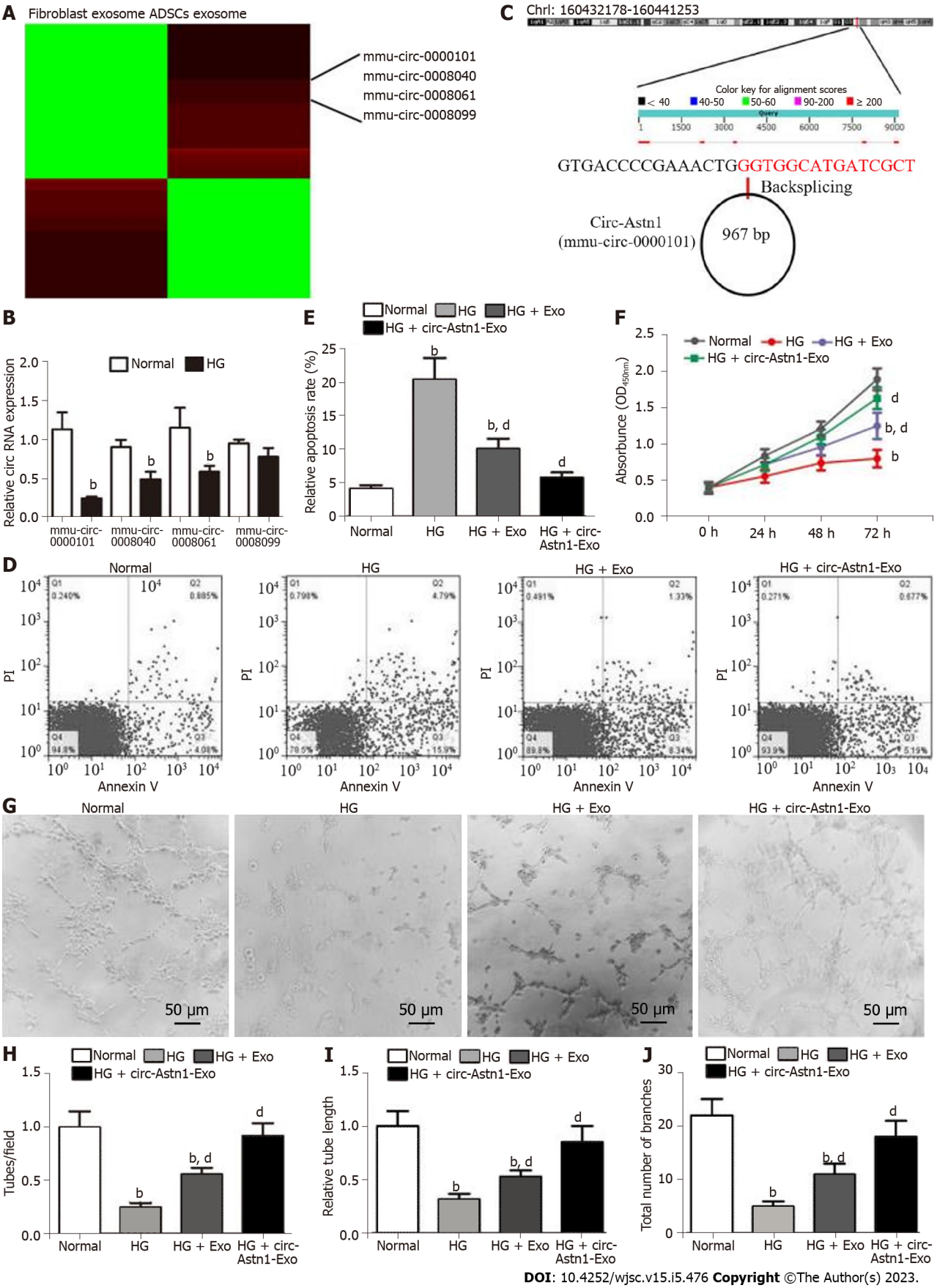

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Figure 2 Exosomes derived from circular RNA astrotactin 1-modified adipose-derived mesenchymal stem cells function importantly in endothelial precursor cell function restoration by decreasing apoptosis under high glucose conditions. A: Heat map regarding all differentially expressed circular RNAs (circRNAs) between adipose-derived mesenchymal stem cells (ADSCs) exosomes and fibroblast exosomes; B: Quantitative polymerase chain reaction giving mmu_circ_0000101 (circular RNA astrotactin 1), mmu_circ_0008040, mmu_circ_0008061, and mmu_circ_0008099 expression in endothelial

precursor cells (EPCs) with or without high glucose (HG) treatment. Data are denoted by the mean ± SD; $^{b}P < 0.001$ *vs* normal; C: The genomic loci of circ-Astn1; D and E: We pretreated EPCs with ADSC exosomes before treatment with exosomes for 1 d under HG conditions. Our team assayed EPC apoptosis *via* flow cytometry after annexin V-FITC staining. $^{b}P < 0.001$ *vs* normal. $^{d}P < 0.001$ *vs* HG; F: EPC proliferation under different treatments, determined by Cell Counting Kit-8 assay. $^{b}P < 0.001$ *vs* normal. $^{d}P < 0.001$ *vs* HG; G-J: Representative photomicrographs of tube-like structures. Scale bar: 50 μm. Technician-counted tube branch points (H), relative tube length (I) and the total number of branches were calculated. $^{b}P < 0.001$ *vs* normal. $^{d}P < 0.001$ *vs* HG. PI: Propidium iodide.

validate the interaction among circ-Astn1, SIRT1, and miR-138-5p, we created a luciferase reporter (LR) vector. The candidate miR-138-5p-binding sites on circ-Astn1 as well as sites with point mutations inserted to prevent binding are shown in Figure 3A. Luciferase activity assay using 293T cells, which we transfected with MUT or WT circ-Astn1, verified that miR-138-5p suppressed circ-Astn1 activity (Figure 3B). RT-qPCR analysis suggested that circ-Astn1 overexpression suppressed miR-138-5p expression in EPCs (Figure 3C). Meanwhile the tubule formation assay showed that upregulation of circ-Astn1 restored angiogenic differentiation ability under HG conditions, but miR-138-5p overexpression destroyed the protective effect of circ-Astn1 (Figure 3D-G).

Next, we created the LR vector. Candidate miR-138-5p-binding sites on SIRT1 3'-UTR and those with point mutations inserted to prevent binding were constructed (Figure 3H). We transfected 293T cells with MUT or WT SIRT1 3'-UTR, which verified that WT miR-138-5p suppressed SIRT1 activity (Figure 3I). RT-qPCR analysis illustrated that miR-138-5p overexpression suppressed FOXO1 and SIRT1 expression at both mRNA and protein levels relating to EPCs (Figure 3J and K). However, overexpression of SIRT1 promoted SIRT1 and downregulated FOXO1 expression even after miR-138-5p overexpression. Analysis of tubule formation verified that miR-138-5p upregulation decreased angiogenic differentiation ability, but overexpression of SIRT1 restored the angiogenic differentiation ability of EPCs (Figure 3L-O).

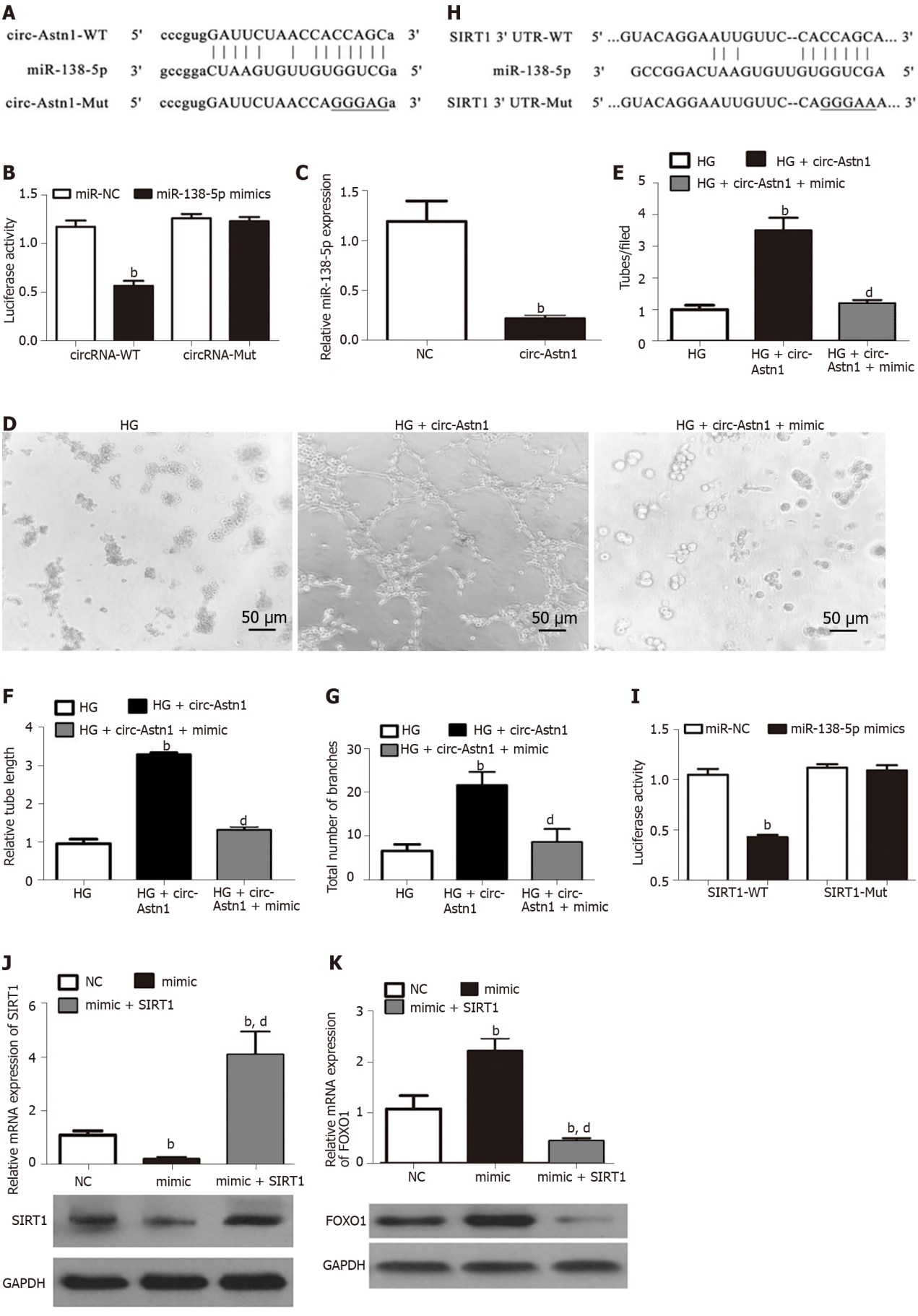
Exos from circ-Astn1-modified ADSCs possess high therapeutic effect, enhancing wound healing

We investigated the influence of ADSC Exos on wound healing in full-thickness cutaneous wounds in mouse feet in a model of STZ-induced diabetes. Mice were treated by subcutaneous injection of Exos from WT or circ-Astn1-modified ADSCs, or an equivalent volume of PBS Exo diluent. Exos with high circ-Astn1 concentration accelerated wound closure significantly compared to PBS-treated control mice. The wounds treated with high circ-Astn1-containing Exos were almost closed by 14 d, while large areas of scarring were visible in both controls and circ-Astn1-knockdown-Exo-treated wounds (Figure 4A). Immunofluorescence with CD31 staining verified that microvascular development was more extensive with Exo treatments, specifically with high-circ-Astn1-containing Exos compared with the control group. However, circ-Astn1-knockdown suppressed the therapeutic effect of Exos (Figure 4B and C). TUNEL staining suggested that circ-Astn1 Exos significantly suppressed skin tissue apoptosis compared with control treatment, but circ-Astn1-knockdown suppressed the therapeutic effect of Exos (Figure 4D and E). Hematoxylin and eosin staining also showed that circ-Astn1 Exos treatment significantly promoted skin tissue wound healing compared with control treatment, but circ-Astn1-knockdown suppressed the therapeutic effect of Exos (Figure 4F). RT-qPCR analysis confirmed that circ-Astn1 Exos significantly suppressed miR-138-5p expression (Figure 4G) but promoted SIRT1 (Figure 4H) and decreased FOXO1 (Figure 4I) expression at both the mRNA and protein levels compared with controls.

DISCUSSION

Vascular deficits are fundamental factors regarding diabetes-related traits. Although former investigations have revealed that the proangiogenic wound healing phase is blunted by diabetes, detailed knowledge of factors regulating skin revascularization as well as capillary stabilization in diabetic wounds was missing[23]. Previous investigations revealed that Exos derived from ADSCs promote diabetic wound healing by regulating the disease microenvironment[4,20]. There is also evidence that circRNAs belong to a new RNA family that has been found to be broadly expressed, and have indispensable biological activities in regulating skin wound healing[18]. In this study, we found a series of circRNAs, which RNA-Seq detection showed were abnormally expressed in ADSC Exos compared with fibroblast Exos. Among the abnormally expressed circRNAs, expression of mmu_circ_0000101 (circ-Astn1), mmu_circ_0008040, mmu_circ_0008061, and mmu_circ_0008099 was all increased significantly in ADSC Exos. Further study showed that circ-Astn1 decreased more significantly in EPCs after exposure to HG conditions. This suggesting that ADSC Exos protected EPCs from HG-induced damage related to circ-Astn1 delivery.

Our *in vitro* experiments revealed that HG conditions promoted EPC apoptosis and destroyed the ability of EPCs to differentiate into blood vessels. Transplantation of ADSC Exos exerted a protective effect in reversing HG-induced EPC damage. Increasing the circ-Astn1 content of Exos increased the protective effect. Bioinformatics analyses identified miR-138-5p as the circ-Astn1 downstream target, and this was confirmed by luciferase reporter (LR) experiments. A previous study revealed that overex-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circ-Astn1-WT 5' cccgugGAUUCUAACCACCAGCa 3'
miR-138-5p 3' gccggaCUAAGUGUUGUGGUCGa 5'
circ-Astn1-Mut 5' cccgugGAUUCUAACCAGGGAGa 3'
H
SIRT1 3' UTR-WT 5' ...GUACAGGAAUUGUUC--CACCAGCA... 3'
miR-138-5p 3' GCCGGACUAAGUGUUGUGGUCGA 5'
SIRT1 3' UTR-Mut 5' ...GUACAGGAAUUGUUC--CAGGGAAA... 3'
B
miR-NC
miR-138-5p mimics
Luciferase activity
circRNA-WT
circRNA-Mut
C
Relative miR-138-5p expression
NC
circ-Astn1
E
HG
HG + circ-Astn1
HG + circ-Astn1 + mimic
Tubes/filed
D
HG
HG + circ-Astn1
HG + circ-Astn1 + mimic
50 μm
F
Relative tube length
G
Total number of branches
I
SIRT1-WT
SIRT1-Mut
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Relative mRNA expression of FOXO1
FOXO1
GAPDH

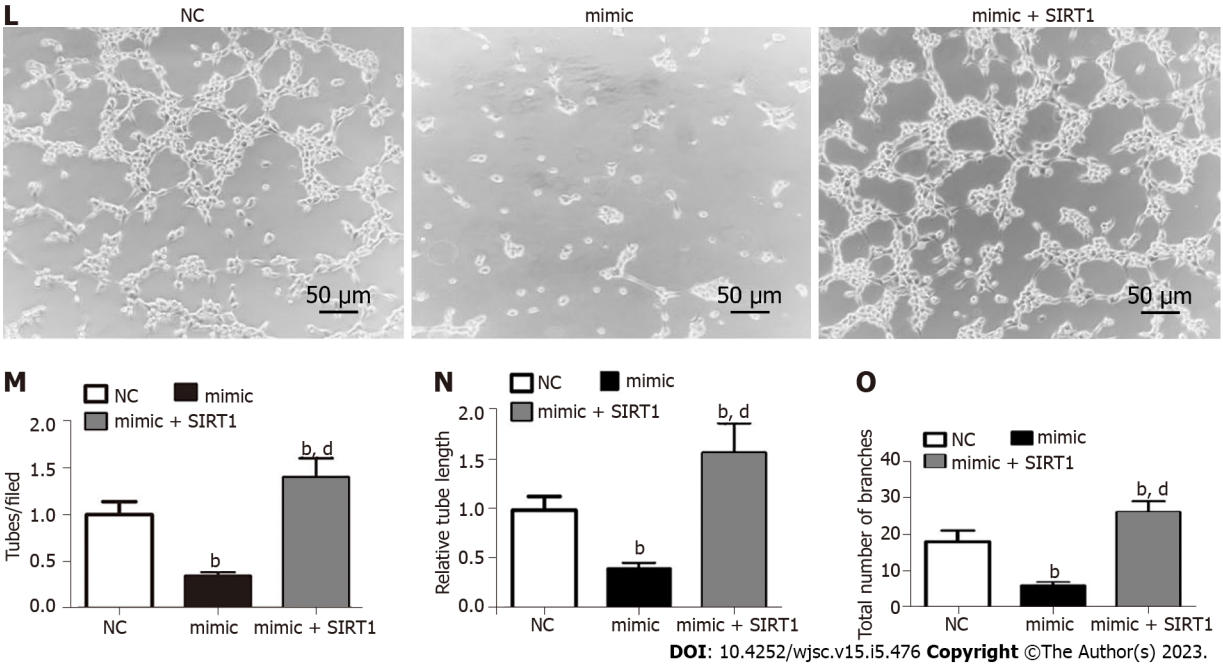

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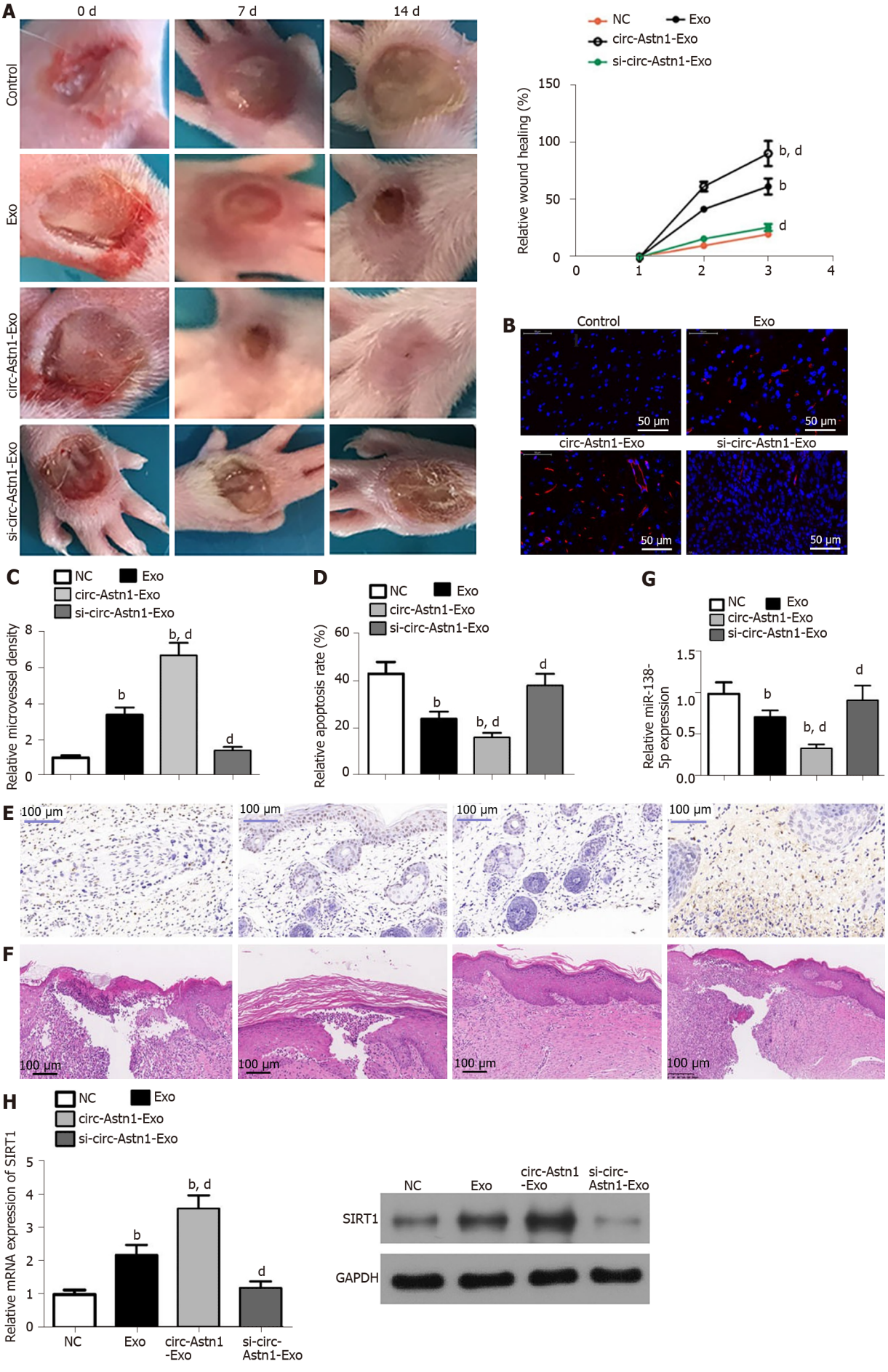
Figure 3 The circular RNA astrotactin 1-mediated mi-138-5p/SIRT1/forkhead box O1 signaling pathway plays an important protective role in endothelial precursor cells under high glucose conditions by promoting angiogenesis. A and B: Luciferase expression levels in HEK293 cells transfected with cloned circular RNA astrotactin 1 (circ-Astn1) wild-type (WT) or mutant (MUT) vector and miR-138-5p mimics. Data are denoted by the mean ± SD. $^{b}P < 0.001$; C: Quantitative polymerase chain reaction (qPCR) detection suggested that miR-138-5p expression was reduced after transfection with circ-Astn1-overexpressing vector in endothelial precursor cells (EPCs). Data are denoted as the mean ± SD. $^{b}P < 0.001$ *vs* NC; D-G: Representative photomicrographs of tube-like structures of EPCs under high glucose (HG) conditions after transfection with negative control or circ-Astn1-overexpressing vector. $^{b}P < 0.001$ *vs* HG. $^{d}P < 0.001$ *vs* circ-Astn1; H and I: Luciferase expression level in HEK293 cells transfected with cloned SIRT1 WT- or MUT-3' UTR vector and miR-138-5p mimics. Data are denoted by the mean ± SD. $^{b}P < 0.001$; J and K: qPCR and western blot analysis indicated that SIRT1 and forkhead box O1 expression were reduced after transfection with miR-138-5p overexpression vector in EPCs. Data are expressed as the mean ± SD. $^{b}P < 0.001$ *vs* NC. $^{d}P < 0.001$ *vs* miR-138-5p mimics; L-O: Representative photomicrographs of EPC tube-like structures under HG conditions after transfection with miR-138-5p mimics combined with or without SIRT1 overexpression vector. Data are denoted by the mean ± SD. $^{b}P < 0.001$ *vs* NC. $^{d}P < 0.001$ *vs* miR-138-5p mimics.

pression of miR-138 aggravates HG-induced vascular cell damage[24]. Our current investigation also found that circ-Astn1 overexpression decreased miR-138-5p expression. Meanwhile miR-138-5p overexpression reduced vascular EPC differentiation, suggesting that circ-Astn1 protected against HG-induced EPC damage by miR-138-5p adsorption.

Additional bioinformatics results showed that SIRT1 was also a miR-138-5p downstream target and this was verified by LR experiments. SIRT1 is a highly conserved nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase, which plays a regulatory role in metabolism and aging[25]. miR-138-5p overexpression reduced SIRT1 expression. Overexpression of SIRT1 restored vascular differentiation of EPCs after miR-138-5p upregulation. Previous studies have suggested that the SIRT1/FOXO1 pathway activity improves the stress microenvironment[26-28]. SIRT1 correlates to and deacetylates FoxO1. Moreover, previous studies have confirmed that SIRT1, a deacetylase that suppresses FoxO1 acetylation which is a crucial negative blood vessel development regulator, restrains anti-angiogenic activity[22,29,30]. Recently, it was reported that oxidative stress induces FoxO1 nuclear translocation which plays an important role in apoptosis regulation[26]. *In vivo* experiments have confirmed that Exos originating from circ-Astn1-modified ADSCs function indispensably in restoring EPC function and promoting wound healing by promotion of angiogenesis and suppression of apoptosis. RT-qPCR analysis demonstrated that treatment with Exos containing high levels of circ-Astn1 reduced miR-138-5p expression and promoted SIRT1. This increase in SIRT1 level suppressed FOXO1 expression, suggesting that Exos derived from circ-Astn1-modified ADSCs enhanced wound healing in a diabetic mouse model *via* miR-138-5p/SIRT1/FOXO1 axis regulation.

CONCLUSION

In conclusion, our research indicated that Exos derived from circ-Astn1-modified ADSCs enhanced wound healing in a diabetic mouse model *via* miR-138-5p/SIRT1/FOXO1 axis induction. Our study verified the therapeutic effects of circ-Astn1-Exos on an STZ-induced diabetic wound healing model. However, more in-depth studies are required to determine the actual role of miR-138-5p/SIRT1/FOXO1 in wound healing.


A
0 d
7 d
14 d
Control
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative wound healing (%)
150
100
50
0
0
1
2
3
4
b, d
b
d
B
Control
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
50 μm
C
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative microvessel density
8
6
4
2
0
b
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D
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative apoptosis rate (%)
60
40
20
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b
b, d
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NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative miR-138-5p expression
1.5
1.0
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0.0
b
b, d
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E
100 μm
F
100 μm
H
NC
Exo
circ-Astn1-Exo
si-circ-Astn1-Exo
Relative mRNA expression of SIRT1
5
4
3
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b
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NC
Exo
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SIRT1
GAPDH

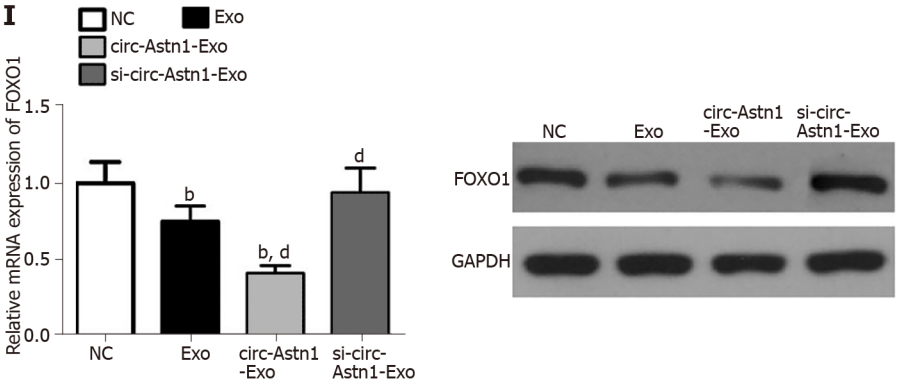

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Figure 4 Exosomes from circular RNA astrotactin 1-modified adipose-derived mesenchymal stem cells have greater therapeutic effect in promoting wound healing in a diabetic mouse model. A: Representative images of full-thickness skin defects after treatment with adipose-derived mesenchymal stem cell (ADSC) exosomes or circular RNA astrotactin 1-modified ADSC exosomes for 0, 1, and 2 wk after wounding; B and C: Microvascular formation evaluated by immunofluorescence staining with cluster of differentiation 31. $^{b}P < 0.001$ *vs* control. $^{d}P < 0.001$ *vs* exosomes; D and E: We assayed apoptosis level *via* terminal deoxynucleotidyl transferase dUTP nick end labeling staining. $^{b}P < 0.001$ *vs* control. $^{d}P < 0.001$ *vs* exosomes; F: Hematoxylin and eosin staining shows wound changes; G-I: Quantitative polymerase chain reaction and western blot analysis showing mi-138-5p (G), SIRT1 (H), and forkhead box O1 (I) expression. $^{b}P < 0.001$ *vs* control. $^{d}P < 0.001$ *vs* exosomes.

ARTICLE HIGHLIGHTS

Research background

Wound healing impairment is a dysfunction induced by hyperglycemia and its effect on endothelial precursor cells (EPCs) in type 2 diabetes mellitus. There is increasing evidence showing that exosomes (Exos) derived from adipose-derived mesenchymal stem cells (ADSCs) exhibit the potential to improve endothelial cell function along with the wound healing process.

Research motivation

The potential therapeutic mechanism of ADSC Exos in wound healing in diabetic mice remains unclear.

Research objectives

To verify the effect of treatment with Exos from circular RNA astrotactin 1 (circ-Astn1)-overexpressing ADSCs on high glucose (HG)-induced EPC dysfunction.

Research methods

In this study, Exos from ADSCs and fibroblasts were used for high-throughput RNA sequencing (RNA-Seq). ADSC-Exo-mediated healing of full-thickness skin wounds in a diabetic mouse model was investigated. We utilized EPCs to investigate the therapeutic function of Exos in cell damage and dysfunction caused by HG. We utilized a luciferase reporter (LR) assay to detect interactions among circ-Astn1, SIRT1 and miR-138-5p. We employed diabetic mice to verify the therapeutic effect of circ-Astn1 on Exo-mediated wound healing.

Research results

High-throughput RNA-Seq detection showed that circ-Astn1 expression was increased in ADSC Exos compared with Exos from fibroblasts. Exos containing high concentrations of circ-Astn1 had enhanced therapeutic effect in restoring EPC function under HG conditions by promoting SIRT1 expression. Circ-Astn1 expression enhanced SIRT1 expression through miR-138-5p adsorption, which was validated by LR assay along with bioinformatics analyses. Exos containing high concentrations of circ-Astn1 had better therapeutic effect on wound healing *in vivo* compared to wild-type ADSC Exos. Immunofluorescence and immunohistochemical investigations suggested that circ-Astn1 enhanced angiopoiesis through Exo treatment of wounded skin as well as suppressing apoptosis through promotion of SIRT1 and decreased FOXO1 expression.

Research conclusions

In summary, we concluded that circ-Astn1 promoted the therapeutic effect of ADSC-Exos and thus improved wound healing in diabetes *via* miR-138-5p absorption and SIRT1 upregulation. Based on our data, we advocate targeting the circ-Astn1/miR-138-5p/SIRT1 axis as a potential therapeutic alternative for treatment of diabetic ulcers.

Research perspectives

More in-depth studies are required to determine the actual role of miR-138-5p/SIRT1/FOXO1 in wound healing.

FOOTNOTES

Author contributions: Wang Z and Wang YB designed the project and drafted the paper based on feedback from the other authors; Feng C, Liu H, and Meng T performed all experiments and analyses; Huang WQ and Song KX took part in the analyses and draft revision.

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Institutional animal care and use committee statement: The Animal Care and Use Committee of Peking Union Medical College Hospital approved the investigation protocol (No: XHDW-2020-01). We carried out all postoperative animal care along with surgical interventions following the NIH Guide for Care and Use of Laboratory Animals. All surgery and euthanasia were performed under sodium pentobarbital anesthesia (30 mg/kg) by intraperitoneal injection, and all efforts were made to minimize suffering.

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

Data sharing statement: The datasets used or/and analyzed during the current study are available from the corresponding author on reasonable request.

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

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ORIGINAL ARTICLE

Basic Study

Stromal cell-derived factor-1α regulates chondrogenic differentiation *via* activation of the Wnt/β-catenin pathway in mesenchymal stem cells

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Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) have been applied to treat degenerative articular diseases, and stromal cell-derived factor-1α (SDF-1α) may enhance their therapeutic efficacy. However, the regulatory effects of SDF-1α on cartilage differentiation remain largely unknown. Identifying the specific regulatory effects of SDF-1α on MSCs will provide a useful target for the treatment of degenerative articular diseases.

AIM

To explore the role and mechanism of SDF-1α in cartilage differentiation of MSCs and primary chondrocytes.

METHODS

The expression level of C-X-C chemokine receptor 4 (CXCR4) in MSCs was assessed by immunofluorescence. MSCs treated with SDF-1α were stained for alkaline phosphatase (ALP) and with Alcian blue to observe differentiation. Western blot analysis was used to examine the expression of SRY-box transcription factor 9, aggrecan, collagen II, runt-related transcription factor 2, collagen X, and matrix metalloproteinase (MMP)13 in untreated MSCs, of aggrecan, collagen II, collagen X, and MMP13 in SDF-1α-treated primary chondrocytes, of glycogen synthase kinase 3β (GSK3β) p-GSK3β and β-catenin expression in SDF-1α -treated MSCs, and of aggrecan, collagen X, and MMP13 in SDF-1α-treated MSCs in the presence or absence of ICG-001 (SDF-1α inhibitor).

RESULTS

Immunofluorescence showed CXCR4 expression in the membranes of MSCs. ALP stain was intensified in MSCs treated with SDF-1α for 14 d. The SDF-1α treatment

promoted expression of collagen X and MMP13 during cartilage differentiation, whereas it had no effect on the expression of collagen II or aggrecan nor on the formation of cartilage matrix in MSCs. Further, those SDF-1α-mediated effects on MSCs were validated in primary chondrocytes. SDF-1α promoted the expression of p-GSK3β and β-catenin in MSCs. And, finally, inhibition of this pathway by ICG-001 (5 μmol/L) neutralized the SDF-1α-mediated up-regulation of collagen X and MMP13 expression in MSCs.

CONCLUSION

SDF-1α may promote hypertrophic cartilage differentiation in MSCs by activating the Wnt/β-catenin pathway. These findings provide further evidence for the use of MSCs and SDF-1α in the treatment of cartilage degeneration and osteoarthritis.

Key Words: Stromal cell-derived factor-1α; Mesenchymal stem cells; Chondrogenic differentiation; Wnt/β-catenin; C-X-C chemokine receptor 4

Core Tip: In this study, we investigated the effect of stromal cell-derived factor-1α (SDF-1α) on the differentiation of bone marrow mesenchymal stem cells (MSCs) and primary chondrocytes *in vitro*. We demonstrated that SDF-1α promotes the chondrogenic differentiation of MSCs, and similar results were observed in primary chondrocytes. In addition, SDF-1α also activates the Wnt/β-catenin pathway to regulate chondrocyte hypertrophy and maturation in MSCs.

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INTRODUCTION

Osteoarthritis (OA) is a chronic, multifactorial disease characterized by progressive degradation of articular cartilage[1]. The underlying molecular mechanism responsible for the pathogenesis of OA is not yet fully elucidated; as such, a disease-modifying therapy remains elusive[2], although a potential therapeutic strategy of cell-based cartilage regeneration using mesenchymal stem cells (MSCs) has been proposed[3,4]. It is known that following cartilage injury, MSCs undergo proliferation to form new cartilage and repair damage. During this process, chemokines play a role in targeted cell recruitment[5]. The chemokine stromal cell-derived factor-1α [SDF-1α, also known as C-X-C chemokine ligand (CXCL) 12 α][6] binds to the CXC receptor 4 (CXCR4) present in synovial fluid and cartilage tissues[7]. SDF-1α plays an important role in the targeted recruitment and chemotaxis of MSCs[8], and increased SDF-1α levels promote the entry of CXCR4-positive MSCs into damaged cartilage[9]. In addition, MSC recruitment mediated by the SDF-1α/CXCR4 axis has been shown to play an important role in other tissue repair processes[10]. Indeed, a previous study showed that intra-articular injection of meniscus progenitor cells promoted cartilage regeneration and improved OA *via* the SDF-1α/CXCR4 axis and by inducing progenitor cell homing[11]. Earlier, Hitchon *et al*[12] had reported the finding of upregulated expression levels of CXCR4 mRNA and protein in chondrocytes of rats with post-traumatic OA, while Kanbe *et al*[13] reported high SDF-1α expression in human chondrocytes of rheumatoid arthritis and OA joint fluid. This latter study also indicated that synovectomy significantly reduced SDF-1α and matrix metalloproteinase (MMP) concentrations in serum. Finally, Xiang *et al*[14] reported their study of human OA cartilage and *in vitro* SDF-1-induced OA chondrocytes, which demonstrated that inhibition of SDF-1α signaling was able to attenuate OA.

MSCs can differentiate into chondrocytes, which are characterized by SRY-box transcription factor 9 (Sox9), aggrecan, and collagen II expression[15]. *In vivo*, human MSCs used for cartilage repair undergo hypertrophic differentiation, which is characterized by an increase in cell volume and in the expression levels of several markers of hypertrophy, including runt-related transcription factor 2 (RUNX2), collagen X, MMP13, Indian hedgehog homolog, and alkaline phosphatase (ALP)[16]. Under physiological conditions *in vivo*, hypertrophic chondrocytes exhibit endochondral ossification. Furthermore, SDF-1α mediates several changes in the bone and cartilage[17], with roles in both physiologic and pathogenic processes. For example, SDF-1α/CXCR4 signaling regulates the bone morphogenetic protein-2-induced chondrogenic differentiation of MSCs and enhances chondrocyte

proliferation and maturation[18]. However, it also increases the expression of MMP3 in chondrocytes, leading to mechanical destruction of the bound matrix[19]. Therefore, despite its role in MSC recruitment, the direct effect of SDF-1α on cartilage differentiation by MSCs requires further clarification.

The present study focused on the direct role of SDF-1α in chondrocyte differentiation and demonstrated that SDF-1α participated in chondrocyte differentiation in MSCs. In addition, the Wnt/β-catenin pathway mediated the effects of SDF-1α on cartilage differentiation.

MATERIALS AND METHODS

MSC isolation and culture

MSCs were obtained from Sprague-Dawley (SD) rats. Ten male 4-8-wk-old SD rats weighing 150-200 g were housed in standard housing conditions with a 12-h light/dark cycle. The rats were euthanized using 20 mg/kg of ketamine intraperitoneally. Bone marrow was flushed from femurs of the SD rats using a 10-mL injector filled with Dulbecco's modified eagle medium (DMEM) and Ham's F12 medium containing 10% fetal bovine serum (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, United States), 100 IU/mL penicillin, and 100 IU/mL streptomycin (Boster Biological Technology, Pleasanton, CA, United States). The cultures were maintained at 37 °C in an atmosphere of 5% CO_2. The cells were grown for 48 h, and the medium was replaced. The cells were allowed to reach 70%-80% confluence and passaged by trypsinization using 0.05% trypsin/ ethylene diamine tetraacetic acid (Boster Biological Technology). The culture medium was replaced every 2 d. Rat MSCs cultured to passage 3 were used for the experiments.

Isolation and culture of primary chondrocytes

Ten male 3-d-old SD rats were euthanized by intraperitoneal ketamine, and their cartilage samples were soaked in a beaker containing 75% alcohol for 15 min. The cartilage surface of the proximal tibia was removed to a depth of 1.0-1.5 mm^3 using the micro-shear method and digested with 0.25% trypsin at 37 °C for 30 min. Following 10 min of centrifugation at 500 × *g*, the tissue pieces were collected and incubated with 0.25% collagenase II (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 37 °C for 24 h. After a second centrifugation, the chondrocytes were cultured under the same conditions as described for the MSCs.

Multilineage differentiation of MSCs

To confirm that the isolated cells were MSCs, their differentiation into bone, cartilage, and adipose cell lineages was induced. For bone differentiation, passage 3 cells were cultured with osteogenic medium (RASMX-90021; Cyagen Biosciences, Inc., Santa Clara, CA, United States). After 21 d, the cells were stained with 0.5% alizarin red S at room temperature. In brief, the cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min at room temperature, and then stained with alizarin red S solution for 30 min at room temperature. Morphology was evaluated using an inverted microscope (Leica DM IRM; Leica Microsystems, Wetzlar, Germany). Chondrogenic differentiation was achieved by pelleting 2.5 × 10^5 passage 3 cells in a 15-mL centrifuge tube at 500 × *g* for 5 min then resuspending the cells in 0.5 mL of chondrogenic induction medium [DMEM high-glucose, 100 nmol/L dexamethasone, 10 ng/mL transforming growth factor (TGF)-β 3, 50 mg/mL ascorbic acid 2-phosphate, 100 mg/mL sodium pyruvate, 40 mg/mL proline and insulin transferrin selenous acid-supplement][20]. The medium was replaced every 3 d. After 21 d, the pellets were fixed with 4% paraformaldehyde for 1 h at room temperature, then embedded in paraffin, cut into 5-μm sections, and stained with Alcian blue. Adipogenesis of MSCs was induced by culturing the cells in 6-well culture plates containing adipogenic medium (Cyagen Biosciences, Inc.). After 21 d, the cultures were fixed with 4% paraformaldehyde, stained with oil red O working solution (60% of 0.5% oil red O/ isopropanol in distilled water) for 1 h at room temperature, and observed using light microscopy (Leica DM IRM; Leica Microsystems).

Fluorescence staining

MSCs cultured in 12-well plates were prepared for immunofluorescence analysis (performed at room temperature). First, MSCs were fixed with 4% paraformaldehyde for 15 min at room temperature. The fixed cells were then permeabilized by incubating in 0.1% Triton (Boster Biological Technology, Inc.) in PBS for 10 min. After the cells were blocked with 3% bovine serum albumin (BSA; Boster Biological Technology, Inc.) in 0.1% Triton/PBS for 1 h at room temperature. The cells were initially incubated with anti-CXCR4 antibody (1:200; Abcam, Cambridge, United Kingdom) overnight at 4 °C and subsequently with an fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibody (H + L) (1:200; Beyotime Institute of Biotechnology, Jiangsu, China) for 30 min at room temperature. The labeled cells were mounted with 4',6-diamidino-2-phenylindole (DAPI) at room temperature and observed by fluorescence microscopy.

MSC micromass culture

MSCs were first resuspended in F12-DMEM medium containing 10% fetal bovine serum, 0.25% penicillin-streptomycin, and 0.25% L-glutamine, and plated at a density of 2.5 × 10^5 cells/10 μL. After incubation for 4 h, a micromass culture medium supplemented with 1 mmol/L β-glycerophosphate and 0.25 mmol/L ascorbic acid with or without SDF-1α (PeproTech, Inc., Rocky Hill, NJ, United States) was added. The cells were cultured in chondrogenic induction medium that was replaced every other day. On day 7, the cells were stained with Alcian blue, and the absorbance of the supernatant was measured at 600 nm.

Chondrogenic differentiation assays

MSCs and primary chondrocytes were seeded in 6-well plates containing the chondrogenic induction medium. The following three conditions were assessed: Control (cytokine-free); 50 ng SDF-1α; and 100 ng SDF-1α[21]. The expression levels of collagen II, collagen X, aggrecan, MMP13, Sox9, and RUNX2 were determined. The expression levels of Wnt/β-catenin were measured in cells incubated for 24 h with 100 ng SDF-1α and ICG-001, an inhibitor of the Wnt/β-catenin pathway in MSCs.

Protein isolation and western blotting

Collagen II (1:2000), collagen X (1:2000), aggrecan (1:2000), MMP13 (1:1000), Sox9 (1:5000), and RUNX2 (1:2000) antibodies were purchased from Abcam, whereas the p- glycogen synthase kinase 3β (GSK3β) (1:2000), GSK3β (1:2000) and β-catenin (1:2000) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, United States). Secondary mouse IgG (1:10000) or rabbit IgG (1:10000) antibodies were purchased from Abcam, and the anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000) antibody was from Boster Biological Technology. Protein was extracted from the cells using 100 mL radio immunoprecipitation assay buffer (Boster Biological Technology, Inc.) supplemented with protease and phosphatase inhibitors. After microcentrifugation for 20 min at 10000 × *g*, the lysates were prepared as described above. The cell protein concentration was detected with a bicinchoninic acid kit (Boster Biological Technology, Inc.). Briefly, a total of 20 μg of cellular protein *per* sample was loaded onto a 10% Bis-Tris gel according to the protocol provided by the manufacturer. The separated proteins were then transferred to polyvinylidene fluoride membranes (Thermo Fisher Scientific), which were blocked for 1 h at room temperature with 5% BSA (Boster Biological Technology, Inc.) in Tris-buffered saline containing 0.1% Tween-20 (TBST). The blots were probed overnight at 4 °C with rabbit antibodies against GAPDH, collagen II, collagen X, aggrecan, MMP13, Sox9, RUNX2, p-GSK3β, GSK3β and β-catenin. Following three washes with TBST, the blots were incubated for 1 h at room temperature with anti-mouse or anti-rabbit IgG-horseradish-peroxidase-labeled secondary antibodies and washed three times with TBST. Finally, immunoreactivity was detected with enhanced chemiluminescence, and densitometry was performed using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, United States).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, United States). The results were summarized as mean ± standard deviation. Every experiment contained ≥ 3 replicate and was performed three independent times, unless otherwise stated. One-way analysis of variance and Fisher's least significant difference post hoc test were performed to compare differences between multiple groups. $P < 0.05$ indicated a statistically significant difference. we use 1 to express $P < 0.05$ and 2 to express $P < 0.01$.

RESULTS

MSC culture and multilineage differentiation potential

The cells were initially quiescent but began to proliferate rapidly after day 3. Growth yielded a monolayer structure, composed of fibroblasts (Figure 1A). At passage 3, the isolated cells were successfully differentiated into the three skeletal cell lineages: Bone, cartilage, and adipose tissue. After culture in the osteogenic medium, nodules formed that were positive for alizarin red S staining, indicating calcium-bearing mineral deposits (Figure 1B). After culture with cartilage induction medium, cartilage microspheres were positive for Alcian blue staining. Blue granules were also noted in MSCs (Figure 1C). After culture in the adipogenic induction medium, lipid accumulation in the form of lipid droplets was noted in some of the cells, which were stained red by oil red O (Figure 1D).

Expression of CXCR4 in rat MSCs

CXCR4 expression was detected in the membrane of the rat MSCs, while DAPI staining was confined to the nuclei of the MSCs (Figure 2).

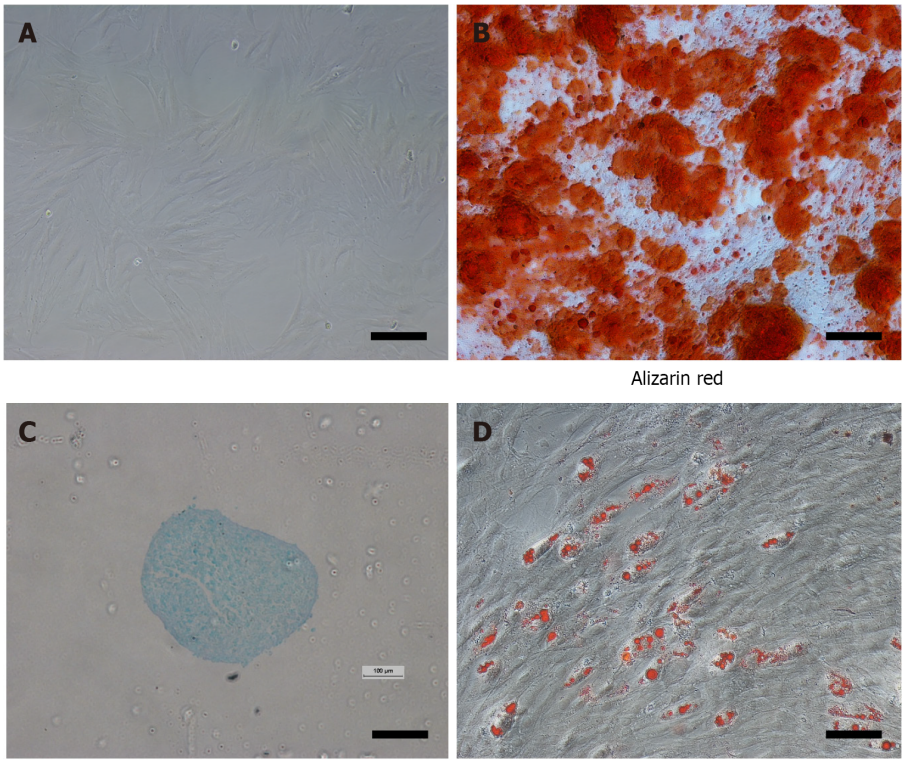

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Figure 1 Characterization of mesenchymal stem cells. A: At passage 3, the cells resembled fibroblasts. Scale bar = 100 μm; B: Differentiation into bone cells was demonstrated by alizarin red S staining. Scale bar = 100 μm; C: Alcian blue staining indicated that the cells had successfully transformed into chondrocytes. Scale bar = 500 μm; D: Oil red O staining confirmed differentiation of the cells into adipose cells. Scale bar = 100 μm.

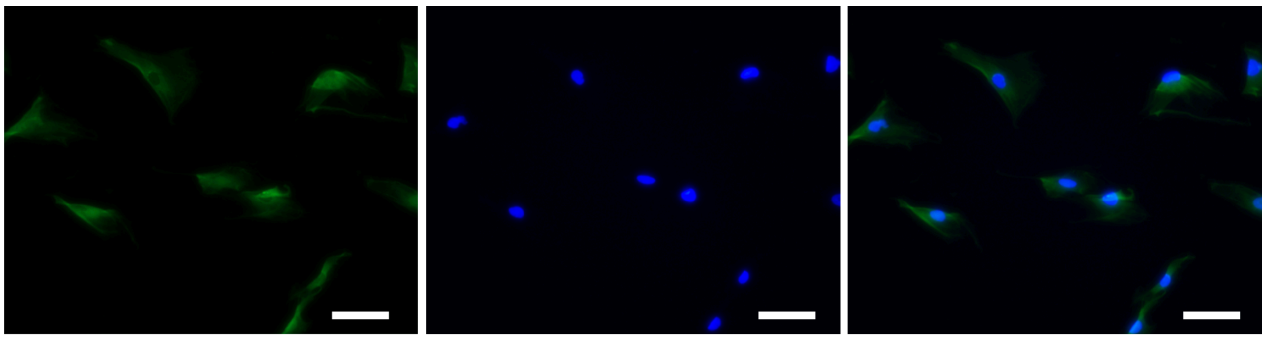

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Figure 2 Expression of C-X-C chemokine receptor type 4 on rat mesenchymal stem cells. Representative image of the expression of C-X-C chemokine receptor type 4 (green fluorescence) on mesenchymal stem cell membranes. CXCR4: C-X-C chemokine receptor type 4; DAPI: 4',6-diamidino-2-phenylindole.

SDF-1a exerted no effect on early cartilage formation of MSCs but enhanced hypertrophic differentiation

No significant differences were noted between control (untreated) cells and cells treated with 50 ng SDF-1α or 100 ng SDF-1α in regards to the size of the cartilage micelles or the absorbance of Alcian blue (Figure 3A and B). ALP expression and activity levels were increased after 14-d SDF-1α treatment compared to control cells (Figure 3C and D).

Effect of SDF-1a on MSCs during cartilage differentiation

Western blotting indicated no significant differences in the expression levels of early chondrocyte differentiation markers (Sox9, aggrecan, and collagen II) between MSCs treated with SDF-1α and untreated MSCs on day 7 (Figure 4A). On day 14, however, the expression levels of chondrocyte hypertrophy markers (RUNX2, collagen X, and MMP13) were increased in a dose-dependent manner in the SDF-1α-

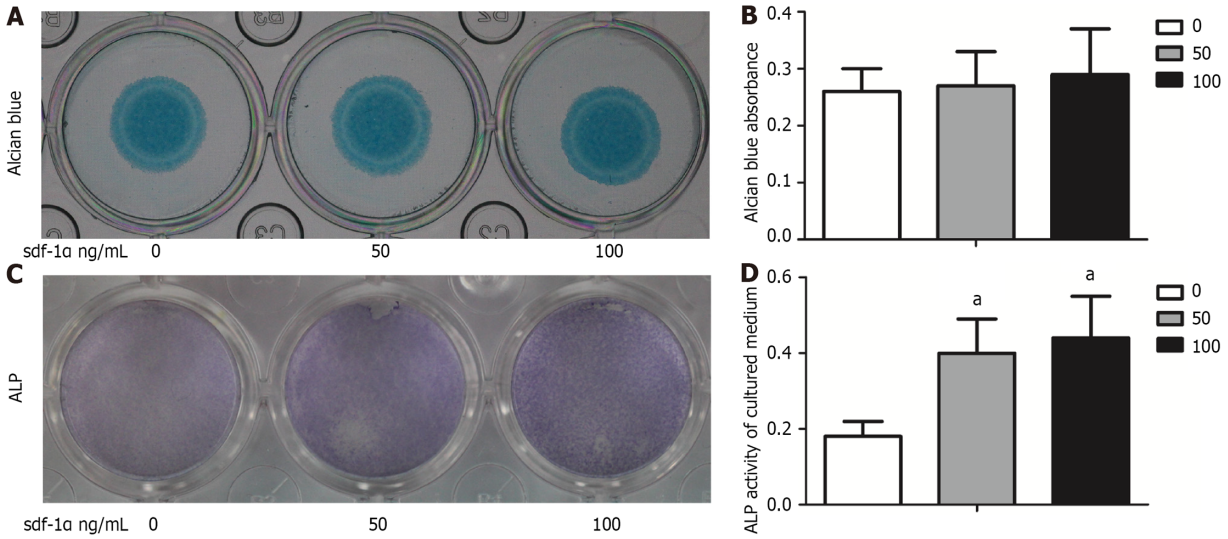

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Figure 3 Alkaline phosphatase activity levels in mesenchymal stem cells treated with stromal cell-derived factor-1α were noted in the absence of an effect on cartilage formation. A: Mesenchymal stem cells (MSCs) were cultured *in vitro* and stained with Alcian blue following 7 d of culture with or without stromal cell-derived factor-1α treatment; B: Alcian blue staining was measured after chemical extraction by measuring the absorbance of the supernatant at 600 nm; C: MSCs were positive for alkaline phosphatase (ALP; light purple staining); D: ALP expression was quantitatively analyzed. The values were representative of the mean ± standard deviation (n = 3). $^{a}P < 0.05$ *vs* control. ALP: Alkaline phosphatase; sdf-1α: Stromal cell-derived factor-1α.

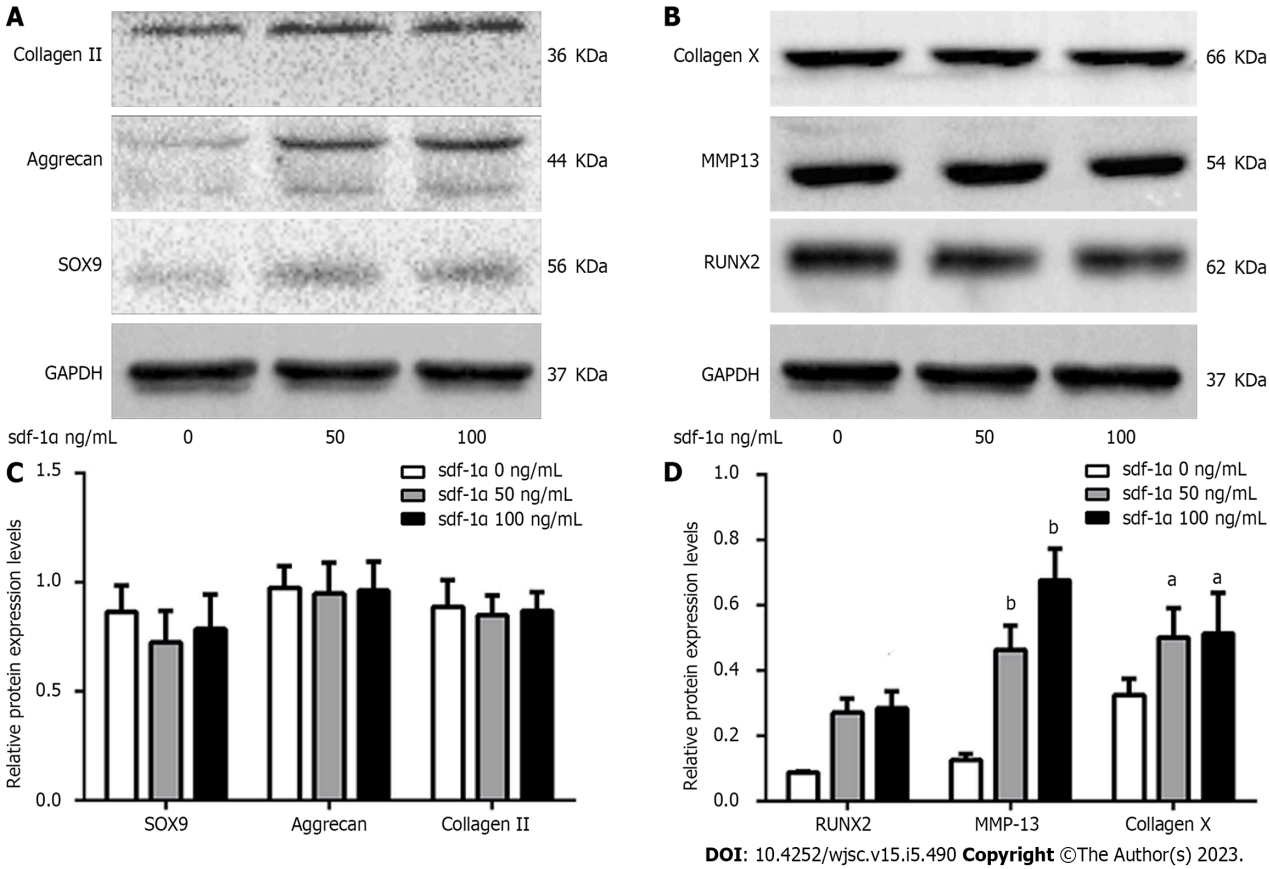

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Figure 4 Effects of stromal cell-derived factor-1α on cartilage differentiation of mesenchymal stem cells. Representative images of western blot analysis of rat mesenchymal stem cells treated with stromal cell-derived factor-1α. A: No changes in the expression levels of SRY-box transcription factor 9 (Sox9), aggrecan, and collagen II were observed; B: Increased expression levels of Runt-related transcription factor 2 (RUNX2), collagen X, and matrix metalloproteinase 13 (MMP13) were observed; C: Relative Sox9, aggrecan, and collagen II protein expression; D: Relative RUNX2, collagen X, and MMP13 protein expression. $^{a}P < 0.05$ *vs* control (Student's *t*-test). $^{b}P < 0.01$ *vs* control (Student's *t*-test). sdf-1α: Stromal cell-derived factor-1α; Sox9: SRY-box transcription factor 9; RUNX2: Runx family transcription factor 2; MMP13: Matrix metalloproteinase 13.

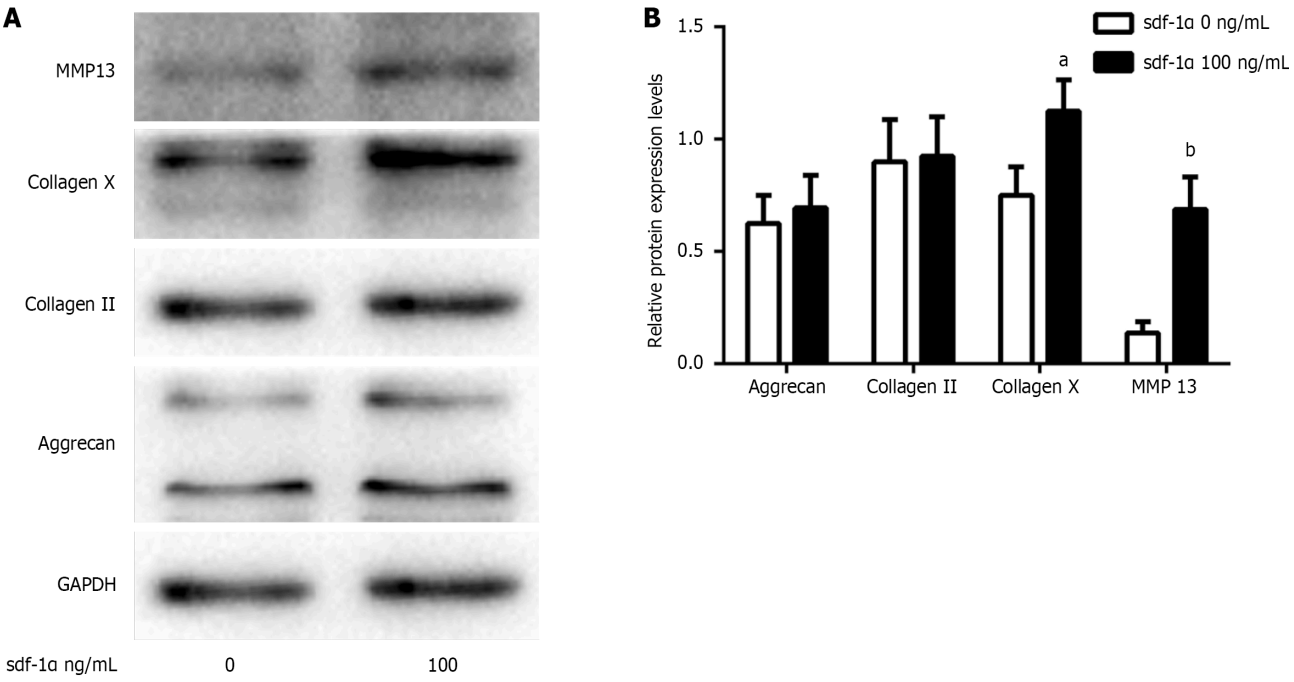

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Figure 5 Effects of stromal cell-derived factor-1α on the cartilage phenotype of primary rat chondrocytes. A: Expression levels of collagen II, aggrecan, collagen X, and matrix metalloproteinase 13 (MMP13) were determined by western blotting in primary chondrocytes treated with stromal cell-derived factor-1α (100 ng/mL); B: Relative collagen II, aggrecan, collagen X, and MMP13 protein expression. $^{a}P < 0.05$ *vs* control (Student's *t*-test), $^{b}P < 0.01$ *vs* control (Student's *t*-test). MMP13: Matrix metalloproteinase 13; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; sdf-1α: Stromal cell-derived factor-1α.

treated group (Figure 4B).

Effects of SDF-1a on the cartilage phenotype of primary chondrocytes.

Western blotting showed that SDF-1α treatment did not affect the expression levels of collagen II and aggrecan in primary chondrocytes, whereas it significantly increased the expression levels of collagen X and MMP13 in the MSCs (Figure 5).

Wnt/β-catenin pathway was involved in the effect of SDF-1a on cartilage differentiation.

SDF-1α promoted the expression of p-GSK3β, decreased degradation of β-catenin, and a gradual increase in β-catenin expression were demonstrated (Figure 6A). Upon blockade of the Wnt/β-catenin pathway *via* ICG-001, the SDF-1α-mediated increase in the expression levels of collagen X and MMP13 was neutralized (Figure 6B).

DISCUSSION

In the present study, rat MSCs, which were successfully differentiated into the three skeletal cell lineages and were positive for the expression of the CXCR4 receptors on the cell membrane, were used to assess the effects of SDF-1α on cartilage formation. The results indicated that the size of the cartilage micromass, the absorbance of Alcian blue, and the expression levels of Sox9, aggrecan, and collagen II did not significantly change in response to SDF-1α. However, the expression and activity levels of ALP and the expression levels of RUNX2, collagen X, and MMP13 were significantly increased. These results demonstrated that SDF-1α promoted hypertrophic cartilage differentiation in MSCs, while not affecting the early differentiation of cartilage. Similar results were obtained in primary chondrocytes. The data further indicated that SDF-1α caused a gradual increase in the expression levels of p-GSK-3β *in vitro* and activated the Wnt/β-catenin pathway, leading to increased collagen X and MMP13 expression levels. These findings demonstrated that the SDF-1α/CXCR4 axis was required in the cartilage differentiation process. Previous studies have implicated other chemokine types, including CXCL8 and CXCL1, as capable of promoting chondrocyte hypertrophy and calcification[22,23].

The Wnt/β-catenin pathway is a classical Wnt signaling pathway involved in tissue development and cell proliferation, differentiation, and apoptosis[24,25]. The signal transduction of the Wnt/β-catenin pathway is well defined and proceeds as follows. Initially, the extracellular Wnt proteins (Wnt-3a, Wnt-4, Wnt-8c, and Wnt-9a) combine with the frizzled and LRP proteins on the cell membrane to form an activation complex. Subsequently, the phosphorylation of GSK-3β blocks the phosphorylation and

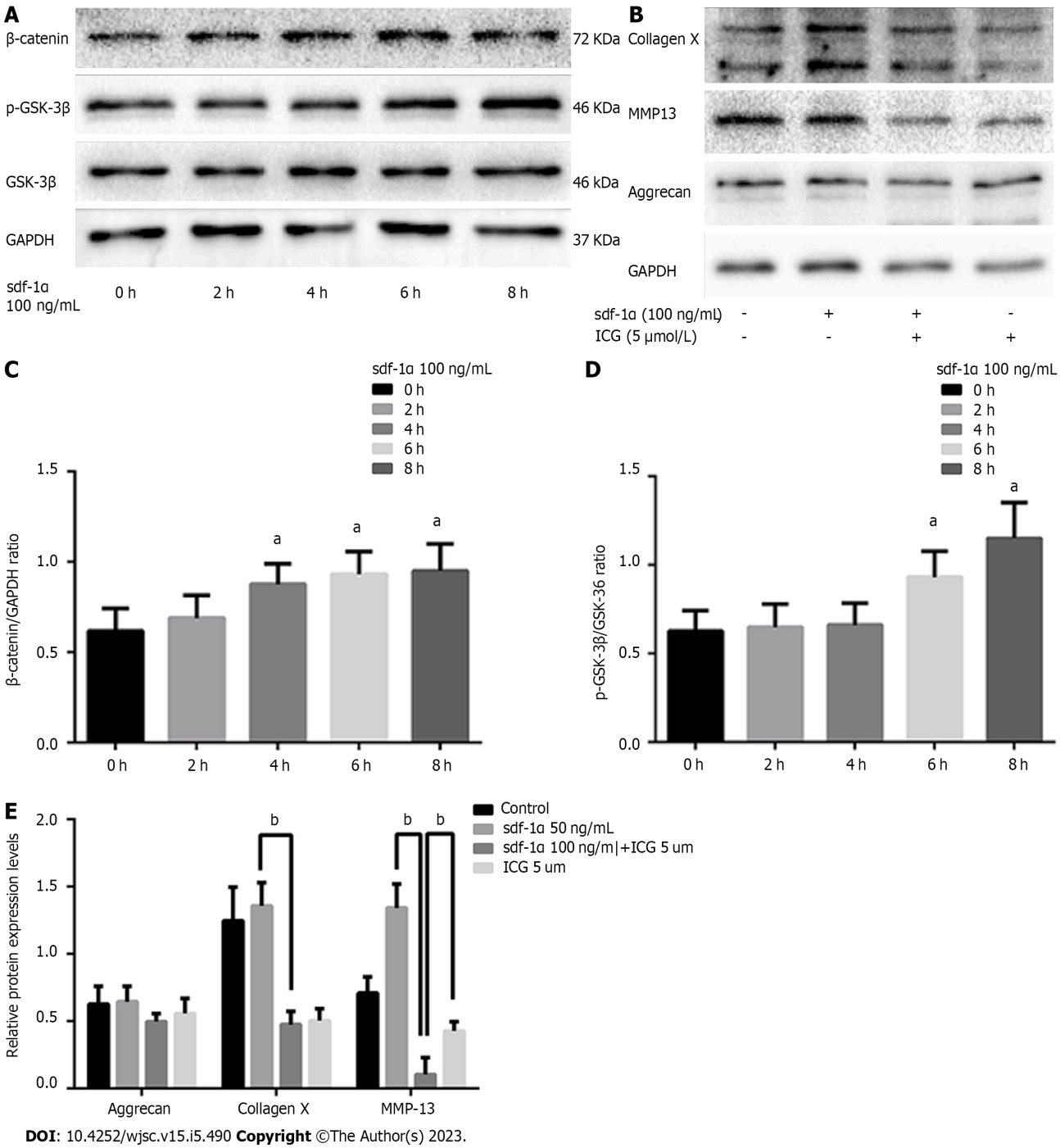

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Figure 6 Wnt/β-catenin pathway involvement in the effects of stromal cell-derived factor-1α on cartilage differentiation. A: Expression levels of β-catenin, p-glycogen synthase kinase 3β (p-GSK-3β), and GSK-3β by western blotting; B: Blockage of the Wnt/β-catenin pathway with ICG-001 inhibited the expression levels of collagen X and matrix metalloproteinase 13; C: Relative β-catenin protein expression; D: Ratio of relative protein expression of p-GSK-3β to relative protein expression of GSK-3β (p-GSK-3β/GSK-3β); E: Relative aggrecan, collagen X, and MMP13 protein expression. $^{1}P < 0.05$, $^{b}P < 0.01$, Student's *t*-test. p-GSK-3β: p-glycogen synthase kinase 3β; GSK-3β: Glycogen synthase kinase 3β; MMP13: Matrix metalloproteinase 13; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; sdf-1α: Stromal cell-derived factor-1α.

degradation of β-catenin. Finally, β-catenin enters the cell nucleus and modulates T cell factor/Lymphoid enhancer factor binding, which initiates the transcription of downstream genes, thus causing biological changes[26-30]. Several Wnt signaling components regulate the hypertrophic maturation of chondrocytes. Specifically, Wnt can induce the accumulation of β-catenin, which then enters the nucleus and binds to cell factor/lymphoid enhancer-binding factor to promote the transcription of the collagen X and MMP13 genes. Ultimately, P-GSK3β can add phosphate groups to the serine/threonine residues at the β-catenin N terminus to promote its degradation[4.31].

Overexpression of the Wnt receptor frzb-1 was shown to hinder chondrocyte maturation and mineralization[32]. In a subsequent study, knock-out of the secreted frizzled-related protein 1, a Wnt signaling antagonist, led to a reduced height of the growth plate and increased calcification of

hypertrophic areas, indicating that activation of the Wnt signaling pathway accelerated endochondral ossification[33]. The findings of the present study are consistent with the collective previous results indicating that the SDF-1α/CXCR4 axis activates the Wnt/β-catenin signaling pathway in MSCs, which in turn increases the production of collagen X and MMP13. Conversely, when we treated the MSCs with the Wnt/β-catenin inhibitor ICG-001, the effects of SDF-1α were no longer observable, which confirmed the regulatory role of Wnt/β-catenin. Thus, the present study indicates that SDF-1α does not promote the early stages of cartilage differentiation nor increase the expression of Sox9, which is similar to the results of Kim *et al*[34].

Hypertrophic differentiation of chondrocytes is the primary barrier preventing the use of MSCs in therapeutic cartilage repair[35,36]. Hypertrophy is sometimes noted in OA[37,38]. However, SDF-1α also mediates MSC recruitment and can exert a positive role in OA[31]. The identification of cytokines that block cartilage hypertrophy caused by SDF-1α, promote physiological endochondral ossification, prevent mineralization of the extracellular matrix, and mediate chondrocyte apoptosis will contribute to an improved understanding of the pathogenesis of OA and provide targets for development of future treatment strategies for this disease[39].

There were some limitations in this study, which must be considered when seeking to generalize our findings. First, measuring the stimulation with SDF-1α in MSCs is challenging because the only verification technique is overexpression or knockdown of the CXCR4 receptor. Second, this study primarily used cell experiments and lacked an *in vivo* perspective to the experimental research. Regardless, through this study, we were able to adequately demonstrate effects of SDF-1α on cartilage differentiation in MSCs and primary chondrocytes.

CONCLUSION

The present study demonstrated a role of SDF-1α in promoting hypertrophic cartilage differentiation in MSCs and primary chondrocytes *in vitro*. SDF-1α activated the Wnt/β-catenin pathway in MSCs. Identification of the novel molecular mechanism by which SDF-1α promotes cartilage differentiation in MSCs suggests a therapeutic approach to OA and cartilage repair.

ARTICLE HIGHLIGHTS

Research background

Stromal cell-derived factor-1α (SDF-1α) has a chemotactic effect on mesenchymal stem cells (MSCs), and SDF-1α and MSCs are used together to treat cartilage degeneration and cartilage defects. The specific effects of SDF-1α on cartilage differentiation in MSCs need to be clarified.

Research motivation

Understanding the effects of SDF-1α on MSCs will provide a new theoretical basis for the use of MSCs in the repair of cartilage degeneration.

Research objectives

To explore the role and mechanism of SDF-1α on cartilage differentiation in MSCs and primary chondrocytes.

Research methods

MSCs were treated with SDF-1α and subsequently stained for alkaline phosphatase and with Alcian blue to demonstrate chondrogenic differentiation. Western blot analysis was used to examine the expression of cartilage differentiation-related and Wnt/β-catenin pathway proteins in MSCs and primary chondrocytes.

Research results

After extraction and incubation with the appropriate differentiation media, MSCs differentiated into the three skeletal lineages. SDF-1α exerted no effect on early cartilage formation but enhanced hypertrophic differentiation in MSCs. SDF-1α had no effect on the expression of SRY-box transcription factor 9, aggrecan, and collagen II but increased the expression of runx family transcription factor 2, collagen X, and matrix metalloproteinase 13 in MSCs and primary chondrocytes. SDF-1α increased the expression of p- glycogen synthase kinase 3β and β-catenin.

Research conclusions

SDF-1α enhanced hypertrophic differentiation in MSCs and primary chondrocytes. This effect was achieved by activating the Wnt/β-catenin pathway.

Research perspectives

These findings provide a new theoretical basis for the treatment of cartilage degeneration with MSCs.

FOOTNOTES

Author contributions: Chen X acquired the data; Zheng J and Dong YH designed the experiments; Chen X and Liang XM analyzed the data and wrote the manuscript; Liang XM and Chen X supervised the study; all authors read and approved the final manuscript.

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