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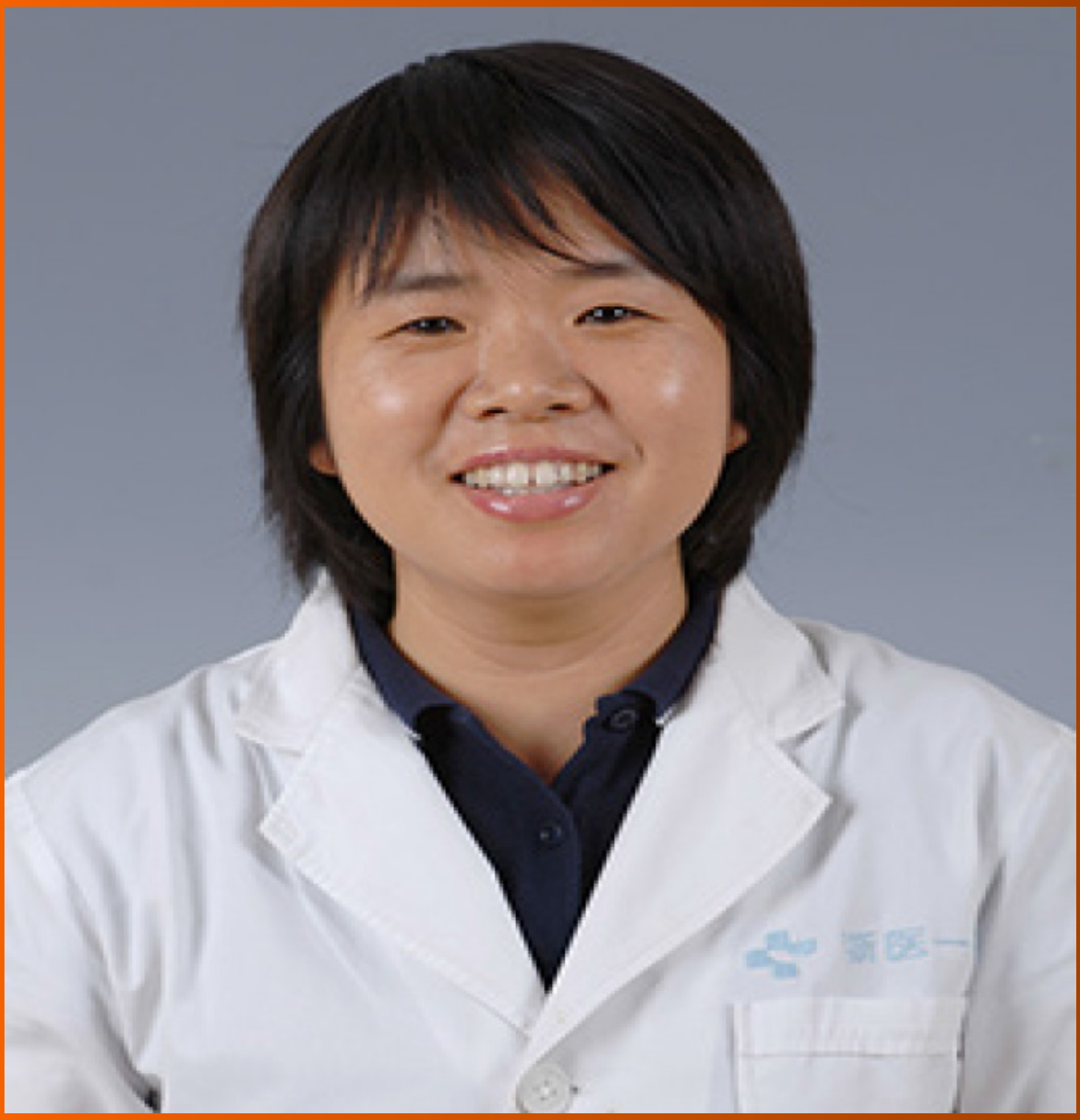

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REVIEW

Molecular modulation of autophagy: New venture to target resistant cancer stem cells

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Abstract

Autophagy is a highly regulated catabolic process in which superfluous, damaged organelles and other cytoplasmic constituents are delivered to the lysosome for clearance and the generation of macromolecule substrates during basal or stressed conditions. Autophagy is a bimodal process with a context dependent role in the initiation and the development of cancers. For instance, autophagy provides an adaptive response to cancer stem cells to survive metabolic stresses, by influencing disease propagation *via* modulation of essential signaling pathways or by promoting resistance to chemotherapeutics. Autophagy has been implicated in a cross talk with apoptosis. Understanding the complex interactions provides an opportunity to improve cancer therapy and the clinical outcome for the cancer patients. In this review, we provide a comprehensive view on the current knowledge on autophagy and its role in cancer cells with a particular focus on cancer stem cell homeostasis.

Key words: Autophagy; Cancer stem cells; Cancer cells; Cancer therapy; Therapeutic resistance; Cancer metastasis

Core tip: Cancer stem cells (CSCs) are a distinct subpopulation in the tumor bulk that are highly plastic, and autophagy has been suggested to modulate their stemness and development during cancer progression. Autophagy is a pro-survival mechanism used by cancer cells to provide bioenergetic substrates. Therefore, dissecting the role of autophagy in cancer propagation can theoretically lead to a more efficient cancer treatment via the modulation of autophagy, in combination with chemotherapeutics to sensitize and target CSCs. This review summarizes the divergent role of autophagy in CSCs and cancer cells and attempts to elucidate the molecular mechanisms involved.

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INTRODUCTION

Autophagy ("self-consumption") is a conserved catabolic process which assists in the clearance of superfluous, damaged organelles and proteins, and contributes in the recycling of the constituents for the maintenance of metabolic homeostasis and as a pro-survival mechanism[1]. Autophagy is further activated by intrinsic and environmental stressors including nutrient deprivation, oxidative stress, cytokine and growth factor deficiency, hypoxia and exposure to infection[2,3]. It can be noted that basal autophagy acts as a quality assurance mechanism in cells and as a source of metabolites[4]. Dysregulation of autophagy is associated with a variety of inflammatory and infectious conditions, as well as neurodegenerative pathologies, ageing and cancer[5].

Autophagy is a highly regulated mechanism that facilitates the deliverance of cytoplasmic components for lysosomal mediated degradation. There are three distinct forms of autophagy, such as microautophagy, chaperone mediated autophagy (CMA) and macroautophagy. Microautophagy is modulated by the direct sequestration of cytosolic cargo causing engulfment, followed by indentation of the lysosome leading to degradation[6]. In comparison, CMA is a prime example of selective autophagy. In this particular pathway, chaperones are utilized targeting specific proteins containing a pentapeptide KFERQ motif sequence. Once engaged this leads to the translocation across the lysosome membrane mediated by lysosome associated membrane protein 2A[7,8]. In contrast, macroautophagy (herein referred to as autophagy) initiates the degradation of intracellular organelles by delivering them to the lysosome by sequestrating sections of the cytoplasm via double membrane vesicles called autophagosomes. The fusion between these two entities not only promotes degradation but also generates bioenergetic substances for recycling. Emerging studies describe the existence of a cross talk between CMA and macroautophagy that promotes a compensatory mechanism under basal and stressed conditions[7,9].

The regulation and process of canonical autophagy

Autophagy relates genes (*Atg*) are involved in the development and turnover of the autophagosomes. Formation of the autophagosome proceeds through multiple steps that include initiation, nucleation, elongation, maturation and thereafter fusion with the lysosome[8,10]. The mitochondria and the endoplasmic reticulum (ER) are contact sites for the formation of autophagosomes[11,12] (Figure 1).

Upstream signaling pathway such mammalian target of rapamycin (mTOR) is a major negative regulator of autophagy as it senses amino acid availability and initiation of cellular anabolism. 5′-AMP-activated protein kinase (AMPK) is activated during starvation[2]. Under these circumstances, AMPK phosphorylates tuber sclerosis complex 2 which inhibits mTOR[13]. Moreover, Atg7 protein is essential in modulating starvation-induced autophagy as demonstrated in *Atg7* conditional knockout mice[14].

The inhibition of mTOR sequentially leads to the activation of pre initiation complex composed of unc-51-like kinase 1 (ULK1) complex, FAK family kinase interacting protein of 200 kDa, Atg13 and Atg101, causing translocation to the membrane, and triggering the initiation step for the assembly of autophagosomes[10]. The ULK1 complex phosphorylates the class III phosphatidylinositol-3-kinase (PI3K) vacuole protein sorting 34 (VPS34) complex; consisting of VPS15, Beclin-1 (BECN1) and Atg14, which stimulates the generation of phosphatidylinositol-3-phospate 3 (PI3P), an essential lipid molecule required for the nucleation step of the phagophore[15-17]. Atg9 positive vesicles on the ER contribute to the nucleation process by interacting with the ULK1 complex[17]. To promote autophagosomes elongation, WD repeat domain phosphoinositide-interacting protein 2 (WIPI-2) and zinc-finger FYVE domain-containing protein 1 are employed for the recruitment of two ubiquitin like systems[16]. Firstly, Atg7 and Atg10 act as E1 like and E2 like enzymes to covalently conjugate Atg12 to Atg5 and then attach to Atg16L[8,18,19]. In the second conjugation pathway, Atg12-Atg5 conjugate serves as an E3 like enzyme, where Atg8 family member LC3 is attached to phosphatidylethanolamine[2,19]. Atg7 and Atg3 mediate this process. Next, the autophagosome matures by membrane bound LC3.

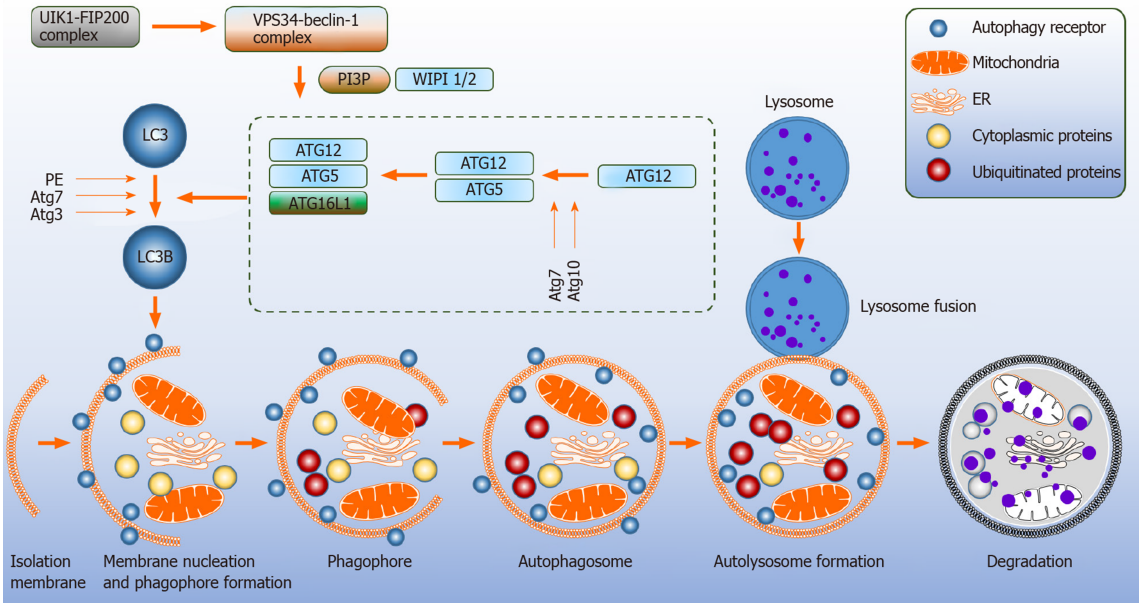

Figure 1 Canonical autophagy pathway. Autophagy is a multistep process that includes the following steps: initiation, nucleation, elongation, maturation and fusion with the lysosome. Several proteins referred to as autophagy related genes regulate this process. Autophagy is stimulated under basal conditions and is induced by stress, for example nutrient deprivation. ATG: Autophagy related genes; ER: Endothelial reticulum; FIP200: FAK family kinase interacting protein of 200 kDa; LC3: Light chain 3; PE: Phosphatidylethanolamine; PI3P: Phosphatidylinositol-3-phospate 3; ULK1: Unc-51-like kinase 1; VPS34: Vacuole protein sorting 34.

NBR1 neighbor of BRAC1 and adaptor protein p62 facilitate in the degradation of misfolded and ubiquitinated substrates by binding to Atg8-LC3[18-20]. The closure of the autophagosome is driven by LC3 causing the Atg12-Atg5-Atg16L complex to dissociate from the autophagosome membrane leaving the lipidated LC3 (LC3B; microtubule-associated proteins 1A/1B light chain 3B) in the autophagosome[16,18]. The degradation of LC3B and p62 are widely accepted markers to measure the autophagic flux.

It should be noted, however, that multiple signaling cascades control autophagy and modify ULK1 and class III PI3K complexes. These include antigen specific receptors (B cell receptor and T cell receptor), CD40 "the co-stimulatory molecule", Toll like receptors, cytokine receptors and nucleotide-binding oligomerization domain protein 2[2]. The VPS34-BECN1 complex can be inactivated by the anti-apoptotic proteins from the B cell lymphoma-2 (BCL-2) family[16]. Here we have discussed the major canonical pathway that utilizes mTOR (Figure 1).

Non-canonical autophagy

Autophagy that precedes the formation of autophagosomes without the involvement of the core machinery is referred to as non-canonical autophagy. An example of non-canonical autophagy would be LC3-associated phagocytosis (LAP) which depends on class III PI3K subunit called RUBICON, a negative regulator of autophagy[2,21]. Unlike canonical autophagy, LAP only requires BECN1 and VPS34 as a pre-initiation complex and downstream conjugation of LC3 to generate NADH oxidase 2[22]. LAP-LC3 is associated to autophagosome maturation and facilitating the degradation of engulfed cells. LAP does not respond to nutrient deficiency or intracellular stressors, unlike canonical autophagy. Additionally, the substrates for this process are extracellular entities including Toll like receptor, pattern recognition receptors and dead cells[22]. LAP occurs in multiple immune cells, such as macrophages, dendritic cells (DCs) and epithelial cells[21]. LAP deficiency in cells and animal models trigger exaggerated inflammation[22].

In the canonical form, it is assumed that the generation of PI3P is essential for the process of autophagy. However, Mauthe *et al*[23] reported resveratrol mediated autophagy did not stimulate PI3P dependent accumulation of WIPI-1 at the autophagosome membrane. This finding was confirmed by PI3P inhibition using wortmannin in combination with resveratrol which led to an increased autophagic flux of LC3B and GFP-LC3 puncta formation. This was promoted in the absence of phagophore formation suggesting an alternative contact site for autophagosome formation. Additionally, the actions of resveratrol were found to be independent of BECN1; however, required Atg7 and Atg5 to induce the LC3 lipidation. It can be concluded that resveratrol induces non-canonical autophagy[23].

The origin of the autophagosome membrane and the formation of the autophagosome remains unclear[24]. Recently, using freeze fracture replica immunolabelling, WIPI-1 puncta were found to be localized on the ER and Plasma membrane and WIPI-2 was detected close to the Golgi cisternae under starvation induced autophagy, exclusively. These findings suggest that WIPI-1 and WIPI-2 are essential components of the autophagosome and the autophagosome membrane site and formation may potentially originate from the ER, Plasma membrane and the Golgi[25]. Interestingly, the deletion of WIPI-2 in the germinal center (GC) B cells enhanced the autophagic activity, suggesting that B cells derived from the GC have the ability to switch from canonical autophagy upon challenge to non-canonical autophagy to meet their metabolic demands[26].

It is believed that Atg5 and At7 are essential for autophagy. However, recent studies have challenged this notion. Atg5/Atg7 independent non-canonical autophagic pathway have been identified, which are able to form autophagosomes mediated in a Rab9 dependent manner from the trans-Golgi network and late endosomes. Autophagy proteins, such as ULK1 and BECN1 were found to regulate this process independent of LC3[27]. The resulting autophagosomes mature and fuse with the lysosome and undergo cargo clearance[28]. Furthermore, ULK1 dependent/Atg5 independent autophagy has been implicated in the removal of the mitochondria from fetal definitive erythroid cells *in vivo*[29]. Additionally, *ULK1*$^{-/-}$ mice models were able to express LC3B under nutrient depleted conditions; indicating the role of ULK1 in the induction of autophagy is dispensable[30]. These reported studies suggest ULK1 is not essential for Atg5/Atg7 dependent canonical autophagy[14,31]. Moreover, ULK1 is upregulated during non-canonical autophagy, and the silencing of *ULK1* inhibits this process[27].

Cross talk between autophagy and apoptosis

It is evident that autophagy participates in catabolism including the breakdown of long-lived proteins, providing bioenergetics material to facilitate in the production of adenosine triphosphate (ATP) and meet the metabolic demands of cells undergoing adverse conditions and rescue them. However, under prolonged metabolic-stressed conditions the pool of bioenergetic substrates will be facilitated to generate ATP dependent apoptosis[32]. Predominately, autophagy has a cytoprotective role. Overall, it can be assumed that autophagy and apoptosis are activated by a common stimulus[19].

Apoptosis "self-killing" is a form of type 1 programmed cell death (PD) and is characterized by the distinct morphological changes causing nuclear condensation (Pyknosis) and fragmentation (Karyorhexis), and membrane blebbing a requisite for the generation of apoptotic bodies (smaller apoptotic cell fragments)[33,34].

Emerging literature indicates a complex network that regulates the interplay between autophagy and apoptosis. This is cell type and stimuli dependent. This dynamic interplay has been described in the following examples: Autophagy and apoptosis can function together in order to induce cell death, autophagy can promote cell survival by antagonizing apoptosis, or autophagy can assist in cell death by activating apoptosis[16,35,36].

Multiple stimuli that can trigger cell death can also induce autophagy. Autophagy as a cytoprotective mechanism is usually induced first, followed by apoptosis[16,37]. Death associated protein kinase (DAPK) signaling is an example when both apoptosis and autophagy are induced either simultaneously or sequentially. Upon stimulation, DAPK phosphorylates BECN1 leading to its dissociation from BCL-2; thus, activating autophagy by binding to VPS34[38,39]. However, activated DAPK is also able to stimulate apoptosis in autophagy deficient conditions[40]. It can be postulated that DAPK regulated autophagy is induced by low levels of stress, however, intense and chronic stress stimuli can initiate apoptosis through DAPK[16].

It has been proposed that autophagy and apoptosis display an inhibitory relationship during the removal of pro-apoptotic proteins in the cytoplasm caused by autophagy, resulting in reduced apoptosis. Caspase-8 activation is a critical step during the extrinsic apoptosis signaling. However, selective autophagy may interfere in an inhibitory manner with the cell-death pathway through the degradation of capsase-8[41]. Furthermore, autophagy can be inhibited by apoptosis *via* numerous mechanisms; for example, autophagy exhaustion during increased intensity levels of stress. In this condition, degradation of autophagic proteins and caspases activity is reduced. For example, BECN1 inactivation occurs after caspase-mediated cleavage, stimulating the release of pro-apoptotic factors, and resulting in autophagy inhibition and induction of apoptosis[42].

Autophagy dependent cell death is defined as a form of cell death distinct from apoptosis or necrosis that mechanistically depends on the autophagic machinery[43]. It is postulated that the formation of autophagosome, and not degradation, leads to the

activation of caspase-8 and the execution of cell death. As reported in mouse embryonic fibroblasts treated with proteasome inhibitor Bortezomib, and pan-sphingosine kinase inhibitor. Pro-caspase- 8 interactions with p62 have been shown to co-localize with the autophagosomes. The surface of the autophagosomes serves as a platform for the maturation of caspase-8 and the initiation of apoptosis[16,43]. Furthermore, the depletion of *Atg5* ablated caspase-8 processing in the presence of Bortezomib leading to a significant reduction in cell death[44].

CANCER STEM CELLS

Tumors are derived from heterogeneous cell types. Cancer stem cells (CSCs; also known as tumor initiating cells) are a small subpopulation of cancer cells within the tumor bulk tissue that retain the capacity for self-renewal, disease propagation, and metastasis, which are decisive for tumor recurrences and are therapy resistance[45-47]. In general, stem cells are characterized by their distinct ability to switch their cell cycle profile from quiescent to proliferative behavior in order to maintain their capability for self-renewal and later multi-potency[48]. Similarly, CSCs have the extraordinary capability to self-renew and differentiate rapidly; accumulating mutations and genetic alterations and transmitting these defects to the proliferating progeny, giving rise to tumor heterogeneity conferring to resistance against anti-cancer therapeutics[49].

Similar to normal stem cells, CSCs reside in dynamic microenvironments known as the stem cell niche, this regulates the fate of adult stem cells by providing signals, such as cell-cell contact and secreting mediators to promote CSCs renewal, tumor invasion and metastasis[24,50]. Normal niches are comprised of heterogeneous collection of cells, such as endothelial, fibroblasts, immune cells, perivascular cells, components of the extracellular matrix, cytokines and growth factors[51]. In comparison, the CSCs niche itself is part of the tumor specific microenvironment that remains distinct from the normal niche[52]. During tumor progression to a malignancy, the CSC state in the primary tumor depends crucially on the microenvironment and potentially on the CSC niche itself[53]. Targeting the CSCs niche is the current subject of research as it is a valuable modality for the treatment and prevention of CSCs growth and downstream signaling[52].

The functional characterization of CSCs in multiple studies have clarified that CSCs are the foundation of tumor formation that can survive treatment with conventional therapies and can cause the recurrence of cancer[54,55]. According to the concept of a stem cell, it can be assumed that even a few surviving CSCs after therapy is sufficient to develop a new tumor leading to a relapse. Due to the ability of CSCs to initiate relapse after conventional cancer therapy, they represent a crucial therapeutic target[46]. CSCs were first identified in acute myeloid leukemia (AML); their presence was confirmed by the isolation of AML-initiating cells based on their phenotypical markers[56]. In solid tumors, breast cancer was one of the first to be characterized, which led to the identification of a specific subpopulation of CSCs marked by $CD44^{+}CD24^{-/Low}$ lineage. This tumorigenic population of cells was able to initiate tumor growth in immunosuppressed mice[57,58]. Furthermore, CSCs have been discovered in several solid cancers, such as lung[55], pancreatic[59], colon[60,61], melanoma[62], ovarian[63,64], brain cancers[65,66] and hematological malignancies of both myeloid and lymphoid origin[67-69].

CSC models of tumorigenesis and plasticity

To date, two paradigms: hierarchical and stochastic have been proposed to account for the tumor origin, progression and heterogeneity. In brief, the hierarchical model is based on a concept that tumor cells are hierarchically arranged cell populations and CSCs represent the top of the arrangement. Carcinogenesis proceeds when a healthy normal stem cell escapes regulation and transforms into a stem cell-like phenotype-CSCs. This in turn gives rise to heterogeneity by generating differentiated and quiescent cells whose proliferation capacity is restricted[52,70]. By contrast, in the stochastic model, cancer is derived from a single somatic cell that initiates tumorigenesis and progression. This paradigm partially relies upon the environment in which the cancer cell is located in, but, fundamentally is defined by hyper proliferation and the acquisition of mutational burden during the cell cycle process contributing to clonal expansion[52].

AUTOPHAGY IN CANCER STEM CELLS

Role of autophagy in the maintenance of CSCs

As highlighted earlier in this review, autophagy is a multifaceted pro-survival mechanism. In cancer, the role of autophagy is context dependent. Autophagy elicits tumor suppressing functions during tumor initiation by limiting inflammation, tissue damage, and genome instability by removing damaged mitochondria and reducing oxidative stress[2]. Extracellular stimuli, such as oxidative stress, nutrient depletion, increased metabolism and hypoxia result to disease propagation; thus, demanding autophagy to meet the high metabolic demands by providing recycled bioenergetic substrates to the CSCs, and whilst doing so, implementing its role as a tumor promotor (Figure 2)[71].

It has been proposed that autophagy is associated to CSC maintenance. *LC3B* gene knockdown in human embryonic stem cells (ESCs) leads to a reduction in pluripotency and due to the accumulation of pluripotency associated proteins suggesting autophagy regulates these proteins[72]. Autophagic flux is upregulated in mammospheres in basal and starvation-induced autophagy and is driven by BECN1 and Atg4A for their survival and expansion. Inhibition of these autophagy genes abolishes the tumor formation[73,74]. Aldehyde dehydrogenase 1-positive ($ALDH1^+$) CSCs isolated from MCF-7 mammospheres presented an increased LC3B dependent autophagic flux with higher rate of p62 degradation compared to the bulk population; indicating an increased synthesis of autophagosomes. In addition, suggesting elevated autophagy is critical for CSCs[74]. Moreover, Antonelli *et al*[75] reports that ataxia-telangiectasia mutated (ATM) kinase modulates breast CSCs through Atg4C. This was validated in an overexpression study of *Atg4C* that was assessed in *ATM* gene silenced cells using sh*ATM*; this led to the rescue of mammosphere formation in *ATM* knockdown cells. These findings correlated with the microarray data of breast cancer samples, however, excluded triple negative tumors[75]. Indeed, these autophagy genes have shown to promote CSC survival and tumorigenicity. RNAi screenings reveal constitutive STAT3 activity is regulated by autophagy and is enriched in the triple negative breast cancer cell lines[73,76]. In those cell lines, Atg7 and BECN1 modulate CD24 expression in $CD44^+CD24^{-/low}$ CSC population and secret interleukin 6 (IL-6) through gp130 and JAK-STAT pathway for CSC maintenance[73,77].

MMTV-PyMT is a well-characterized transgenic murine model for breast CSCs tumorigenesis. Yeo *et al*[78] reported autophagy differentially regulates two distinct breast cancer stem-like cells *ALDH1*+ and $CD29^{hi}CD61^+$ though EGFR/STAT3 and Tgfβ/Smad. Depletion of *FIP200* decreased STAT3 activation by decreasing phosphorylation of EGFR and had consequently impaired the tumor initiating properties of *ALDH1*+ and $CD29^{High}CD61^+$ breast CSCs. Autophagy inhibition led to decreased mRNA levels of *TGFβ2* and *TGFβ3* triggering dysregulation in Smad signaling which is essential for $CD29^{High}CD61^+$ CSCs[78]. The secretion of IL-6 is autophagy dependent and is mediated through STAT3/JAK2 pathway[77]. From these studies, it can be assumed STAT3 signaling may potentially be an important factor in CSCs transformation.

In general, FOXO transcription factors have been associated in the regulation of cellular homeostasis, stem cell maintenance, ageing and tumor suppression. Mice with somatic deletion of *FOXO1, FOXO2* and *FOXO4* resulted in thymic lymphomas and hemangiomas[79]. Upregulation of *FOXO1* promotes self-renewal of t(8;21) pre leukemia cells *in vitro* and *in vivo*, and restricts differentiation of AML cells with t(8;21) translocation; indicating *FOXO1* is not a tumor suppressor, however, plays a crucial role in leukemia stem cells (LSCs) maintenance[80]. Absence of *FOXO3* has been reported to contribute to the expansion of CSC population as well as increase self-renewal and tumorigenesis in prostate[81], colon[82], and glioblastoma[83] and promote tumor initiation in breast cancer[84]. Recently, it has been proposed that DNA methyltransferase 1 mediates *FOXO3a* promoter hyper methylation causing downregulation of *FOXO3a* gene expression in breast CSCs; thus, suppressing CSCs phenotype markers and tumorigenicity[85]. To date, the role of FOXO in CSCs remains controversial. It has been reported FOXOs are implicated in autophagy[86-88]. *FOXO3* overexpression studies reveal this gene directly regulates autophagy related genes involved in the autophagosome pre-initiation complex: *WIPI-1/2*, core initiation complex: *ULK1*, autophagosome formation and elongation: *Atg14*, *GABARAP*, *Atg5*, *Atg10*. *FOXO3* knockout cells downregulated many of these genes and PINK1 (component of mitophagy) and exhibited poor LC3B lipidation turnover; indicating *FOXOs* are required to maintain basal autophagy in neural stem and progenitor cells[87]. *FOXO3A* induced autophagy promotes survival in human pluripotent stem cells[88]. The pro autophagy protein, AMBRA1, modulates the differentiation of regulatory T cells through FOXO3/FOXP3 axis. In the context of immunosurveillance against tumors, AMBRA1 deficiency leads to defective generation of the induced

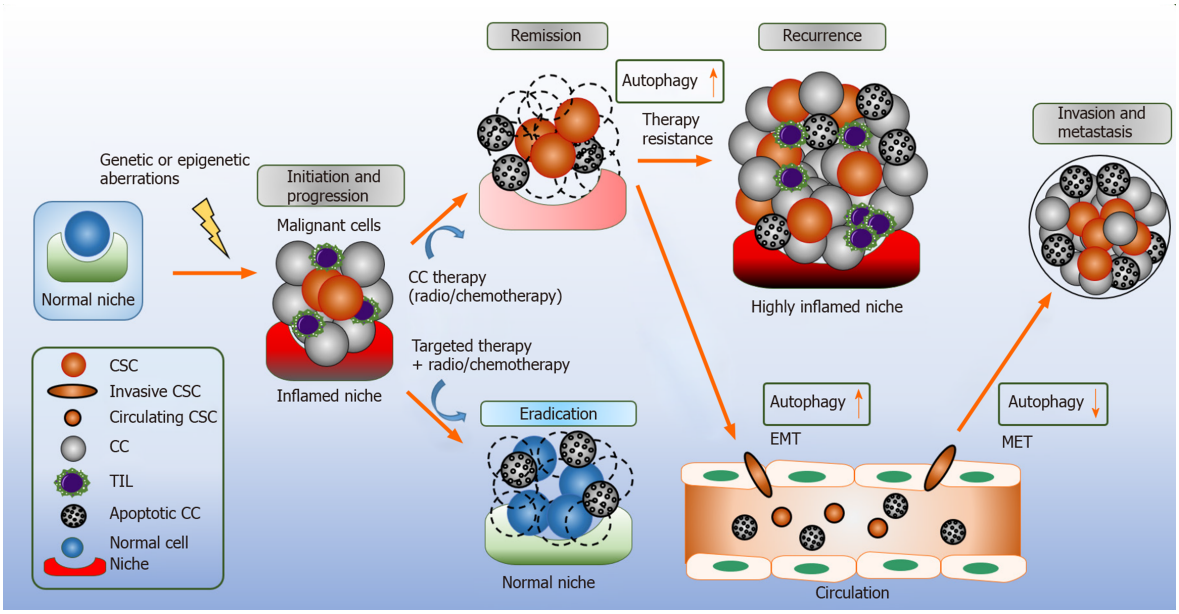

Figure 2 Autophagy in cancer stem cells. Autophagy has a context dependent role in cancer. Cancer stem cells (CSCs) are a heterogeneous collection of different cells types that acquire genetic aberrations/epigenetic modifications and retain the ability to undergo extensive cell proliferation, retain stemness and give rise to differentiated diverse cancer cell lineages. Potentially the CSC niche will provide protective mechanisms for the disease propagation. Autophagy promotes invasion of cancer stem cells through TGF-1β dependent epithelial-mesenchymal transition; however, during mesenchymal-epithelial transition autophagy is downregulated as the circulating CSCs are scavenging an organ to seed for metastasis. Moreover, autophagy reinforces the resilience of CSCs plasticity, remodeling the immunosurveillance and facilitating the acquisition of resistance to conventional chemotherapies which contribute to cancer relapse. By targeting autophagy, cancer cells and CSCs are sensitized to enhancing the efficacy of chemotherapy agents and reducing their toxicity and disease relapse. CSC: Cancer stem cell; CC: Cancer cell; EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition; TIL: Tumor-infiltrating lymphocytes.

regulatory T cells in lymph nodes of tumor bearing mice; influencing the regulatory T cells function in tumor response[89].

Additional FOXO family members are associated to autophagy. It is reported that *FOXA2* knockdown in ovarian CSCs leads to a reduction in the number of spherical clusters of cells, size and the percentage of phenotype surface markers; suggesting FOX2A modulates the ability of self-renewal *in vitro*[90]. Inhibition of autophagy by *Atg5* knockdown, bafilomycin A1 (vacuolar H^+ ATPase inhibitor) or chloroquine (CQ; lysosomotropic agent- late stage autophagy inhibitor) repressed *FOXA2* expression. *FOXA2* overexpression partially rescues these effects; indicating autophagy modulates ovarian CSCs stemness through *FOX2A*[90]. These studies identify a synergy between FOXOs and autophagy. This relationship promotes CSC stemness and tumorigenesis; however, the mechanisms behind these actions remains unclear and require further elucidation. Though it is noteworthy that the regulatory role of autophagy in CSC is very complex.

Autophagy induces metabolic reprogramming in CSCs

The tumor microenvironment (TME) is a critical driver of tumor heterogeneity, encouraging CSCs plasticity, remodeling immune surveillance, and facilitating their metastatic potential and ultimately conferring CSCs resistance to chemotherapy drugs[52,91]. Non neoplastic cells, and their secreted mediators, such as growth factors and the release of cytokines, are found to contribute to the TME[92]. The core regulatory mechanism for oxygen sensing and adaption to hypoxia is hypoxia inducible factor (HIF), in particular HIF-1α and HIF-2α. HIF target genes are able to induce human ESCs markers sufficient to induce pluripotent stem cell inducers: *OCT4*, *NANOG*, *SOX2*, *KLF4*, *MYC* and *miRNA-302* in multiple cancer cell lines. Similar results were reported in prostate tumors[93]. Hypoxia-Notch1-SOX2 signaling axis has been found to activate ovarian CSCs by stimulating self-renewal capacity and drug resistance[94]. Hypoxia activation and upregulation of HIFs has been implicated in aggressive tumor phenotypes, including breast and glioma CSCs, as a result correlating with co-localization studies of these markers with CSCs markers results to poor survival outcome in cancer patients[95,96].

The integration of autophagy in the cancer stem niche provides metabolic plasticity to CSCs from hypoxic conditions, nutrient limitation and acidosis[92]. Immunohistochemistry of pancreatic ductal adenocarcinoma (PDA) tissues reveals co-expression of hypoxia, pancreatic CSC markers (CD44, CD24) and autophagy (BECN1

and LC3B). Hypoxia starvation induced autophagy has been demonstrated to increase clonogenicity and migration of PDA-CSC^{High} cells and the number of autophagosomes formed[97]. In accordance, CD133^{+} pancreatic CSCs is dependent on HIF-1α to induce autophagy for stem cell maintenance[98]. Similarly, CD133^{+} liver CSCs showed higher survival capacity under hypoxic and nutrient deprived conditions[99]. Recently, phosphorylation of EZR at Thr567 residue and activation of PRKCA/PKCα kinase has been suggested to be a responsible candidate for enhanced self-renewal capacity of colorectal CSCs in hypoxia induced autophagy. The blockade of *Atg5*, *BNIP3*, *BNIP3L*, or *BECN1* reversed these effects[100]. Limited literature is available to define the exact interplay between hypoxia, autophagy and the maintenance of the TME.

HIF-1α enhances the secretion of TGF-β1-Smad in mesenchymal stem cells (MSC) which facilitates the propagation of CD44^{+} breast cancer stem-like cells[94], promoting epithelial to mesenchymal transition (EMT)[101]. Autophagy inhibition by *Atg5* silencing and CQ treatment, notably enhanced the transcriptional activation of epithelial marker CD24 whilst repressing EMT marker vimentin in response to TGF-1β, dysregulating cellular ability to migrate and invade[102]. In non small cell lung cells, vimentin was downregulated in the presence of TGF-β1 treatment in *Atg7* knockdown cells, indicating autophagy positively regulates TGF-β1 in EMT[103]. To the contrary, autophagic targeting of EMT transcription factors, such as *Snail* and *TWIST*, through death-effector domain-containing DNA-binding protein-PI3KC3 has been shown to inhibit tumor metastasis and growth in breast cancer[104]. The divergent role of autophagy in EMT has been illiustrated in Figure 2.

Recently, it has been shown that pluripotent transcription factor NANOG, contributes to hypoxia-induced autophagy by directly activating BNIP3L. NANOG promotes resistance to immune mediated actions of cytotoxic T cells[105].

Mitophagy is the selective degradation of defective mitochondria by autophagy to avoid the accumulation of oxygen species and its association to cell death, senescence and malignant transformation. Mitochondria has a central role in generating ATP derived from oxidative phosphorylation (OXPHOS) and the tricarboxylic acid cycle[2]. Human pancreatic CSCs are primarily reliant upon OXPHOS for energy acquisition, as compared to their counterpart; indicating increased mitochondrial activity contributes to CSC stemness[106]. Similar results were observed in mice exhibiting *KRAS* gene ablation in pancreatic adenocarcinoma cells[107]. Moreover, *KRASG12D* mutated pancreatic adenocarcinoma cells have been shown to enter into quiescence in response to oncogene ablation and did not present metabolic stress and induced autophagy. This finding was confirmed by measuring the levels of LC3B by immunoblotting and using flow cytometry to quantify the autophagic flux of *KRAS* mutated cells stably expressing GFP-LC3; and Bafilomycin A1 treatment rescued the GFP signaling. Interestingly, these cancer cells exhibited stem cell-like phenotype[107]. Increased mitophagy is reported in esophageal squamous cell carcinoma CD44^{High} undergoing EMT; the expression of CD44 is rendered during the inhibition of Parkin dependent mitophagy, resulting to cell death[108]. Hepatic CSCs stemness and self-renewal capacity is maintained by the removal of p53 localized to the mitochondria and removed in a mitophagy dependent manner. In contrast, during the suppression of mitophagy, p53 is phosphorylated by PINK1 and translocated to the nucleus to prevent *Oct4, SOX2* and *NANOG* transcription in the hepatic CSC population. These results suggest that the activity of p53 is regulated by mitophagy to promote hepatocarcinogenesis[94]. In LSCs, the loss of p53 simultaneously activates endogenous *KRASG12D* mutation inducing aggressive AML phenotype; thus, enabling abnormal growth[109]. Mitophagy is activated in LSCs by the constitutive activity of AMPK and FIS1; preventing differentiation via GSK3 downstream mechanism and promotes stemness. Inhibition of AMPK-FIS1 axis results to suppression of proliferation and induction of differentiation[110].

THE ROLE OF AUTOPHAGY IN DIFFERENTIATED CANCER CELLS

Bcl-2 binds directly to BECN1 and plays a vital role in the development and differentiation of normal B cells to inhibit autophagy[111-113]. In accordance, immunohistochemistry studies of patients with diffuse large B cell lymphomas (DLBCL) revealed that increased BECN1 levels with reduced levels of Bcl-2 correlated favorably to the clinical survival outcome with better response to the first line treatment of R-CHOP[114,115]. The incidence of breast, ovarian and prostate cancer is higher in 40%-75% patients with monoallelic deletions of *BECN1* gene. Furthermore, in mice with heterozygous deletion of *BECN1* predisposed them to spontaneous malignancies including DLBCL, suggesting *BECN1* is a haplo-insufficient tumor

suppressor gene[116]. Similar findings were reported in the incidence of pre-B acute lymphoblastic lymphoma with elevated expressions of programmed death ligand 1 (PD-L1) and IL-10[117]. A study led by Bertolo *et al*[118], suggests constitutive suppression of autophagy responses in BCL-6 driven GC-derived lymphomas, including DLBCL contribute to lymphomagenesis. In mice, the homozygote deletion of *BECN1* results to embryonic lethality, in comparison *BECN1* heterozygous deletion leads to the establishment of spontaneous tumors and defective autophagy; however, did not impair apoptosis[119].

Enhanced autophagic flux has been attributed significantly to metastatic tumorigenesis and immunosuppression related chemoresistance. In *ex vivo* lung cancer cells, CQ augments carboplatin treatment by sensitizing the lung cancer drug resistant cells and non-resistant cells by limiting the proliferation status and providing synergistic effects with carboplatin to induce apoptosis. These findings corroborated with the decreased LC3B level and BECN1 protein expressions suggesting a decrease in the formation of autophagosomes. The administration of CQ in drug resistant cancer cells, strikingly reduced the drug resistant proteins: MDR1, MRP1 and ABCG2 and mRNA reduction of *MRP1* and *ABCC2*. The combination treatment of CQ and Carboplatin significantly reduced both the protein and the mRNA levels. Furthermore, this decreased the expression of PD-L1 suggesting autophagy has a role in modulating of PD-L1 in cancer evasion and immunosuppression. Interestingly, the combination treatment promoted the infiltration of $CD4^+$, $FOXP3^+$ tumor infiltrating lymphocytes (TILs) indicating autophagy inhibition with carboplatin could mediate lymphocyte infiltration in the tumor and upregulate only specific expression of TILs, leading to immune system activation[120].

THE ROLE OF AUTOPHAGY IN TUMOR DORMANCY

Cancer progression leads to metastatic growth resulting to a majority of cancer related deaths[121]. In many cases, dissemination of tumor cells (DTCs) has already occurred in patients at diagnosis. It is challenging to detect DTCs at secondary sites, as they may have entered into dormancy and become refractory to therapeutic targets[122]. The divergent characteristics of DTCs have emphasized the need to improve this phenomenon. It is postulated that autophagy is activated during the seeding process of DTCs at secondary sites providing an adaptive response to nutrient depletion and environmental stress[123]. For example, the tumor suppressor gene *ARHI* (RAS homologue) is downregulated in 60% of ovarian cancer cases. Studies in ovarian cancer cell lines revealed autophagy induction is mediated by *ARHI* as it inhibits PI3K-mTOR signaling. This is corroborated by co-localization staining of Atg4 and LC3B in autophagosomes suggesting *ARHI* facilitates the autophagosome formation through this signaling. Xenograft model expressing SKOv3-ARHI cells supplemented with ARHI by doxycycline repressed tumor growth, however, the withdrawal of ARHI after 32 or 42 d stimulated rapid tumor growth, indicating that the cancer cells, in particular, CSCs remained viable and dormant during latency. Autophagy inhibition by CQ in this model confirms dormancy requires ARHI mediated autophagy[124]. Accordingly, *Atg7* is essential for the reduction of lung metastatic burden utilizing a non-canonical autophagy pathway independent of BECN1[125]. In contrast, recent microarray analysis of CSCs in breast cancer patients revealed the expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (Pfkfb3), which correlated with an aggressive cancer phenotype coupled with self-renewal ability and metastasis potential. Dormant breast cancer cells display $Pfkfb3^{Low}Autophagy^{High}$ phenotype with elevated levels of LC3B and p62. In contrast, the metastatic breast cancer cells which exhibited $Pfkfb3^{High}Autophagy^{Low}$; suggesting the status of autophagy changes during the phenotypic transition. Knockdown of *Atg3*, *Atg7* or *p62* genes promoted the proliferation and outgrowth restoring the upregulated expression of Pfkfb3 in dormant breast CSCs. The ablation of autophagy related genes gained $CD49f^{High}/CD24^{Low}$ phenotype with increased stemness signature in CSCs. These findings reveal autophagy activation could function to prolong the overall survival of patients by promoting permanent dormancy of CSCs. Additionally, Pfkfb3 protein was found to directly interact with ubiquitin binding domain of p62, suggesting its role as a substrate. Moreover, inactivation of autophagy can facilitate dormancy of breast CSCs to metastatic lesions by stabilizing *Pfkfb3* gene expressions *via* p62[126]. These studies highlight the poorly understood role of autophagy during dormancy in breast CSCs, in which targeting autophagy enables the sensitization of CSCs to chemotherapy by eliminating the adaptive response to autophagy[124,125]. Though it is noteworthy, CSCs are heterogeneous and disease specificity adds complexity to the matter[126].

CLINICAL IMPLICATIONS OF TARGETING AUTOPHAGY IN RESISTANCE CANCER STEM CELLS

Autophagy demonstrates tumor-suppressing actions in early cancer initiation; but fundamentally provides adaptive responses – an advantage to cancer cells and CSC during cellular stress. It remains an open question whether to stimulate or to inhibit autophagy in cancer, specifically, in combination with anti-cancer therapeutics. Autophagy inhibition may provide a reasonable rational to be used; as multiple tumors stimulate autophagy as a source of nutrient replenishment for their increased metabolic demands, survival and disease propagation (Figure 3)[127]. Hydroxychloroquine (HCQ) is an FDA approved drug, with the capability to suppress autophagy at a later stage by inhibiting lysosome acidification and due to these functions it has been used in numerous early phase clinical trials[128]. Meta-analysis data reveals autophagy inhibition based treatment leads to a better therapeutic response as compared to chemotherapy or radiation in the absence of autophagy; suggesting this may provide a new therapeutic strategy for anti-tumoral therapy[129]. However, the activation of autophagy may potentially hold a beneficial role as an anticancer therapy. For example, tat-BECN1 peptide was shown to induce autophagy in *HER2*-positive breast cancer xenografts and prevented tumor growth[131].

In hindsight, CSCs and their counterparts have a unique and complex interaction within the tumor niche which challenges the opportunity to target autophagy directly[132]. Several studies indicate the beneficial impact of combination treatments of chemotherapeutics with autophagy modulators. For instance, the combination of autophagy modulators with chemotherapy showed to stimulate of $CD8^+$ T cell-dependent anticancer immune responses leading to tumor sensitization and cancer cell growth reduction (Figure 2)[133].

TARGETING CANCER CELLS AND CANCER STEM CELLS USING AUTOPHAGY MODULATORS

CSCs are highly tumorigenic and contribute to cancer relapse due to their ability to self-renew and differentiate into heterogeneous cancer cell lineages. Their resilience is demonstrated in the treatment of chemotherapy and radiation therapy[57]. In addition, CSCs are able to remain in a quiescent state and cultivate their ability to become resistant by gaining adaption to their environment[123]. For example, in castration resistant prostate cancer it has been shown that autophagy is induced during Docetaxel treatment and STAT3 contributes to cancer cell survival[134]; suggesting it is important to target autophagy directly or as a combination treatment to sensitize cancer cells.

It should be highlighted that CQ and HCQ exert anti-tumor effects in combination with anti-cancer treatments in clinical trials[135]. In PDA the combination of Gemcitabine with HCQ was assessed[136], this was also evaluated in studies of breast cancer and irradiation[137], or in combination with the autophagy inducer Temsirolimus in patients with various solid cancers including melanoma[138]. In preclinical *in vitro* models of breast cancer, similar results were reported[139]. These findings suggest that autophagy inhibition and activation are promising methods to elicit the sensitization of CSCs to chemotherapy. Moreover, it can be concluded that metastatic cells are preferentially vulnerable to lysosomal inhibition; however, it would be important to assess if these metastatic cancer cells express stem cell-like phenotypical features[140]. For example, autophagy inhibition in breast CSCs expressing Pfkfb3 were found to promote tumor metastasis[126]; suggesting therapeutic strategies involving autophagy modulation in treating CSCs, also depends on the cancer phenotype. As mentioned above, CQ and HCQ have been used as late stage autophagy inhibitors in numerous studies. However, the development of newer generation of lysosome inhibitors are more selective and potent which have been introduced, including Lys05 (analogue of CQ) and dimeric quinacrine (DQ661) - a derivative of Lys05. Both are specific in targeting the lysosome and causing impairment of palmitoyl-protein thioesterase activity by impairing mTOR signaling pathway[141]. Lys05 is a potent autophagy inhibitor in comparison to HCQ. Lys05 has shown to decrease the number of LSCs *in vitro* by promoting their maturation; similar results were seen in patient-derived samples[142]. DQ661 is effective in targeting cancer paradigms of melanoma, colon cancer and PDA by repressing growth and inhibiting autophagy[141]. Inhibitor of V-ATPase called Concanamycin A, protease inhibitor E64d and pepstatin A, have also been introduced[143]. These autophagy modulators are providing an opportunity to explore different combination treatments in different cancer types. Moreover, these lysosomotropic targets are deemed to be effective in

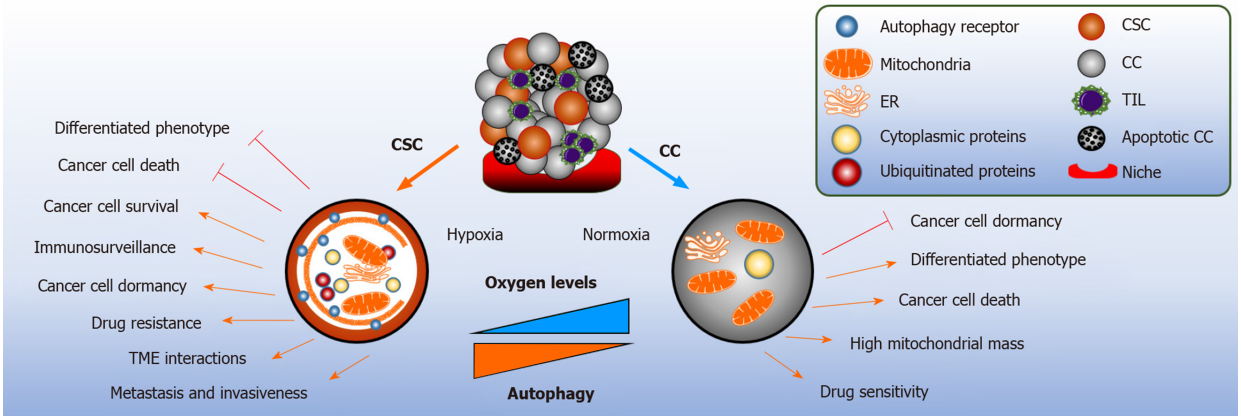

Figure 3 The divergent role of autophagy in cancer stem cells and cancer cells. Cancer stem cells (CSCs) drive the initiation and progression of cancer in multiple tumors. CSCs are reliant on their niches to sustain their self-renewal capacity and plasticity. Hypoxia induced autophagy, provides metabolic plasticity to CSCs. The role of autophagy in hypoxia is to modulate the metabolic remodeling of cancer cells, in particular CSCs. Additionally, autophagy and hypoxia have been implicated in immunosurveillance of CSCs during glucose limitation by increasing the expression of programmed death ligand 1 which results to tumor-infiltrating lymphocytes exhaustion. In addition, autophagy supports tumor dormancy, metastasis and invasion resulting to the treatment of resistant CSCs. CSC: Cancer stem cell; CC: Cancer cell; ER: Endothelial reticulum; TIL: Tumor-infiltrating lymphocytes; TME: Tumor microenvironment.

bulk autophagy degradation, in comparison to selective autophagy, such as mitophagy[2]; in such instances early stage autophagy inhibitors would be considered to be more beneficial. Early stage autophagy inhibitors could target the initiation of autophagy, for example PIK-III (Vps34 inhibitor)[144], MRT68921, SBI-0206965 (ULK inhibitors)[145,146] and SAR405 (PIK3C3/Vps34 inhibitor)[147]. Interestingly, SAR405 and Everolimus (an autophagy inducers) demonstrate significant synergism in renal tumor cells by reducing cancer cell proliferation[147]. Additionally, early stage autophagy inhibitors would be a strategic method to target tumors grown in oxygenated environments, as they use OXPHOS as an alternative source of metabolism.

Autophagy is an adaptive mechanism modulating the TME surrounding CSCs. Several studies defined CSCs inducing autophagy in the TME to support their stemness and cancer propagation by activating the autophagic machinery under nutrient depleted and hypoxic conditions, for example in breast cancer[72-74]. By these actions, autophagy can initiate the development of an aggressive cancer phenotype and develop resistance to cell death. Further investigations are needed to explore the role of autophagy in these cells within the tumor niche in order to tackle the protective surroundings of the TME.

INTERACTION BETWEEN AUTOPHAGY AND IMMUNOTHERAPY

Oncolytic viruses (OVs) therapy is an emerging anti-cancer treatment capable of efficiently killing CSCs and cancer cells in several tumor types[148]. The most commonly used OVs include adenoviruses, herpes simplex virus, measles virus, reovirus, Newcastle disease virus and adenovirus serotype 5[149]. OVs retain the capability to infect, replicate and integrate into tumor cells and potentially in their immunosuppressed TME. Malignant cells overexpressing certain virus receptors, including coxsackie-adenovirus receptor[150,151], CD155[152], CD46[153] and laminin[154] are targeted by OVs. Several studies revealed that autophagy facilitates immunogenic cell death *via* stimulating the release of pathogen associated molecular pattern and damaged associated molecular pattern and initiating their responses in the TME[155]. These responses activate the secretion of ATP from the tumor cells promoting the stimulation of antigen presenting cells, such as DCs to elicit antigens on major histocompatibility I and II molecules which stimulate T cells[13,156,157]. Consequently, pro inflammatory cytokines, including type I interferons induce the stimulator of interferon genes signaling in DCs, further benefiting anti-tumoral T cell responses[158]. In the context of autophagy, OVs employ strategic methods to survive and propagate within the cancer cells by perturbing the core autophagic machinery[159,160].

Autophagy can either be promoted or inhibited during oncolytic adenovirus therapy[161]. The expression of adenovirus oncoprotein triggers the upregulation of Atg1, Atg5 and LC3 proteins[162]. Leukemic cells treated with oncolytic adenovirus

encoding *BECN1* (*SG511-BECN1*) significantly induced autophagic cell death *in vitro*. Similarly, primary blasts isolated from chronic myelocytic leukemia patients with Imatinib resistance and AML patients with relapse disease treated with *SC511-BECN1* showed an increase in BECN1 expression and LC3B accumulation. This led to significantly reduction of colony formation in comparison to *SG511* control[163]. Interestingly, combination treatment of *SG511-BECN1* and Doxorubicin is highly synergistic in chronic myelocytic leukemia cell lines leading to significant cancer cell death. Increased levels of BECN1 and LC3B proteins were observed in comparison with normal mononuclear cells; suggesting the combination of *SG511-BECN1* and Doxorubicin elicits synergistic effects in an autophagy dependent manner[164].

In liver CSCs, oncolytic virus expressing tumor suppressor gene, *TSLC1*, and specifically targeting Wnt signaling, promoted the generation of autophagosomes. This was confirmed by the upregulation of BECN1 and accumulation of total LC3 and led to the reduction of p62 and Survivin. This resulted in cell death in an autophagy dependent manner. The inhibition of autophagy by CQ induced the accumulation of total LC3 and p62, this in turn promoted the survival of the liver CSCs. The hepatic xenograft models treated with this adenovirus induced apoptosis and inhibited tumor metastasis resulting in an improved survival outcome[165]. It has been proposed autophagy activators, such as Rapamycin or Temozolomide synergistically sensitize tumor cells to adenovirus by stimulating autophagy, without modifying the viral replication; thus, inducing autophagy dependent cell death as an antitumor mechanism[166]. In addition, the adenovirus E4 protein suppresses autophagy by activating mTOR signaling and inhibiting ULK1 activity[161].

IMMUNE CHECKPOINT INHIBITORS IN MODULATION OF AUTOPHAGY

The clinical development of immune checkpoint inhibitors (ICIs) is an emerging treatment modality for the reversal of TILs dysregulation phenotype, thereby imposing antitumor responses. Different immune checkpoints, such as T lymphocyte antigen-4 (CTLA-4), PD-1 and PD-L1 could be clinically targeted using ICIs[167].

It is reported that PD-L1 expression on melanoma and ovarian cancer cells elicits tumor growth mainly *via* Akt-mTOR regulated autophagy; this data corroborated with a comparative microarray analysis. Moreover, melanoma PD-L1^{High} expressing tumors demonstrated increased sensitivity to CQ; thus, limiting proliferation *in vitro* and *in vivo*[168]. RNA sequencing data in PD-L1 positive glioma cells promoted cancer invasion in starvation induced autophagy, utilizing the Akt-F-Actin signaling[169]. In gastric cancers the knockdown of *Atg5* and *Atg7* genes inhibited LC3B formation, leading to the upregulation of PD-L1 by the activation of NF-Kb pathway[170]. These accumulating studies confirm intrinsic PD-L1 functions through the activation of Akt-mTOR pathway, however, the mechanisms by which PD-L1 transduces signals remains unknown. The identification of these targets may potentially lead to targeted combinational treatments using autophagic agents. Recently, it is reported that Sigma1 promotes the degradation of PD-L1 using selective autophagy and ablates the functional interaction of PD-1 and PD-L1 in co-cultures of T cells and tumor cells[171]. In accordance, targeting cancer cells expressing CD274 with PD-L1/PD-1 inhibitors can stimulate autophagy and promote sensitization of cancer cells when combined with autophagy inhibitors[172].

CTLA-4 inhibitor is an effective ICI in a subset of patients with metastatic melanoma. In a small cohort of melanoma patients, a subcluster of MAGE-A cancer germline antigens, were found to be overexpressed causing resistance to CTLA-4 inhibition, but not PD-1. Tissue microarray data revealed that the LC3B expression in MAGE-A$^+$ tumors was significantly attenuated as compared to MAGE-A$^-$ tumors. Moreover, immunohistochemistry data indicated MAGE-A and damaged associated molecular pattern protein high-mobility group box 1 (HMGB-1) were mutually expressed in the clinical samples. *In vitro* ubiquitination screening confirmed that autophagy was suppressed by the MAGE-TRIM28 ubiquitin ligase complex[173]. HMGB-1 is a pro autophagic protein that directly interacts with BECN1 by displacing BCL-2; thus, sustaining autophagy and promoting cellular survival[174]. The secretion of HMGB-1 mediates the priming of immune adaptive response[175]. To overcome CTLA-4 therapy resistance in melanoma patients, the induction of autophagy may potentially be relevant in enhancing the effect of CTLA-4 inhibitors; thus, minimizing tumor immune tolerance. Combining CTLA-4 inhibition with Rapamycin *in vivo* during CD8$^+$ T cell priming, led to an increase of Ag-specific memory CD8$^+$ T cells and enhanced their function, which in turn, resulted to tumor growth reduction, rapid bacterial clearance and mediated cytokine production[176]. Taking these findings into

consideration, the induction of autophagy would reinstate the CTLA-4 expression and its suppressive functions, thereby, eliciting antitumoral activity.

CONCLUSION

New therapeutic concepts are needed to improve the prognosis of cancer patients. One possible starting point is the tumor-specific metabolism of cancer cells. Autophagy is a catabolic recycling process exciting different forms of cancer cells and CSCs. In general, CSC maintenance and the development of an aggressive cancer phenotype have strongly been correlated to autophagy. In cancer, the role of autophagy is context dependent as it demonstrates functions both as a tumor suppressor during tumor initiation and as a pro-survival mechanism during cancer propagation by facilitating CSCs and cancer cells adaptive responses during metabolic stresses and dormancy.

Targeting autophagy could potentially represent a promising therapeutic target for preventing the aggressive and resistance cancer phenotypes. There is convincing evidence that the inhibition of autophagy in cancer cells, and specifically in CSCs, augments cytotoxicity leading to antitumoral effects under certain conditions. Therefore, we can expect valuable knowledge regarding suitable autophagy-associated biomarkers in tumor cells and new therapeutic approaches that are specifically directed against autophagy-dependent pathways in cancer cells or CSCs. Additionally, it is increasing evident that autophagy is involved in the maintenance of immune cell homeostasis, activation and function in the TME. However, limited studies are available to interpret whether autophagy enhancement or inhibition my support the effects of immunotherapy. Several additional preclinical studies are necessary to identify them, specifically, in a context dependent manner. This would represent an important step in the direction of improved and individualized cancer therapy.

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REVIEW

Advances in treatment of neurodegenerative diseases: Perspectives for combination of stem cells with neurotrophic factors

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Abstract

Neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis, are a group of incurable neurological disorders, characterized by the chronic progressive loss of different neuronal subtypes. However, despite its increasing prevalence among the ever-increasing aging population, little progress has been made in the coincident immense efforts towards development of therapeutic agents. Research interest has recently turned towards stem cells including stem cells-derived exosomes, neurotrophic factors, and their combination as potential therapeutic agents in neurodegenerative diseases. In this review, we summarize the progress in therapeutic strategies based on stem cells combined with neurotrophic factors and mesenchymal stem cells-derived exosomes for neurodegenerative diseases, with an emphasis on the combination therapy.

Key words: Neurodegenerative diseases; Stem cells; Brain-derived neurotrophic factor; Glial cell line-derived neurotrophic factor; Nerve growth factor; Combination therapy

Core tip: Neurodegenerative diseases are currently incurable and the therapeutic strategies have been disappointing. Stem cells and neurotrophic factors are promising therapeutic agents, with the combination of the two being more attractive. This review focuses on the advances in such combination therapies in the treatment of

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neurodegenerative diseases. The combination of stem cells with neurotrophic factors can not only replenish the target neurons but also provide secreted neurotrophins to improve the microenvironment for nerve repair and regeneration, which might represent a new approach in the treatment of neurodegenerative diseases.

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INTRODUCTION

Neurodegenerative diseases, mainly involving gradual and progressive neuron loss and neuronal function decline, usually lead to cognitive and behavioral dysfunctions and severe life quality impairment of the patients. Currently, there remains a lack of effective therapeutic agents due to the obscure cause of the neuronal death and the impeded early diagnosis of neurodegenerative diseases. Stem cells and neurotrophic factors are promising therapeutic agents with neural differentiation and neuroprotective effects for neurodegenerative diseases[1-3]. Figure 1 illustrates the possible effects of mesenchymal stem cells (MSCs) and neurotrophic factors for each disorder described in this paper.

Stem cells have emerged as one of the most actively researched potential therapeutic tools for a wide range of diseases. They can be divided into pluripotent stem cells and adult stem cells. The former encompasses the embryonic stem cells and induced pluripotent stem cells; the latter includes the neural stem cells (NSCs), hematopoietic stem cells, MSCs, and olfactory ensheathing stem cells. All stem cells have the potentiality of continuous self-renewal, high proliferation, and multidirectional differentiation into various cell types to replace degenerated or dead cells[4]. They also act as neuroprotection and neurodifferentiation promoters by secreting neurotrophic factors (NTFs) and extracellular vesicles (EVs, so called exosomes) containing NTFs. These abilities make stem cells a promising therapeutic choice for neurodegenerative diseases. In particular, MSCs appear to be the most suitable, due to their availability, low immunogenicity, multiple differentiation ability, and secretion of NTFs and exosomes[5-8].

The NTF protein family, mainly consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), neurotrophic factor 3 (NT3) and neurotrophic factor 4 (NT4), are necessary for neuronal development, health and survival, as well as for stem cell proliferation and differentiation into target neurons. Some NTFs are protective to cell survival and neuronal degeneration, which show promise as therapeutic agents for neurodegenerative diseases[2,3]. However, some serious problems, *e.g.*, rapidly degraded NTFs need to be frequently delivered and recombinant NTFs protein cannot pass through the blood-brain barrier (BBB), must be confronted[1,9,10].

Gene transduction by recombinant viral vectors makes it possible for a sustained supply of therapeutic factors after single transfection of target cells. But, the vector systems-associated drawbacks, including toxicity and inflammation, non-relevant cell infection and risk of genome insertional mutagenesis, still prompt alternative therapeutic strategies, such as transplantation of NTF-releasing cells. The effectiveness of this construct has been demonstrated in *in vivo* neuronal disease models, in which cell-delivered BDNF has shown the same or even better neuroprotective effect than recombinant BDNF[11]. MSCs have been considered as the optimal delivery platform for sustained delivery of therapeutically relevant amounts of NTFs to degenerative neuronal structures, because of their secretion of various factors that can reduce inflammation, cell toxicity and cell death, and can enhance neurons connections[12]. Moreover, when compared with MSCs alone, MSCs-NTFs showed better results in several rodent neurodegenerative models[1].

EVs are phospholipid bilayer enveloped spherical particles categorized into exosomes, microvesicles, and apoptotic bodies based on their origin and size. Exosomes are 30–100 nm in diameter and involved in cells communications by transferring genetic material including mRNA and miRNA, proteins, lipids and

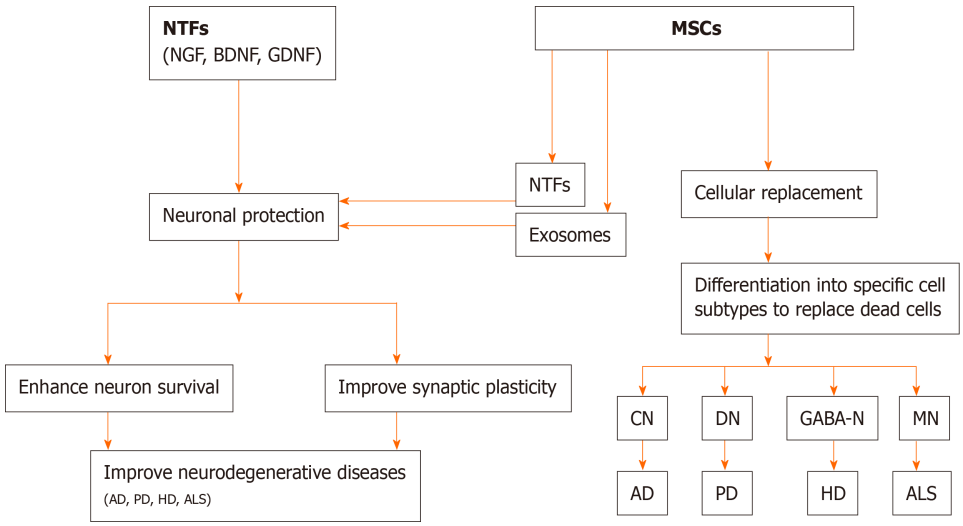

Figure 1 Possible effects of mesenchymal stem cells and neurotrophic factors for Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis. AD: Alzheimer's disease; ALS: Amyotrophic lateral sclerosis; BDNF: Brain-derived neurotrophic factor; CN: Cholinergic neurons; DN: Dopamine neurons; GDNF: Glial cell line-derived neurotrophic factor; GABA-N: Striatal GABAergic medium-sized spiny neurons; HD: Huntington's disease; MN: Motor neurons; MSCs: Mesenchymal stem cells; NGF: Nerve growth factor; NTFs: Neurotrophic factors; PD: Parkinson's disease.

membrane receptors[13]. The unability to cross the BBB of most drugs is a great challenge for the treatment of neurodegenerative diseases. Thus, the ability to cross the BBB of exosomes makes it a promising delivery system to transport therapeutical signals or drugs into the brain for neurological diseases like neurodegenerative diseases. Furthermore, sophisticated techniques makes it possible to engineer more precisely targeted exosomes to a desired tissue or region[6,14]. Exosomes can be obtained from different cell types, MSCs can secrete a higher amount of exosomes than other cell types, and MSC-derived exosomes show promising effects in multiple conditions by triggering regeneration responses[15,16]. There is accumulating evidence showing the neurotherapeutic potentiality and successful application of exosomes secreted by various stem cell types, especially MSCs for the treatment of neurodegenerative diseases. MSC-exosomes is currently considered as an alternative non-cell therapy to stem cell therapy. Moreover, the development of genetically modified MSCs-exosomes might provide a new perspective for developing therapeutic strategies for neurodegenerative diseases in the future[6,17].

In summary, stem cells, NTFs and MSC-exosomes are promising therapeutics for neurodegenerative diseases with their own distinctive advantages and disadvantages. The combination therapy might not only have enhanced effect but also play a complementary role in overcoming deficiencies of single therapy. Since excellent comprehensive reviews of stem cell-based therapy and NTFs-based therapy for neurodegenerative diseases have been published[1-3,5,7,10], in this review, the combination of stem cells with NTFs and the MSC-exosomes for the treatment of neurodegenerative diseases is discussed, with an emphasis on the combination therapy.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most common type of dementia, affecting approximately 55 million people worldwide[18]. AD, including the familial type and sporadic type, manifests with cognitive impairment. AD pathologies include senile plaques caused by excessive deposition of beta-amyloid (Aβ) due to abnormal degradation of extracellular amyloid precursor protein, neurofibrillary tangles formed by intracellular hyper-phosphorylated Tau, loss of cholinergic neurons, neuroinflammation, oxidative stress, and changes in such NTFs as NGF and BDNF[19,20]. Currently, drug therapies such as acetylcholinesterase inhibitors (donepezil, galantamine) and NMDA receptor antagonists (memantine) can only delay symptoms, but not relieve disease pathology or progression[21,22]. Studies have demonstrated that neurons derived from stem cells can integrate with existing neural networks and repair damaged neurons in the host brain, yielding improvements in learning and memory deficits[23], and that NTFs can improve symptoms and provide neuroprotective effects in AD[24,25].

NTFs such as NGF and BDNF play important roles in neuron survival and differentiation, synapse plasticity, learning, and memory[26,27]. NGF is secreted by the postsynaptic cortex and hippocampal neurons in precursor form (proNGF), which converts to the mature form (mNGF) upon interaction with the extracellular protease plasmin. Upon the NGF molecule binding to the receptor tropomyosin receptor kinase (Trk) A/p75, the complex is internalized and retroactively transported to cholinergic cell bodies in the basal forebrain, triggering cholinergic function and promoting the release of acetylcholine[28-30]. Both proNGF and mNGF can induce neurotrophic effects through TrkA, but proNGF can induce apoptotic signals by interacting with p75[31,32]. Interestingly, changes in NGF metabolism, accumulation of proNGF level, and reduction of mNGF level have been observed in the pathological process of AD. Higher proNGF levels not only induce pro-apoptotic signaling but also affect the receptors binding to mNGF, leading to retrograde atrophy of cholinergic neurons in the basal forebrain[32-34]. Since cholinergic cell bodies retain their sensitivity to NGF, NGF delivery is a potential method to restore cholinergic signaling in the cortex and hippocampus. BDNF, on the other hand, is a neurotrophic protein that is highly expressed in the brain and plays important roles in neuronal survival and differentiation, synaptic formation, and hippocampal long-term potentiation. These BDNF effects in the hippocampus are mediated by the Trk B receptor[35,36]. ProBDNF is a precursor form of BDNF that interacts with the p75 receptor to induce apoptosis. It has been demonstrated that in the AD brain, proBDNF and p75 receptors are increased, while BDNF and TrkB receptor are decreased, a situation conductive to apoptosis signaling[37-40]. Moreover, studies have shown that higher serum BDNF levels are associated with a slower rate of cognitive decline in AD patients[41].

NGF and BDNF have low stability and short half-life, and as such cannot effectively pass through the blood-brain barrier. Additionally, repeated direct delivery of NTFs may have serious peripheral side effects[42]. Stem cells can secrete neurotrophins to a certain degree to improve the survival of neurons, despite their lower cell survival, limited lifespan, and majority dying before they affect the injured area[30,43]. Recently, it has been reported that using stem cells as carriers to deliver NTFs to the AD brain can increase the survival rate of neurons, improve learning and memory, reduce Aβ deposition, promote neurogenesis, and inhibit neuron apoptosis and glial cell activation[25,44-50] (Table 1).

Transplantation of NSCs combined with NGF into AD rats led to significant improvement in learning and memory and supplemented basal forebrain cholinergic neurons[25]. Hippocampus transplantation of bone marrow stromal cells (BMSCs)-NGF also significantly improved learning and memory of AD rats, as compared with the BMSC-implanted group, suggesting that BMSCs were effective carriers for NGF delivery[44]. Lateral cerebral ventricle transplantation of human BDNF-modified NSCs elicited a better improvement in learning and memory than that achieved in the NSCs-implanted AD rats[45]. NSC transplantation into transected rat basal forebrain followed by BDNF injection into the lateral ventricle also led to better improvements in the number of cholinergic neurons and the ability of learning and memory than implantation of NSCs alone[46]. Lateral ventricle transplantation of the *BDNF* gene-modified BMSCs into the AD rat model significantly attenuated the nerve cell damage in the CA1 region of the hippocampus, leading to significant improvement in learning and memory[47]. The protective effect of MSCs on AD pathology was enhanced by MSCs-BDNF, suggesting that the BDNF supply from MSCs-BDNF was enough to prevent AD pathology[48]. Treatment of AD with BDNF-overexpressing NSCs has also shown to improve the vitality of NSCs, to increase the therapeutic potential of implanted NSCs, and to alleviate AD cognitive deficits[49]. Our previous study showed that transplanting BDNF-modified human umbilical cord MSCs-derived cholinergic neurons not only improved memory and learning but also reduced the expression of amyloid-associated protein Aβ levels and promoted neurogenesis in AD rats[50].

MSC-exosomes showed similar effects to MSCs on the stimulation of neurogenesis and alleviation of learning and memory impairment evaluated by Morris water maze and novel object recognition tests in AD mice bilaterally dentate gyrus injected with $A\beta_{1-42}$, suggesting the possibility of developing MSC-exosomes as a cell-free candidate of MSCs for AD treatment[51]. Hypoxia-preconditioned MSC-exosomes restored synaptic dysfunction, decreased amyloid plaque deposition and the Aβ levels, and reduced inflammatory responses, leading to learning and memory improvement in the APP/PS1 AD mice[52]. Human umbilical cord MSC-exosomes injection alleviated neuroinflammation by modulating the microglia activation and cleared Aβ deposition in the brains of AD mice, leading to cognition repairment[53]. Neocortex injection of BM-MSC-EVs effectively reduced the Aβ burden and the number of dystrophic neurites in the hippocampus and cortex of 3 to 5-mo-old (early stages) APPswe/PS1dE9 AD mice, indicating a potentiality to intervent AD in early stages[54].

Table 1 Combination therapy of stem cells with neurotrophic factors in Alzheimer's disease

Cell types	Neurotrophic factors	Study design and outcome	Ref.
NSCs	NGF	Embryonic rat NSCs were separated and induced by NGF-PEG-PLGA-NPs *in vitro*, and were transplanted into AD rats(lateral ventricular injected with 192IgG-saporin). The Morris water maze was used to evaluate learning and memory, followed by immunohistochemical staining for basal forebrain cholinergic neurons, hippocampal synaptophysin, and AchE fibers. The rats in the combined treatment group showed significant improvement in spatial learning as compared to the untreated AD model animals. The treated rats also showed significantly higher number of basal forebrain cholinergic neurons and fibers with AchE positivity, and higher expression of hippocampal the rats in the NSCs group.	Chen *et al*[25], 2015
BMSCs	NGF	When compared with BMSCs transplantation alone, BMSCs-NGF transplantated into the hippocampus of AD rats (bilaterally injected with Aβ) significantly improved learning and memory. The findings suggested efficient NGF delivery by BMSCs.	Li *et al*[44], 2008
NSCs	BDNF	The AD rat model was established by cutting the unilateral fibria-fornix of male rats. Lateral cerebral ventricle transplantation of the NSCs and NSCs-hBDNF provided behavioral amelioration of AD rats assessed *via* the Morris water maze, and the effect of NSCs-hBDNF was better than that of NSCs.	Zhao *et al*[45], 2005
NSCs	BDNF	Transected rat basal forebrain BrdU-labeled NSCs transplantation followed by lateral ventricle BDNF injection led to labeled NSCs differentiation into neurons and astrocytes in the basal forebrain. The rats in the NSCs and BDNF combination group showed better improvement in the number of cholinergic neurons, and learning and memory as compared to the other groups.	Xuan *et al*[46], 2008
MSCs	BDNF	*BDNF* gene-modified BM-MSCs were transplanted into the lateral ventricle of an AD rat model. Nerve cell damage in the CA1 region of the hippocampus was significantly attenuated. BDNF tyrosine kinase B mRNA and protein levels were significantly increased, and learning and memory were significantly improved.	Zhang *et al*[47], 2012

MSCs	BDNF	A unique neuronal culture of familial-type AD neurons was made from the 5x familial-type AD mouse, an amyloid precursor protein/PS1 transgenic mouse model, to investigate progressive neurodegeneration associated with AD pathology and the efficacy of MSCs-BDNF. Analyses of the expression of BDNF, synaptic markers and survival/apoptotic signals indicated that pathological features of cultured neurons could accurately mimic AD pathology. The protective effect of MSCs was enhanced by MSCs-BDNF. The BDNF supplied from MSCs-BDNF was sufficient to prevent AD pathology.	Song *et al*[48], 2015
NSCs	BDNF	Hippocampus transplanted NSCs-BDNF integrated into the local brain circuits of the 16-mo-old Tg2576 mice, improved the engrafted cells' viability, neuronal fate, neurite complexity, the synaptic density, and the cognitive deficits of the AD mice.	Wu *et al*[49], 2016
hUC-MSCs	BDNF	Right hippocampus transplantation of BDNF-modified hUC-MSCs-derived cholinergic-like neurons significantly improved spatial learning and memory in the AD rats assessed by Morris water maze testing, increased the release of acetylcholine, enhanced the activation of astrocytes and microglia, reduced the expression of Aβ and BACE1, and inhibited neuronal apoptosis detected by Western blotting, immunohistochemistry, immunofluorescence assay, and TUNEL assay.	Hu *et al*[50], 2019

Aβ: Beta-amyloid; AchE: Acetylcholine esterase; AD: Alzheimer's disease; BDNF: Brain-derived neurotrophic factor; BM-MSCs: Bone marrow-mesenchymal stromal cells; BMSCs: Bone marrow stromal cells; BMSCs-NGF: Nerve growth factor gene-modified bone marrow stromal cells; BrdU: 5'-Bromo-2'-deoxyuridine; hUC-MSCs: Human umbilical cord-mesenchymal stem cells; MSCs: Mesenchymal stem cells; MSCs-BDNF: Brain-derived neurotrophic factor-modified mesenchymal stem cells; NGF: Nerve growth factor; NGF-PEG-PLGA-NPs: Nerve growth factor-poly(ethylene glycol)-poly (lactic-co-glycolic acid)-nanoparticles; NSCs: Neural stem cells; NSCs-hBDNF: Human brain-derived neurotrophic factor-modified neural stem cells; TUNEL: TdT-mediated dUTP nick end labeling.

PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common neurodegenerative disorder. The motor symptoms of PD mainly include rest tremor, rigidity, bradykinesia and postural instability, while common nonmotor symptoms include neuropsychiatric and sleep disorders as well as sensory and autonomic dysfunction[55]. The pathological feature of PD is progressive degeneration and loss of dopamine (DA) neurons in the midbrain substantia nigra. Symptoms arise when 50% of the DA neurons are lost[56]. Unfortunately, there is no cure or disease-modifying therapy available for PD at present. Commonly used symptom-relief medications include levodopa, carbidopa, DA agonists, anticholingeric agents, amantadine, and DA metabolism inhibitors. However, the currently available drugs often provide only partial symptom control and elicit frequent side effects, such as motor complications (known as dyskinesia and wearing-off[57]) and gastrointestinal and neuropsychiatric dysfunctions[55]. Considering that these therapies for PD do not treat the underlying pathology, alternative therapies are still intensively pursued, including those based on stem cells and NTFs[49,55].

The goal of stem cell-based therapy to treat PD is to replace degenerated and lost DA neurons in the substantia nigra with healthy ones or to prevent further neuron loss[7]. Moreover, investigations into the use of NTFs as therapeutic options for PD were prompted by their role in neuronal survival, differentiation and plasticity, their correlation with the disease (namely NTFs' deficiency), and the findings of replacement or enhancement of NTF signals providing neuronal protection in PD

models[58,59]. The first identified potential NTF to treat PD was GDNF, which is able to increase DA uptake and the survival of embryonic midbrain DA neurons[60]. GDNF has since received the most attention for clinical trials[55]. Cell-based GDNF delivery is currently recognized as an appropriate alternative for treatment of PD, following clinical trials of GDNF alone yielding mixed results[61]. MSCs are the most promising cellular vehicle to deliver NTFs for PD treatment, and MSCs engineered to overexpress GDNF or BDNF have received much attention[62-68] (Table 2).

In the PD rat model established by the injection of 6-hydroxydopamine (6-OHDA) , dopaminergic neuron sprouting increased as a result of striatum transplantation (at 4 d prior to injury) of MSCs transfected with a retrovirus to express GDNF[62]; in addition, unilateral striatum transplantation of GDNF-overexpressing human MSCs decreased amphetamine-induced rotations and improved DA fibers' rejuvenation[63]. In the lactacystin-induced neurotoxicity(in the medial forebrain bundle) PD rat model, intrastriatal injection (at 1 wk prior to injury) of BMSCs transduced with lentivirus to overexpress GDNF was protective against the neurotoxicity and led to significantly increased striatal DA levels and behavior recovery, as assessed by apomorphine-induced rotations[64]. In a MPTP-treated non-human primate PD model, striatum and substantia nigra transplantation of BM-MSCs genetically modified to overexpress GDNF resulted in increased striatum DA levels and improved contralateral limb function[65]. In a lipopolysaccharide-induced PD model, unilateral striatal transplantation of MSCs-GDNF provided local neuroprotection of dopaminergic terminals in the striatum of PD rats[66]. Transplantation of human (h)MSCs-BDNF into the unilateral 6-OHDA-lesioned substantia nigra also resulted in remarkable nigral tyrosine hydroxylase-positive cell hypertrophy, striatal tyrosine hydroxylase-staining increase, and amphetamine-induced motor symptom stabilization[67]. In another study of the 6-OHDA-lesioned PD rat model, prior to transplantation, MSCs were first induced to NTF-secreting cells by *in vitro* exposure to nystatin, L-glutamine, human epidermal growth factor, human basic fibroblast growth factor (hbFGF) and N2 for 72 h, then dibutyryl cyclic AMP, isobutylmethylxanthine, human platelet-derived growth factor, human neuregulin 1-β1/HRG1-β1 EGF domain, and hbFGF for 3d, resulting in aquintupled increase in BDNF expression and doubled increase in GDNF expression. The striatum transplantation of these induced MSCs improved the amphetamine-induced rotations behavior, and ameliorated DA deficits more efficaciously than uninduced MSCs[68]. A study investigating the combination of human umbilical vein mesenchymal stem cells (HUVMSCs)-derived dopaminergic-like cells with NGF in a PD rat model found that as compared to cell grafting only, combination therapy significantly promoted the survival of the grafted cells and increased the dopaminergic content, leading to significant motor function improvement[69].

A study investigating the therapeutic effects of MSC-secretome on the physiological recovery in a 6-OHDA rat PD model underwent substantia nigra and striatum injection of MSC-secretome and rotarod and staircase tests, and observed increased dopaminergic neurons and neuronal terminals in the injected areas and recovery in the motor performance of PD rats, indicating that MSC-secretome is a novel therapeutic strategy for PD[70]. In another 6-OHDA rat PD model, the injection of hBMSC- secretome induced higher levels of neuronal differentiation, led to the rescue of DA neurons and the recovery of behavioral performance in the staircase test[71].

HUNTINGTON'S DISEASE

Huntington's disease (HD) is a fatal inherited neurodegenerative disorder; its hallmark motor, cognitive and psychiatric dysfunctions manifest upon the progressive deterioration of striatal GABAergic medium-sized spiny neurons caused by mutations in the huntingtin (*HTT*) gene, leading to increased polyglutamine repeats in the HTT protein[72,73]. Multiple possible neurodegenerative mechanisms of HD are currently under investigation, and this knowledge is anticipated to serve as a basis for the development of new HD therapies. The abilities of stem cells to rescue or replace the damaged and dying neurons, and to prevent further cell damage and death, make stem cell-based therapies promising for treatment of this neurodegenerative disease[74].

In HD, BDNF has been demonstrated to mediate striatal neuronal function and survival by providing neurotrophins and neuroprotection[75]. Studies have also revealed a reduction in BDNF levels in HD patients, which may contribute to the clinical manifestations[76]. In the striatum, the reduced levels of BDNF are partially due to function loss of the wild-type HTT protein, which assists in vesicle transport of BDNF, while the mutation of which has adverse effects on BDNF transcription,

Table 2 Combination therapy of stem cells with neurotrophic factors in Parkinson's disease

Cell types	Neurotrophic factors	Study design and outcome	Ref.
MSCs	GDNF	MSCs-GDNF transplantation induced a pronounced local trophic effect in the denervated striatum of the 6-OHDA PD rat model.	Moloney *et al*[62], 2010
MSCs	GDNF	Striatum transplantation of GDNF-releasing Notch-induced BM-MSCs(SB623 cells) significantly decreased amphetamine-induced rotation and promoted DA fiber activation of the 6-OHDA PD rat model.	Glavaski-Joksimovic *et al*[63], 2010
MSCs	GDNF	The intrastriatal transplantation of BMSCs-GDNF significantly rescued the DA neurons from lactacystin-induced neurotoxicity, with regard to behavioral recovery and striatal dopamine level increase of the lactacystin-lesioned PD rat model, established by intrastriatum transplantation of BMSCs-GDNF followed by lactacystin induction of a lesion at the median forebrain bundles 1 wk later.	Wu *et al*[64], 2010
MSCs	GDNF	MSCs-GDNF were transplanted into the unilateral striatum and SN of cynomolgus monkeys (PD monkey model) to investigate the protective function of MSCs-GDNF against MPTP-induced injury. Monkeys in the MSCs-GDNF group showed spared contralateral limbs' motor function and had higher dopamine level and enhanced dopamine uptake in the striatum of the grafted hemisphere.	Ren *et al*[65], 2013
MSCs	GDNF	The lipopolysaccharide-lesioned PD rat model was used to assess the ability of MSCs-GDNF to protect against lipopolysaccharide-induced neuroinflammation, neurodegeneration, and behavioral impairment. Both experimental groups received a unilateral intrastriatal transplantation of either MSCs-GDNF or MSCs-green fluorescent protein. Protection and/or sprouting of the dopaminergic neuron terminals was induced by the secreted GDNF in the striatum of PD rats.	Hoban *et al*[66], 2015
MSCs	BDNF	The signals and/or molecules that regulate BDNF expression/delivery were investigated in hMSCs cultures and the effect of epigenetically generated BDNF-secreting hMSCs were evaluated for their impact on intact and lesioned SN. Results showed that the amphetamine-induced motor symptoms were stabilized.	Somoza *et al*[67], 2010
MSCs	BDNF; GDNF	The intrastriatum transplantation of NTF-SCs posterior to the 6-OHDA lesion led to an obvious amelioration of amphetamine-induced rotations, and the damaged striatal dopaminergic nerve terminal network was regenerated.	Sadan *et al*[68], 2009

HUVMSCs	NGF	As compared to HUVMSCs-derived dopaminergic-like cells alone, combination with NGF significantly promoted the cell survival, increased the dopaminergic content, and improved motor function of PD rats.	Li *et al*[69], 2010

6-OHDA: 6-Hydroxydopamine; BDNF: Brain-derived neurotrophic factor; DA: Dopamine; GDNF: Glial cell line-derived neurotrophic factor; hMSCs: Human mesenchymal stem cells; HUVMSCs: Human umbilical vein mesenchymal stem cells; MSCs: Mesenchymal stem cells; NGF: Nerve growth factor; NTF: Neurotrophic factor; NTF-SCs: Neurotrophic factor-secreting stem cells; PD: Parkinson's disease; SN: Substantia nigra.

proper transport, and secretion[77]. BDNF administration was shown to be neuroprotective *in vitro*, to rat neurons containing mutant HTT, and *in vivo*, to the striatum of R6/1 mice[75,78]. Therefore, BDNF administration is considered another hopeful candidate for HD treatment. To this end, an interesting and widely characterized candidate therapy in which MSCs were engineered to secrete BDNF was developed and found to promote neuron survival and regeneration in HD[79-81] (Table 3).

Retrovirus-*BDNF*/*NGF* gene-modified MSCs were shown to produce a 6.8-fold and 4.6-fold increase in the expression of BDNF in stem cells and in cell culture media, respectively. All 4-mo-old YAC 128 mice bilateral striatum transplanted with unmodified MSCs or NGF/BDNF (alone or combination)-overexpressing MSCs, showed reduced clasping; in addition, mice transplanted with the BDNF-overexpressing MSCs showed the longest rotarod latencies and the least amount of striatum neuronal loss, restored striatum NeuN-positive cell counts to the level detected in wild-type (non-HD) mice. These findings demonstrated that BDNF-modified MSCs facilitated behavioral and histological recovery of YAC 128 HD mice[79]. Intrastriatal administration with human MSCs-BDNF to YAC128 and R6/2 transgenic HD mice demonstrated that the MSCs-BDNF treatment significantly reduced anxiety, attenuated striatal atrophy in the YAC128 mice, and increased the mean lifespan and neurogenesis-like activity of the R6/2 mice. These improvements were attributed to the enhancement of endogenous neurogenesis stimulation and maturation promoted by BDNF and various complementary therapeutic factors secreted by the MSCs[80]. Transplantation of embryonic stem cell-derived BDNF-overexpressing neural progenitors to three different HD mouse models - the quinolinic acid-lesioned model and the two genetic models R6/2 and N171-82Q - led to motor function improvement in the quinolinic acid-lesioned model, which may be due to enhanced neuronal and striatal differentiation, while only subtle effects were shown in the two genetic models. The difference in the behavior improvement can be attributed to the different cell survival rates in different models; this is in agreement with the finding that neural progenitor cells (NPCs) transplanted into the two transgenic mice lines usually show lower cell survival rate[81].

In an *in vitro* HD model of R6/2 mice-derived neuronal cells, exosomes derived from adipose stem cells (ASC-exo) significantly decreased the mHtt aggregates, reduced abnormal apoptotic protein level, mitochondrial dysfunction and cell apoptosis, suggesting a therapeutic potentiality of ASC-exo for HD[82].

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is one of the neurodegenerative disorders involving progressive degeneration of both upper and lower motor neurons, leading to palsy and death ultimately in 3-5 years from onset[83]. Multiple underlying mechanisms are involved in ALS pathology, including glutamate excitotoxicity, oxidative and endoplasmic reticulum stress, mitochondrial dysfunction, microglial and astrocyte function abnormality, and neurotrophic support impairment[84]. There are currently only two available disease-modifying medicines - riluzole and edaravone - that have shown benefit, albeit slight and to a limited set of patients[85]. Given the complex ALS pathogenesis and limited drug efficacy, there is a remarkable urgency to find new therapies for ALS. Stem cell-based therapy holds great promise for treating ALS by providing both cell replacement and NTF delivery to target the multiple pathologies[86,87]. Stem cells available for ALS treatment include NSCs, MSCs, embryonic stem cells, induced pluripotent stem cells, and olfactory ensheathing stem cells[88].

NTFs might benefit ALS patients by protecting motor neurons and preventing disease progression[89]. Besides the replacement of degenerated motor neurons by stem cells, neurotrophic support also plays an important role in the motor neurons'

Table 3 Combination therapy of stem cells with neurotrophic factors in Huntington's disease

Cell types	Neurotrophic factors	Study design and outcome	Ref.
MSCs	BDNF; NGF	BM-MSCs were genetically engineered to overexpress BDNF and/or NGF, and were then injected into the striata of 4-mo-old YAC128 transgenic and wild-type mice to determine the effects on motor function. Transplantation of MSCs-BDNF may slowdown neurodegenerative processes and provide behavioral sparing in the YAC128 mouse model of HD.	Dey *et al*[79], 2010
MSCs	BDNF	MSCs-BDNF were intrastriatially transplanted into YAC128 and R6/2 transgenic (immune-suppressed HD model) mice. MSCs-BDNF transplantation reduced anxiety, decreased striatal atrophy in the YAC128 mice and prolonged the mean lifespan and increased neurogenesis of the R6/2 mice.	Pollock *et al*[80], 2016
ESCs-derived NPCs	BDNF	ESCs-derived BDNF-overexpressing NPCs were transplanted into a quinolinic acid-lesioned model and two transgenic mouse lines (R6/2 and N171-82Q). NPCs-BDNF had a significant effect on motor function recovery in quinolinic acid-lesioned mice, while the genetic mouse model had only slight improvement. Adult neurogenesis was preserved in a BDNF-dependent manner.	Zimmermann *et al*[81], 2016

BDNF: Brain-derived neurotrophic factor; BM-MSCs: Bone marrow-mesenchymal stem cells; ESCs: Embryonic stem cells; HD: Huntington's disease; MSCs: Mesenchymal stem cells; NGF: Nerve growth factor; NPCs: Neural progenitors.

survival and function[90]. Thus, it is reasonable to combine stem cells and NTFs for the treatment of ALS, especially by transplanting stem cells engineered to overexpress NTFs[91]. Indeed, it has been shown that transplantation of stem cells combined with specific growth factors can markedly preserve neuromuscular junctions, attenuate motor neuron death, delay onset, improve motor function, and prolong survival of the SOD1^{G93A} rat ALS model[92-100] (Table 4).

It has been reported that lumbar spinal cord transplantation of human NPCs genetically modified to secrete GDNF only limited motor neuron degeneration in the SOD1^{G93A} ALS rats[92,93], while cortex transplantation also prolonged the lifespan[94]. On the other hand, bilateral intramuscular transplantation of human(h)MSCs-GDNF led to survival of the hMSCs and release of GDNF into the muscle of the SOD1^{G93A}ALS rats, which increased the number of neuromuscular connections and prevented the loss of motor neurons in the spinal cord, leading to delayed disease progression and increased lifespan (by 28 d)[95]. Similarly, intrathecal transplantation of human NSCs overexpressing VEGF into the SOD1^{G93A} ALS mice delayed disease onset and prolonged lifespan[96]. In addition, combination therapy of intranasal NGF administration with lateral ventricle NSCs transplantation also delayed disease onset, improved motor function and extended survival of the SOD1^{G93A}ALS mice[97].

In order to determine whether the effect of hMSCs-GDNF on slowing the progression of the disease could be enhanced by multiple NTFs, hMSCs-GDNF, hMSCs-VEGF, hMSCs-IGF-1, or hMSCs-BDNF were transplanted bilaterally into muscles of the SOD1^{G93A}ALS rats. Compared to individual NTF delivery, intramuscular delivery of hMSCs-GDNF combined with hMSCs-VEGF demonstrated synergic and greater effects on increasing survival rate, preventing motor neuron degeneration, and protecting neuromuscular junction[98]. In addition, transplantation of muscle progenitor cells-MIX (a mixture of muscle progenitor cells expressing BDNF, GDNF, VEGF, or IGF-1) into the hind legs of the SOD1^{G93A}ALS mice, decreased neuromuscular junction degeneration and increased axonal survival, leading to delayed disease onset (by 30 d) and prolonged survival (by 13 d). These results demonstrated that continuous delivery of the mixture of NTFs by engineered muscle progenitor cells might be a beneficial therapy for ALS[99]. In 2016, there were a phase 1/2 and a phase 2a clinical trials transplanting NTF-secreting BM-MSCs to small

Table 4 Combination therapy of stem cells with neurotrophic factors in amyotrophic lateral sclerosis

Cell types	Neurotrophic factors	Study design and outcome	Ref.
hNPCs	GDNF	hNPCs-GDNF were transplanted into the lumbar spinal cord of SOD1^{G93A} ALS rats. Genetically-modified hNPCs were able to survive, integrate, and release GDNF in the spinal cord of SOD1^{G93A} rats.	Klein *et al*[92], 2005
hNPCs	GDNF	hNPCs-GDNF were unilaterally transplanted into the spinal cord of SOD1^{G93A} ALS rats. There was robust cellular migration into degenerated areas, efficient delivery of GDNF and remarkable preservation of motor neurons at early and end stages of the disease.	Suzuki *et al*[93], 2007
hNPCs	GDNF	hNPCs-GDNF were unilaterally transplanted into the motor cortex of SOD1^{G93A} ALS rats. The hNPCs-GDNF matured into astrocytes, and released GDNF, which protected motor neurons, delayed disease pathology, and extended survival of theSOD1^{G93A} rats.	Thomsen *et al*[94], 2018
hMSCs	GDNF	hMSCs-GDNF were transplanted bilaterally into three muscle groups of a fALS rat model. Transplanted cells survived within the muscle, released GDNF, and increased the number of neuromuscular connections. Direct muscle delivery of hMSCs-GDNF ameliorated motor neuron loss within the spinal cord, delayed disease progression, and increased overall lifespan by 28 d.	Suzuki *et al*[95], 2008
hNSCs	VEGF	hNSCs overexpressing VEGF were IT transplanted into SOD1^{G93A} mice. Intrathecal hNSCs-VEGF transplantation significantly delayed disease onset and prolonged survival of the SOD1^{G93A} mice.	Hwang *et al*[96], 2009
NSCs	NGF	Intranasal NGF administration combined with lateral ventricle NSCs transplantation to the SOD1^{G93A}ALS mice delayed onset, improved motor function and prolonged lifespan.	Zhong *et al*[97], 2017
hMSCs	GDNF; VEGF; IGF-I; BDNF	To determine whether multiple NTFs played a synergistic role of slowing disease progression, SOD1^{G93A} rats were bilaterally muscularly transplanted with hMSCs-GDNF, hMSCs-VEGF, hMSCs-IGF-I, or hMSCs-BDNF. hMSCs-GDNF and hMSCs-VEGF prolonged survival and slowed the loss of motor function, and the combined delivery of GDNF and VEGF showed a strong synergistic effect.	Krakora *et al*[98], 2013
MPCs	BDNF; GDNF; VEGF; IGF-1	Hind legs transplantation of MPCs-MIX, a mixture of MPCs expressing BDNF, GDNF, VEGF, or IGF-1,decreased neuromuscular junction degeneration, increased axonal survival, delayed onset and prolonged lifespan of the SOD1 ^{G93A} mice.	Dadon-Nachum *et al*[99], 2015

MSCs	GDNF; BDNF; VEGF; HGF	To determine the safety and possible clinical efficacy of autologous MSCs-NTF cells transplantation in ALS patients. All patients were followed for 3 mo before the transplantation and for 6 mo after the transplantation. In the phase 1/2 part of the trial, 6 patients with early-stage ALS were injected IM and 6 patients with more advanced disease were transplanted IT. In the second stage, a phase 2a dose-escalating study, 14 patients with early-stage ALS received a combined IM and IT transplantation of autologous MSCs-NTF. Treatment of ALS patients with autologous MSCs-NTF cells by IT, IM, or combined (IT+IM) administration was safe and well tolerated. The rate of progression of forced vital capacity and ALS Functional Rating Scale-Revised score in the IT (or IT+IM)-treated patients were reduced.	Petrou *et al*[100], 2016 (clinical trials)

ALS: Amyotrophic lateral sclerosis; BDNF: Brain-derived neurotrophic factor; fALS: Familial amyotrophic lateral sclerosis: GDNF: Glial cell line-derived neurotrophic factor; HGF: Hepatocyte growth factor; hMSCs: Human mesenchymal stem cells; hNPCs: Human neural progenitor cells; hNPCs-GDNF: Lentivirus-modified hNPCs secreting GDNF; hNSCs: Human neural stem cells; IGF-1: Insulin-like growth factor-1; IM: Intramuscularly; IT: Intrathecally; MPCs: Muscle progenitor cells; MSCs-NTF: Neurotrophic factor secreting mesenchymal stem cells; NGF: Nerve growth factor; NSCs: Neural stem cells; NTF: Neurotrophic factor; VEGF: Vascular endothelial growth factor.

groups of ALS patients. Different administration methods were evaluated for patients in different stages of the disease, with early patients transplanted intramuscularly and progressive ones transplanted intrathecally. Results showed reduced progression rate of forced vital capacity and ALS Functional Rating Scale-Revised score in the intrathecal (or intrathecal plus intramuscular)-treated patients. Clinical trials have since shown that both routes of administration are safe, but the possible clinical benefits need to be confirmed by a larger cohort study[100].

In *in vitro* ALS models, adipose-derived stromal cells derived exosomes (ASC-exosomes) showed neuroprotection through oxidative damage protection, mitochondria function restoration and anti-apoptosis effects, indicating that ASC-exosomes is a promising approach to treat ALS[101-103].

CONCLUSION

Neurodegenerative diseases are a large group of neurological disorders characterized by progressive neuronal degeneration and loss, leading to motor and cognition impairment and ultimately death of affected patients. There is currently a lack of effective treatments for all neurodegenerative diseases because of their obscure pathogeneses. However, studies have revealed the considerable therapeutic promise of stem cells and NTFs, and especially when used in combination. The combination therapy of stem cells with NTFs – generated by engineering stem cells to overexpress NTFs, that is, using stem cells as a delivery platform for NTFs - can not only replenish the target neurons but also secrete neurotrophins to improve the microenvironment for nerve repair and regeneration. However, different neurodegenerative diseases exhibit specific neuron type loss, with cholinergic neurons in AD, dopaminergic neurons in PD, projection neurons in HD, and motor neurons in ALS. Thus, future research should give priority to the use of stem cell-derived disease-specific cell types in combination with cell-specific NTFs. Given the great promise of stem cells in combination with NTFs in clinical application, this novel treatment avenue is expected to provide benefit to patients suffering from neurodegenerative diseases in the future.

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REVIEW

Current and future uses of skeletal stem cells for bone regeneration

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Abstract

The postnatal skeleton undergoes growth, modeling, and remodeling. The human skeleton is a composite of diverse tissue types, including bone, cartilage, fat, fibroblasts, nerves, blood vessels, and hematopoietic cells. Fracture nonunion and bone defects are among the most challenging clinical problems in orthopedic trauma. The incidence of nonunion or bone defects following fractures is increasing. Stem and progenitor cells mediate homeostasis and regeneration in postnatal tissue, including bone tissue. As multipotent stem cells, skeletal stem cells (SSCs) have a strong effect on the growth, differentiation, and repair of bone regeneration. In recent years, a number of important studies have characterized the hierarchy, differential potential, and bone formation of SSCs. Here, we describe studies on and applications of SSCs and/or mesenchymal stem cells for bone regeneration.

Key words: Skeletal stem cell; Mesenchymal stem cell; Bone regeneration; Periosteum; Bone marrow; Skeleton

Core tip: Stem cell-based therapies have multiple applications in the field of bone regeneration. Recent research has demonstrated the advantageous use of skeletal stem cells (SSCs) and mesenchymal stem cells for bone modeling and remodeling. Our analysis indicates the hierarchy, self-renewal and differential potential of SSCs and the functions of SSCs, mesenchymal stem cells, and circulating progenitor cells on bone regeneration.

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INTRODUCTION

The bones in our body are living tissues. They are composed of two types of tissues: (1) The cortical (compact) bone as a hard outer layer, which is dense, strong, and tough; and (2) The trabecular (cancellous) bone as a spongy inner layer[1]. Long bones, such as the tibia and femur, consist of articular cartilage, epiphyses, growth plate, metaphysis, diaphysis, periosteum, endosteum, and a marrow cavity[1]. Bones provide protection for vital organs and structural support for the body due to their tough and rigid structures resulting from a mineralized matrix[2]. Bones also act as a storage area for minerals (*e.g.*, calcium) and provide a microenvironment for bone marrow (where blood cells are produced in long bones)[3].

During life, bones undergo organogenesis, modeling, and remodeling[4]. Bone modeling occurs when bone formation and bone resorption occur on separate surfaces, which means these two processes are not coupled during long bone increases in diameter and length[5]. Bone remodeling, the replacement of old bone by new bone, occurs primarily in the adult skeletal system to maintain bone mass[5]. This process involves the coupling of bone resorption and bone formation. Bone formation occurs by two distinct developmental processes. Intramembranous ossification, which occurs by the direct differentiation of mesenchymal progenitors into osteoblasts, involves the replacement of connective tissue membrane with bone tissue[6]. Endochondral ossification involves the replacement of a hyaline cartilage model with bone tissue[7]. Bone repair or fracture healing proceeds through four phases: inflammation, intramembranous ossification, endochondral ossification, and bone remodeling[8]. Bone repair depends on the function of specific cell types, such as mesenchymal stem cells (MSCs) and osteoblasts[9,10]; the expression of soluble molecules (cytokines and growth factors)[11-13]; the scaffold (hydroxyapatite and extracellular matrix molecules)[14,15]; and various mechanical stimuli during the entire repair process[16,17].

Stem cells are defined as cells with the ability to self-renew and differentiate into different cell types[18]. According to their differentiation capacity, stem cells can be categorized as totipotent, pluripotent, multipotent, or unipotent[8]. Totipotent stem cells are capable of generating all of the cell types in animals, such as early blastomeres[19]. Pluripotent stem cells are capable of generating embryonic tissues from all three primary germ layers. Induced pluripotent stem cells experimentally derive from adult somatic cells, and embryonic stem cells (ESCs) originate from the inner cell mass of the blastocyst[20-24]. Multipotent stem cells can differentiate into multiple specific cell types in a specific tissue or organ[25] and are located in specialized niches, where they can interact with the local microenvironment to maintain the stemness or differentiation potential. The musculoskeletal system contains many multipotent stem cells. The most studied multipotent stem cells in the musculoskeletal system are the hematopoietic stem cells (HSCs)[26], which are the source of all types of blood cells, and bone marrow mesenchymal stem cells (BMMSCs), also known as bone marrow stromal cells (BMSCs)[27]. Unipotent stem cells can develop into only a single cell type[28,29].

The skeletal system contains multiple tissue types including bone, cartilage, blood vessels, nerves, and fat. Each tissue in the skeletal system is generated and maintained by the accurate management of specific stem cells. Among the most well-known stem cells in the skeleton are the HSCs, defined as having the critical role of the long-term maintenance and production of all mature blood cell lineages during life[30,31]. The isolation of non-hematopoietic stem cells in the bone marrow relies on the ability of the cells to attach to plastic plates, which are thought to be ''mesenchymal stem cells'' or "skeletal stem cells." These stem cells contain heterogeneous mixtures of cells with different potencies, such as bone, cartilage, adipo-tissue, endothelial cells, fibroblasts, and stroma. At this time, the MSCs have two opposing descriptions. MSCs can be the self-renewing, postnatal, and multipotent stem cells for bone tissue, which are considered a specific type of bone marrow perivascular cell. In contrast, MSCs can be ubiquitous in connective tissues and are defined by *in vitro* characteristics, such as adipose tissue[32,33], periosteum[34,35], the synovial joint[36-38], and muscle tissue[39,40]. In 2006, the International Society for Cellular Therapy proposed minimal criteria for defining the concept of human MSCs: They must be plastic-adherent; highly express CD105, CD73, and CD90 while lacking expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules; and be able to differentiate to

osteoblasts, chondroblasts, and adipocytes *in vitro*[41]. This set of standards for the definition of human MSCs is consistent with laboratory-based scientific investigations and preclinical studies. However, the relationships between MSCs and SSCs are still not definitively known.

ORIGIN OF SSCs

The SSC concept derives from experiments conducted by Friedenstein *et al*[42], who found that heterotopic transplants of bone marrow form reticular tissue and bone[42,43]. They confirmed the presence of colony-forming unit fibroblasts in the tissue culture plastic (TCP), adherent, non-hematopoietic cells in the bone marrow. However, there remained considerable heterogeneity within the TCP-adherent cell population. The formation of the ectopic ossicle was ascribed to a specific cell population in the TCP-adherent cells. Subsequently, the generation of an ossicle has been assigned to multipotent clonogenic progenitor cells, which give rise to cartilage, bone, and adipocytes[44]. These progenitor cells were first termed as osteogenic by Friedenstein *et al*[42] or as stromal stem cells by Owen *et al*[44]; they were then named MSCs by Caplan[45] and Pittenger *et al*[46]. Finally, they were considered SSCs by Bianco *et al*[47].

In past decades, several studies have attempted to identify cell surface markers that are expressed by SSCs, including the STRO-1 antigen, CD73, CD44, CD166, CD105, CD90, CD146, and CD271, or by negative selection for hematopoietic markers, such as CD45, CD34, CD14, CD79a, CD19, CD11b, and HLA-DR surface markers[48,49]. However, due to variation in certain markers, there is still a lack of consensus regarding the cell surface markers unique to SSCs. The absence of a set of specific surface markers may have contributed to the presence of confusing data in the literature related to the identification of SSCs. Concerning the present controversy, the definition of SSCs states that the SSC population should have the capacity to produce four distinct lineages: bone, cartilage, adipo-tissue, and hematopoiesis-supportive stroma *in vivo*. Nevertheless, a list of specific surface markers, which could be extensively studied, would be widely accepted.

SSCs

In 2013, Chan *et al*[50] reported a lineage-restricted and self-renewing skeletal progenitor that was isolated from the skeletal elements of fetal, neonatal, and adult mice and could form bone, cartilage, and bone marrow; it was named bone-cartilage-stromal progenitors (BCSPs). However, the main aim of the study was to focus on the regulation of the vascularization and hematopoiesis of HSCs by BCSPs, and they did not intensively study the role of BCSPs in bone regeneration or repair.

In 2015, two reports published in *Cell* helped to advance the SSC field and provide insight into the cell hierarchy[51,52]. A study by Worthley *et al*[51] used the secreted bone morphogenetic protein (BMP) agonist, Gremlin 1 (Grem1), to label skeletal progenitor cells. They found Grem1 positive cells beside the growth plate and determined that the trabecular bone could self-renew and generate diverse cells, such as osteoblasts, reticular marrow stromal cells, and chondrocytes but not adipocytes. They later named them osteo-chondro-reticular (OCR) stem cells. In the femoral fracture callus, they found that Grem1$^+$ OCR stem cells contributed to the expansion and differentiation into osteoblasts and chondrocytes. In another study, Chan *et al*[52] found clonal regions in the bone, especially at the growth plate, that encompassed bone, stromal tissue, and cartilage in mice. Subsequently, they showed that the CD45- Ter119- Tie2- AlphaV + Thy- 6C3- CD10- CD200+ cell population in the growth plate could self-renew *in vitro* and generate other subpopulations, such as pre-BCSP and BCSP. These cell populations could specify their differentiation toward bone, cartilage, or stromal cells but not toward fat or muscle, which are regulated by soluble factors. They concluded that the CD45- Ter119- Tie2- AlphaV+ Thy- 6C3- CD105- CD200+ cell population represented SSCs in postnatal skeletal tissues. Furthermore, they found that the SSC number increased in the callus of a femoral fracture more than in the uninjured femur with enhanced osteogenic capacity. In a similar study, Marecic *et al*[53] found that BCSP expansion preceded ossified callus formation in femoral fractures and that irradiation reduced the fracture-induced BCSP expansion. The fracture-induced BCSPs (f-BCSPs) possessed greater plating efficiency, viability, alkaline phosphatase (ALP) activity, and Alizarin Red staining (ARS) than did the uninjured femur BCSPs (u-BCSPs). The f-BCSPs formed significantly larger bone specimens compared with u-BCSPs when transplanted under the renal capsules of immunodeficient mice. Although the hierarchy of stem cells and the differential

capacity were studied in depth in these studies, little is known about the involvement of SSCs in bone development, modeling, and remodeling. As mentioned above, SSCs are multipotent cells that differentiate into bone, cartilage, and stromal niches; however, they are unable to differentiate into other cell types, such as adipocytes, fibroblasts, muscle cells, or hematopoietic cells.

Chan *et al*[54] published another study in 2018, which focused on the human SSC. Using single cell RNA sequencing, fluorescence-activated cell sorting, and *in vivo* differentiation assays, they showed that the PDPN+ CD146- CD73+ CD164+ fetal growth plate cells produced the most colony-forming units *in vitro* and determined that they possessed self-renewal and multipotency, which were thought to be putative human SSCs. Further hierarchical studies showed that this cell population was capable of the linear generation of osteogenic and chondrogenic subpopulations and was at the top of the differentiation tree. These studies established an ingenious human bone xenograft mouse model, transplanting human fetal phalangeal grafts with intact periosteum into immunodeficient mice; they found that fracture of the implanted bone induced the expansion of human SSCs near the fracture site. Furthermore, they found that human SSCs favored hematopoiesis and, conversely, that HSCs supported the human SSC lineage.

Another study published in 2018 by Mizuhashi *et al*[55] reported that SSCs were generated from PTHrP-positive chondrocytes in the resting zone of the growth plate in a mouse model. Mouse SSCs (41.6% ± 4.4%), pre-BCSP (31.7% ± 6.2%), and BCSP (53.4% ± 16.9%) were positive for PTHrP. The analysis showed that PTHrP-positive chondrocytes, which are considered a unique SSC class in the resting zone, were multipotent and could longitudinally form columnar chondrocytes, which underwent hypertrophy, then became multiple types of cells, such as osteoblasts and marrow stromal cells, beneath the growth plate. Additionally, these stem cells were able to send a signal to the transit-amplifying chondrocytes to maintain their proliferation so that they could maintain the integrity of the growth plate; transit-amplifying chondrocytes sent cues to determine the cell differentiation fates of PTHrP-positive chondrocytes in the resting zone.

The SSCs were derived from the growth plate in most of the abovementioned studies, which focused on their multipotency by transplanting stem cells under the renal capsules of immunodeficient mice involved in endochondral ossification. Duchamp found that periosteal cells (PCs) and BMSCs were derived from the same embryonic Prx1-mesenchymal lineage and that postnatal PCs had an enhanced clonogenicity, growth, and differentiation capacity compared to BMSCs[56]. Although they did not identify the SSCs in the periosteum, they concluded that the presence of SSCs in the periosteum was associated with greater regenerative potency. Another study, from Weill Cornell Medical School, identified SSCs, periosteal stem cells (PSCs), which were present in the periosteum of the long bones and calvarium of mice[57]. The PSCs displayed self-renewal and multipotent capacities and possessed different transcriptional signatures compared to the other SSCs. As previously mentioned, other SSCs form bones through endochondral ossification, whereas PSCs form bones *via* a direct intramembranous pathway in the long bone or cranial bone. The differentiation capacity of PSCs for bone formation would therefore be enhanced in response to a fracture.

MSCs

In 1991, Caplan[45] introduced the term "mesenchymal stem cells" to define the putative stem cells of skeletal tissues (bone and cartilage). The concept of MSCs extended to include bone marrow[58,59], adipose tissue[33,60], the periosteum[61], the synovial lining[62], muscle tissue[63], the umbilical cord[64], and different types of dental tissues[65]. Among them, BMMSCs were one of the well-studied sources. It is currently thought that BMMSCs show an essential role in supporting bone healing through the secretion of nutritional and immunomodulatory factors rather than *via* a direct effect on the formation of the bone callus. BMMSCs secrete growth factors and cytokines to influence bone regeneration *via* paracrine and autocrine systems; this process includes vascular endothelial cell growth factors, platelet-derived growth factors, BMPs, fibroblast growth factors, insulin-like growth factor, and epidermal growth factor[65,66]. Inflammation is essential for any wound healing including bone repair. The first phase of fracture repair is the inflammation phase. Besides the trophic role, BMMSCs are critical regulators of the local inflammation micro-environment during bone repair. Macrophages are a key cell population that contributes to the inflammatory environment, whereas BMMSCs show an immunomodulatory effect on macrophages[67,68]. These inflammation factors include prostaglandin-E2[69], monocyte

chemoattractant proteins (MCP-1 and MCP-3)[70], tumor necrosis factor-α[71], transforming growth factor-β[72], and numerous interleukins (IL-1, IL-3, IL-4, IL-6, and IL-10)[73,74].

Zuk *et al*[75] first described the isolation of adipose tissue-derived MSCs (ADSCs) from adipose tissue and characterized their phenotype and multipotency. Although ADSCs do not have superior osteogenic potential compared to BMMSCs *in vitro*[76-79], ADSCs are easier to acquire than BMMSCs. ADSCs have been reported to exhibit high angiogenesis with either the ability to differentiate into endothelial cells or to secrete angiogenic factors, which favor osteogenesis and bone healing[80]. Moreover, ADSCs have a favorable effect on bone regeneration *in vivo*[81] and are widely used in clinical trials.

The periosteum is a tough layer of dense connective tissue that surrounds the bone surface, which contains different bone cells that enable bone to grow in thickness, which favors fracture repair and nourishes bone tissues[82]. The innermost layer contains stem cells that contribute to bone homeostasis and fracture healing, which respond to bone injury within 48 h through rapid proliferation. The stem cells from the periosteum have enhanced clonogenicity, growth, and differentiation capabilities[56,57]. Studies using reporter mice have identified Prx1 as a periosteal marker[83,84]. Studies in adult animals have shown that Prx1 is expressed in the periosteum and contributes to the formation of fracture callus[85]. Although only a limited number of studies have focused on the identification of MSCs in the periosteum, it is generally accepted that the periosteum plays an essential role in bone modeling and remodeling and is an important trophic pool for fracture healing.

Synovial tissue-derived mesenchymal stem cells (SMSCs) are obtained by a minimally invasive procedure and have been used for cartilage repair[86-89]. They are effective in regenerating critically sized bone defects when combined with polyether ketone[90], although few studies of SMSCs have focused on bone regeneration. Muscle-derived MSCs also had high osteogenic potential in a mouse model[91] but need to be further characterized. Umbilical cord MSCs (UCMSCs) show a favorable osteogenic potential, similar to that of BMMSCs, and are able to contribute to bone and vessel regeneration[92]. UCMSCs also show great potential for bone regeneration in the presence of secretion factors[93-95], biomaterials[96-98], exosomes[99], and gene modification therapy[100,101]. Dental tissue-derived MSCs have been well-characterized and have shown features originally ascribed to BMMSCs. At least six different dental tissue-derived mesenchymal stem cell types have been isolated and have been described by Bartold *et al*[65]. Briefly, dental pulp stem cells and periodontal ligament stem cells exhibit considerable bone regenerative capabilities, whereas human apical papilla stem cells, dental follicle stem cells, exfoliated deciduous teeth stem cells, and gingival mesenchymal stem cells require further study[65].

CIRCULATING PROGENITOR CELLS

Although hematopoietic cells are developmentally derived from the mesoderm in a manner similar to osteoblasts, they have no direct role in fracture healing or heterotopic ossification[102]. Other circulating cells, such as CD34+ cells from endothelial progenitor cells (EPCs), exhibit accelerated bone healing[103,104]. The EPCs, induced into the peripheral circulation by trauma, contribute to neovascularization and are involved in fracture healing[105,106]. CD31+ cells from peripheral blood facilitate bone endogenous regeneration by supporting immunomodulation and vascularization[107]. The circulating osteogenic progenitor cells, a type I collagen+/CD45+ subpopulation of mononuclear adherent cells in bone marrow, serve as osteogenic precursors for heterotopic ossification[108]. AMD3100, an antagonist of the chemokine receptor 4 that rapidly mobilizes stem cell populations into the peripheral blood, exerts significant beneficial effects, involving improved neovascularization and osteogenesis, on bone healing[109-111]. Using surgically conjoined transgenic mice which constitutively express green fluorescent protein (GFP) in no erythroid tissue and syngeneic wild-type mice models, circulating osteogenic connective tissue progenitors (GFP+ cells) from transgenic mice are mobilized to fracture sites in wild-type mice and contribute to osteogenic differentiation in the early stage of fracture healing[112]. Additionally, exposure to young cells, by heterochronic parabiosis, rejuvenates bone repair in aged animals[113]. Taken together, these results demonstrate that circulating progenitor cells play an important role in bone regeneration.

CLINICAL TRANSLATION

Bone defects and fracture nonunion can be caused by skeletal abnormalities, tumor resection, or infection, and they remain a major challenge in trauma and orthopedic surgery. Current treatments recommend the use of autologous and allogenic bone to repair these defects. For large bone defects, bone transfer techniques, membrane induction techniques, and vascularized fibula can be clinically adopted, but most of these methods involve treatment in stages, with long treatment cycles, injury in the blood supply area, complicated surgery, and other possible complications[114]. Tissue engineering is an attractive approach for the current treatments and could minimize these limitations. The easy accessibility of MSCs from bone marrow and their multi-differentiation potency have driven the use of BMMSCs in the clinic.

Many studies currently use autologous bone marrow cells harvested during orthopedic procedures, and most of them use stem cells in combination with biomaterials[115-118]. Autologous MSCs combined with β-tricalcium phosphate graft material as a carrier can promote the healing of femoral bone defects[116]. Using autologous BMMSCs grown in a serum cross-linked scaffold is an alternative therapy for maxillary bone defects[117]. Another trial confirmed that autologous BMMSCs successfully induced significant formation of new bone in patients with severe mandibular ridge resorption[119]. Moreover, peripheral blood CD34+ cells and bone marrow aspirate concentrates have been effectively used in bone defects and bone nonunion[120,121].

Translational studies using stem cells are ongoing. Table 1 details 12 trials, which were completed or currently underway and are recorded at clinicaltrials.gov, maintained by the National Institutes of Health. Randomized clinical trials using defined SSC populations are needed to evaluate the efficacy of SSC-based therapies in future clinical trials.

LIMITATIONS AND DISADVANTAGES

In recent years, significant progress has been made in the study of SSCs. However, there is still a distance between basic research and clinical translation. The main reason is that there is currently no precise definition of SSCs, and they are relatively difficult to obtain. SSCs in most studies are obtained from growth plates, which is difficult and impractical for clinical translation. Although there is a lot of research on circulating progenitor cells, there is also a lack of a unified definition of circulating progenitor cells. Most of the studies do not focus on a unique class of cells but a group of mixed cells. Subsequent research needs to accurately classify circulating progenitor cells and study the specific functions of each group. Most of the circulating progenitor cells can be more easily obtained through the blood system than other SSCs, and its clinical translation has broad application prospects.

We recorded the relevant clinical trials from clinicaltrials.gov; however, it is still not comprehensive enough. In the future, we should search for the clinical research registration websites from different countries, and pay attention to the progress of the trials on time. At present, MSCs are the most widely used in clinical trials, and in the future scientists should expand clinical research on different types of SSCs.

CONCLUSION AND FUTURE PERSPECTIVES

Cell-based therapy has been widely used in recent decades to treat a variety of physiological defects. A number of stromal stem cells harvested from different tissues have exhibited therapeutic characteristics *in vivo* and *in vitro*. Among them, BMMSCs and ADSCs are widely considered to be the more usable candidates for regenerative medicine due to their easy accessibility and expansion. For bone tissue regeneration, SSCs and/or BMMSCs have positive differential potentials and therapeutic functions. This will ensure the availability of SSCs and BMMSCs for animal research and clinical applications in the future.

As previously mentioned, SSCs at the growth plate and periosteum can differentiate into bone, cartilage, and bone marrow but not into adipose tissue. In the future, it will be important to identify an original SSC population that can differentiate into all bone tissues. The hierarchy of the original SSCs needs to be clarified, and the precise definition of SSCs requires international consensus. Furthermore, the angiogenic ability of SSCs favoring bone repair needs to be thoroughly studied, and the effect of cell homing on bone repair should be a major focus of future research.

Table 1 Clinical trials employing mesenchymal stem cells for bone healing

Title	Conditions	Interventions	Phase	Enrollment	Status
Allogeneic mesenchymal stem cell transplantation in tibial closed diaphyseal fractures	Tibial fracture	Mesenchymal stem cell injection	2	40	Completed
The efficacy of mesenchymal stem cells for stimulating the union in treatment of non-united tibial and femoral fractures in Shahid Kamyab Hospital	Nonunion fracture	Injection of mesenchymal stem cell in non-union site	2	19	Completed
Bone regeneration with mesenchymal stem cells	Mandibular fractures	Application of autologous mesenchymal stem cells	3	20	Completed
Stem cells and tibial fractures	Tibial fractures	CD34+ hematopoietic stem cells	1	9	Completed
Autologous implantation of mesenchymal stem cells for the treatment of distal tibial fractures	Tibial fractures	Autologous mesenchymal stem cells implantation	2	24	Completed
Autologous stem cell therapy for fracture non-union healing	Non-union of fractures	Carrier plus *in vitro* expanded autologous BMSCs	Not applicable	35	Completed
Treatment of non-union of long bone fractures by autologous mesenchymal stem cells	Nonunion fractures	Cell injection	1	6	Completed
Percutaneous autologous bone-marrow grafting for open tibial shaft fracture	Tibial fractures; fractures, open	Osteosynthesis	Not applicable	85	Completed
Use of adult bone marrow mononuclear cells in patients with long bone nonunion	Long bone nonunion	Osteosynthesis	2	7	Completed
A comparative study of 2 doses of BM autologous H-MSC+ biomaterial *vs* iliac crest autograft for bone healing in non-union	Non-union fracture	Cultured mesenchymal stem cells; autologous iliac crest graft	3	108	Recruiting
Clinical trial of intravenous infusion of fucosylated bone marrow mesenchymal cells in patients with osteoporosis	Osteoporosis; spinal fractures	Fucosylated MSCs for osteoporosis	1	10	Recruiting
Reconstruction of jaw bone using mesenchymal stem cells	Bone atrophy	BCP with autologous MSCs	1	13	Enrolling by invitation

BMSCs: Bone marrow mesenchymal stem cells; BM: Bone marrow; MSCs: Mesenchymal stem cells.

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MINIREVIEWS

DNA methylation and demethylation link the properties of mesenchymal stem cells: Regeneration and immunomodulation

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Abstract

Mesenchymal stem cells (MSCs) are a heterogeneous population that can be isolated from various tissues, including bone marrow, adipose tissue, umbilical cord blood, and craniofacial tissue. MSCs have attracted increasingly more attention over the years due to their regenerative capacity and function in immunomodulation. The foundation of tissue regeneration is the potential of cells to differentiate into multiple cell lineages and give rise to multiple tissue types. In addition,the immunoregulatory function of MSCs has provided insights into therapeutic treatments for immune-mediated diseases. DNA methylation and demethylation are important epigenetic mechanisms that have been shown to modulate embryonic stem cell maintenance, proliferation, differentiation and apoptosis by activating or suppressing a number of genes. In most studies, DNA hypermethylation is associated with gene suppression, while hypomethylation or demethylation is associated with gene activation. The dynamic balance of DNA methylation and demethylation is required for normal mammalian development and inhibits the onset of abnormal phenotypes. However, the exact role of DNA methylation and demethylation in MSC-based tissue regeneration and immunomodulation requires further investigation. In this review, we discuss how DNA methylation and demethylation function in multi-lineage cell differentiation and immunomodulation of MSCs based on previously published work. Furthermore, we discuss the implications of the role of DNA methylation and demethylation in MSCs for the treatment of metabolic or immune-related diseases.

Key words: Mesenchymal stem cells; DNA methylation and demethylation; Multi-lineage differentiation; Regeneration; Immunomodulation; Immune disease

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Core tip: Mesenchymal stem cells (MSCs) harbor the capacity to regenerate diverse tissues and can also perform key immunomodulatory functions. DNA methylation and demethylation are known to modulate stem cell maintenance and differentiation in embryonic stem cells. However, the role of DNA methylation and demethylation in MSC-based tissue regeneration and immunomodulation requires further investigation. In this review, we discuss how DNA methylation and demethylation function in multi-lineage cell differentiation and immunomodulation of MSCs based on previously published work. In addition, we discuss the implications of the role of DNA methylation and demethylation in MSCs for the treatment of metabolic or immune-related diseases.

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INTRODUCTION

DNA methylation and demethylation are two vital epigenetic regulatory mechanisms for gene expression. DNA cytosine methylation is a frequently occurring process that is orchestrated by DNA methyltransferases (DNMTs), which generate 5-methylcytosine (5mC)[1]. The methylation process at the 5th cytosine can be reversible, which is called DNA demethylation. This process has received increased attention over recent years. Increasingly more researchers began to identify enzymes that could generate 5-hydroxymethylcytosine (5hmC) from 5mC in mammalian cells. For the first time, in 2009, TET1 was shown to convert 5mC into 5-hmC[2]. Thereafter, all three of the TET family proteins (TET1, TET2, and TET3) were demonstrated to catalyze a similar hydroxymethylation reaction[3]. TET family proteins are also receiving increased attention because of their function in DNA demethylation.

In addition to their function in multi-lineage differentiation and tissue regeneration[4], mesenchymal stem cells (MSCs) also display profound immunomodulation capacity *via* a sophisticated molecular network[5]. DNA methylation and demethylation are known to modulate stem cell maintenance and differentiation by activating or suppressing an array of genes[6]. Previous research on DNA methylation and demethylation has primarily focused on embryonic stem cells and neural systems. Nevertheless, how DNA methylation and demethylation impact MSC function remains elusive. Here, we discuss recent studies concerning the effect of DNA methylation and demethylation on MSC-based regeneration and immunomodulation.

OSTEOGENIC DIFFERENTIATION OF MSCS IS REGULATED BY DNA METHYLATION AND DEMETHYLATION

MSCs hold promising potential for regenerative medicine due to their capacity for self-renewal and multi-lineage differentiation into tissue-specific cells, which include osteoblasts, chondrocytes, and adipocytes. During osteogenic differentiation of MSCs, osteogenic-specific genes such as *RUNX2*, *OPN*, *COX2*, *ALP*, and *OCN*[7-11], which are regulated by DNA methylation, showed increased expression and decreased DNA methylation. Demethylation was observed at specific CpG regions in the promoters of osteogenic lineage-specific genes, including *Runx2, Dlx5, Bglap*, and *Osterix*, during osteogenic differentiation in adipose-derived MSCs (Ad-MSCs). Upon demethylation inhibition, osteogenic gene expression became down-regulated[12]. On the other hand, Daniunaite *et al*[13] found that genes encoding the main pluripotency factors, such as Nanog and Sox2, showed decreased gene expression along with decreased 5hmC levels during the osteogenic differentiation of Ad-MSCs.

In another study on Ad-MSCs, an age-related decline in proliferation was observed. Ad-MSCs isolated from old donors showed significantly impaired osteogenic differentiation capacity compared to young donors. Furthermore, decreased expression of *Nanog, Oct4*, and *Lin28A* and increased expression of *Sox2* were observed. A simultaneous decrease of global 5hmC in Ad-MSCs from old donors also

occurred. When 5-azacytidine (5-Aza), a DNMT inhibitor, was used to treat Ad-MSCs from old donors, increased global 5hmC and increased TET2 and TET3 expression were observed, which was accompanied by an increase in osteogenic differentiation capacity[14]. These results suggest that global DNA demethylation levels correlate with the osteogenesis capacity of MSCs, and that DNMT inhibitors could down-regulate DNA methylation to improve osteogenesis. Notably, an additional study by Kornicka *et al*[15] drew similar conclusions.

Bone marrow MSCs (BMMSCs) are a population of multipotent stem cells isolated from bone marrow that harbor the capacity for self-renewal and multi-lineage differentiation. The osteogenic differentiation of BMMSCs is also regulated by dynamic changes, as well as a balance of DNA methylation and demethylation. Bone loss caused by mechanical unloading is partially due to the impaired regeneration capacity of BMMSCs[16]. When mechanical stimuli were rescued, Dnmt3b was released from the *Shh* gene promoter, thus leading to promoter demethylation and up-regulated gene expression. Hedgehog signal was then activated by Shh, promoting BMMSCs to differentiate into osteoblasts[17]. Yang *et al*[18] found that in *Tet1* and *Tet2* double knockout mice, 5hmC levels of the *P2rX7* promoter were down-regulated, leading to miR-293a-5p, miR-293b-5p, and miR-293c-5p accumulation, and a decrease in BMMSC osteogenic differentiation capacity. Upon re-activating P2rX7, microRNA secretion from *Tet* double knockout BMMSCs was increased, thus partly rescuing both the osteopenia phenotype and BMMSC function.

Mechanisms of TET-mediated DNA demethylation in distinct MSCs vary due to their diverse sources. When small hairpin RNA lentiviral vectors were transfected to knock down TET1, the proliferation rate and odontogenic differentiation capacity of human dental pulp stem cells were significantly suppressed. This indicated that TET1 plays an important role in dental pulp repair and regeneration[19]. In another study focusing on human BMMSCs, TET1 recruited other epigenetic modifiers, including SIN3A and EZH2, to inhibit the osteogenic differentiation of BMMSCs in an indirect manner. On the other hand, TET2 was found to directly promote the osteogenic differentiation of BMMSCs[20]. The underlying mechanisms of how the TET family proteins regulate MSC function from distinct sources require further investigation.

ADIPOGENIC DIFFERENTIATION OF MSCS IS RELATED TO DNA METHYLATION AND DEMETHYLATION

Noer *et al*[21] reported that in undifferentiated Ad-MSCs, the promoters of adipogenic genes, including *LEP*, *PPARγ2*, *FABP4* and *LPL*, are hypomethylated, in contrast to myogenic or endothelial genes. During adipogenic differentiation, although specific CpG sites of the *LEP* promoter undergo demethylation, the global methylation status of *LEP, PPARG2, FABP4*, and *LPL* promoters across different Ad-MSC clones remains stable. Yang *et al*[18] showed that *Tet*1 and *Tet*2 small interfering RNA treatment does not alter the adipogenic differentiation capacity of BMMSCs.

Barrand *et al*[22] showed that in adipose MSCs, the promoter of *OCT4* was hypermethylated consistent with its repression. Melzner *et al*[23] found that the promoter of leptin underwent extreme demethylation (9.4% ± 4.4%) during the maturation of human preadipocytes toward terminally differentiated adipocytes. What's more, methyl-CpG binding proteins could bind to specific sites in the promoter and repressed leptin expression. Fujiki *et al*[24] reported that during the differentiation of 3T3-L1 preadipocytes to adipocytes, the hypermethylated *PPARγ2* promoter was progressively demethylated, while 5-Aza could increase the expression of *PPARγ2*, indicating that the methylation of its promoter inhibited the gene expression.

Overall, additional research on the dynamics of DNA methylation and demethylation during adipogenesis from different MSC sources is necessary.

CHONDROGENIC DIFFERENTIATION IS REGULATED BY DNA METHYLATION AND DEMETHYLATION

DNA methylation and demethylation status also change during MSC differentiation into chondrocytes. Chondrogenic differentiation of Ad-MSCs and BMMSCs was associated with a < 50% reduction in methylation rates at two specific CpG sites in the *COL10A1* gene, and transcription of this gene was strongly induced[25]. Ito *et al*[26] discovered that 5hmC increased during chondrogenic differentiation of C3H10T1/2, a MSC line, and that *TET1* expression was significantly up-regulated. Furthermore, *Tet1*

knockdown resulted in a marked decrease in the expression of chondrogenesis markers such as Col2 and Col10. In addition, 5hmC in the *Igf1* promoter is a preferable binding site for TET proteins in chondrocytes. Additional targets of *Tet* genes, as well as other enzymes that function in DNA methylation and demethylation, need to be identified in order to reveal the underlying mechanisms of chondrogenic differentiation of MSCs.

Lin *et al*[27] found that stepwise preconditioning–manipulated BMMSCs showed improved cell proliferation and chondrogenic differentiation potential *in vitro* and enhanced therapeutic effect on the progression of osteoarthritis *in vivo*, and one mechanism of that is the reduction in CpG methylation at the promoters of *Nanog* and *Oct4*. Pollock *et al*[28] demonstrated an experimental DMSO-free formulation which could improve post-thaw function of MSCs including chondrogenesis, as DMSO is a strong inducer of demethylation which may affect the potential of MSCs for therapeutic use in treatment of human diseases. These studies reminded us that epigenetic modification of MSCs could be a promising approach to improve their therapeutic effects.

These results regarding DNA methylation and demethylation indicate that hypomethylation of specific genes, such as *Runx2*, *Opn*, *Dlx5*, *Osterix*, *Col2*, and *Col10*, play important roles in multi-lineage differentiation of and tissue regeneration by MSCs (Figure 1).

MYOGENIC DIFFERENTIATION ASSOCIATED WITH DNA DEMETHYLATION

Cardiogenic differentiation is another important property of MSCs, and stem cell therapy for cardiovascular diseases is now in clinical trial[29]. Bhuvanalakshmi *et al*[30] found that in differentiated cardiomyocytes from MSCs, six out of the ten CpG islands of the promoter regions of *Nkx2.5*, the early cardiac gene, underwent demethylation. What's more, the CpG promoter demethylation of *sFRP4*, a Wnt antagonist, was also observed. This result is consistent with the previous findings that 5-Aza treatment of BMMSCs inhibited the ventricular scar from thinning and expanding, minimized left ventricular chamber dilatation, and thus improved myocardial function[31]. Antonitsis *et al*[32] treated hBMMSCs with 5-Aza *in vitro* to induce them to differentiate towards a cardiomyogenic lineage. Nakatsuka *et al*[33] also used 5-Aza to investigate the myogenic differentiation potential of mouse dental pulp stem cells. DNA demethylation induced by 5-Aza and forced expression of Myod1 upregulated the muscle-specific transcriptional factors such as Myogenin and Pax7.

IMMUNOMODULATION OF MSCS ASSOCIATED WITH DNA METHYLATION AND DEMETHYLATION

Aside from tissue regeneration, MSCs play an important role in immunomodulation, which may prove critical for treating a variety of immune diseases such as colitis, arthritis, and systemic lupus erythematosus[34-36]. Immunomodulation by MSCs relates to the secretion of extracellular matrix proteins[37] as well as a variety of cytokines including IL-2, IL-4, IL-10, TNF-α, and INF-γ[38-40]. MSC immunoregulation can also occur through cellular contacts[40-42]. B cell proliferation was found to be inhibited by human MSCs, not through the induction of apoptosis but through G0/G1 cell cycle arrest[43]. MSCs may suppress T cell proliferation, cytokine release, cytotoxicity, and Th1/Th2 balance[44,45].

Of late, how DNA methylation and demethylation regulate MSC-induced immunomodulation has received increasingly greater attention. Yang *et al*[46] found that *Tet*1- and *Tet*2-mediated *Foxp3* demethylation plays a significant role in the differentiation of regulatory T cells as well as the maintenance of immune homeostasis. Khosravi *et al*[47] reported that MSCs could enhance the demethylation of the Treg-specific demethylated region upon cell-cell contact, and MSC-based induction of regulatory T cells is associated with direct modifications of the RUNX complex genes (*RUNX1*, *RUNX3*, and *CBFB*). Yu *et al*[48] found that the down-regulation of both *TET1* and *TET2* leads to hypermethylation of the *DKK-1* promoter, which leads to activation of the Wnt/β-catenin signaling pathway and thus up-regulates Fas ligand (the *FasL* gene) expression in periodontal ligament stem cells. This in turn enhances their immunomodulatory ability, which is demonstrated by their elevated capacity to induce T cell apoptosis. Taken together, these results demonstrate a significant role for TET-mediated DNA demethylation in MSC-based

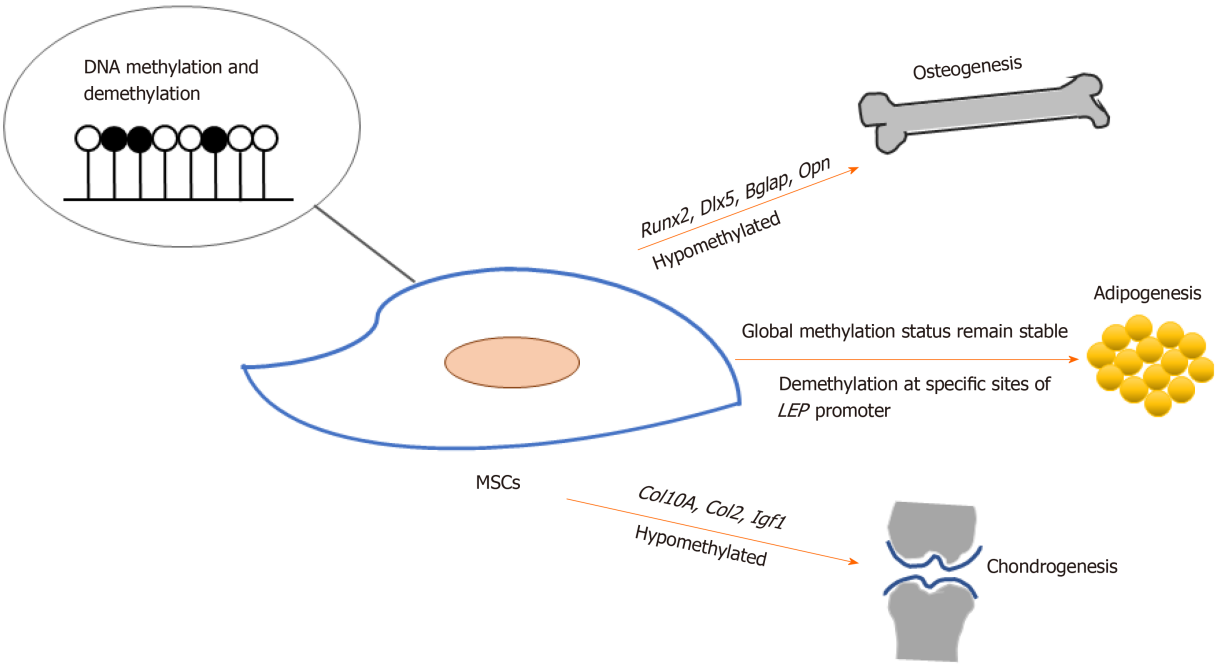

Figure 1 Hypomethylation of specific genes in mesenchymal stem cells drives multi-lineage differentiation and tissue regeneration. MSCs: Mesenchymal stem cells.

immunomodulation (Figure 2). Nevertheless, further investigations are required to reveal whether the methylation of MSCs is involved in regulation of other immune cells such as macrophages and natural killer cells and the underlying mechanisms.

IMPLICATIONS FOR DISEASE TREATMENT

As previously mentioned, DNA methylation participates in the regulation of gene expression, which may contribute to metabolic diseases when there is an imbalance in DNA methylation *vs* demethylation. García-Ibarbia *et al*[49] compared bone tissue samples from patients with osteoporotic hip fractures and osteoarthritis. Their results showed that Wnt pathway activity is reduced in patients with hip fractures compared with those with osteoarthritis. Additionally, six genes, including *FZD10*, *TBL1X*, *CSNK1E*, *SFRP4*, *CSNK1A1L*, and *WNT8A*, showed significantly different methylation rates between both groups. *FZD10*, *CSNK1E*, *TBL1X*, and *SFRP4* are hypermethylated in osteoarthritis, while *WNT8A* and *CSNK1A1L* are hypomethylated compared with fractures. This result may help explain the distinctions in Wnt pathway activity between the two groups. MSCs from spinal ligaments with ectopic ossification largely differentiated into osteogenic lineage. Chiba *et al*[50] found that MSCs isolated from the spinal ligaments of ossification from yellow ligament patients showed higher expression of *GDNF* and *Wnt5a*, which are hypomethylated compared with the control group. This result indicates that osteogenic features of MSCs from patients with ossification of the yellow ligaments are promoted by *GDNF* and *Wnt5a* demethylation.

In 2002, Bartholomew *et al*[51] first reported that MSCs harbored immunosuppressive functions by demonstrating their ability to inhibit a mixed lymphocyte response *in vitro* as well as prevent rejection in a baboon skin allograft model *in vivo*. The immunosuppressive capacities of MSCs have therein provided new therapeutic insights into immune-mediated disease treatments. Centeno *et al*[52] reported that autologous MSCs and physiologic doses of dexamethasone could increase meniscus volume of the human knee. In addition, MSCs can relieve symptoms of multiple sclerosis, multiple system atrophy, and amyotrophic lateral sclerosis in varying degrees[53,54]. How DNA methylation and demethylation function in MSC therapy for immunological diseases necessitates further exploration.

CONCLUSION AND PERSPECTIVE

Although a wealth of research has investigated MSC therapy, including hundreds of

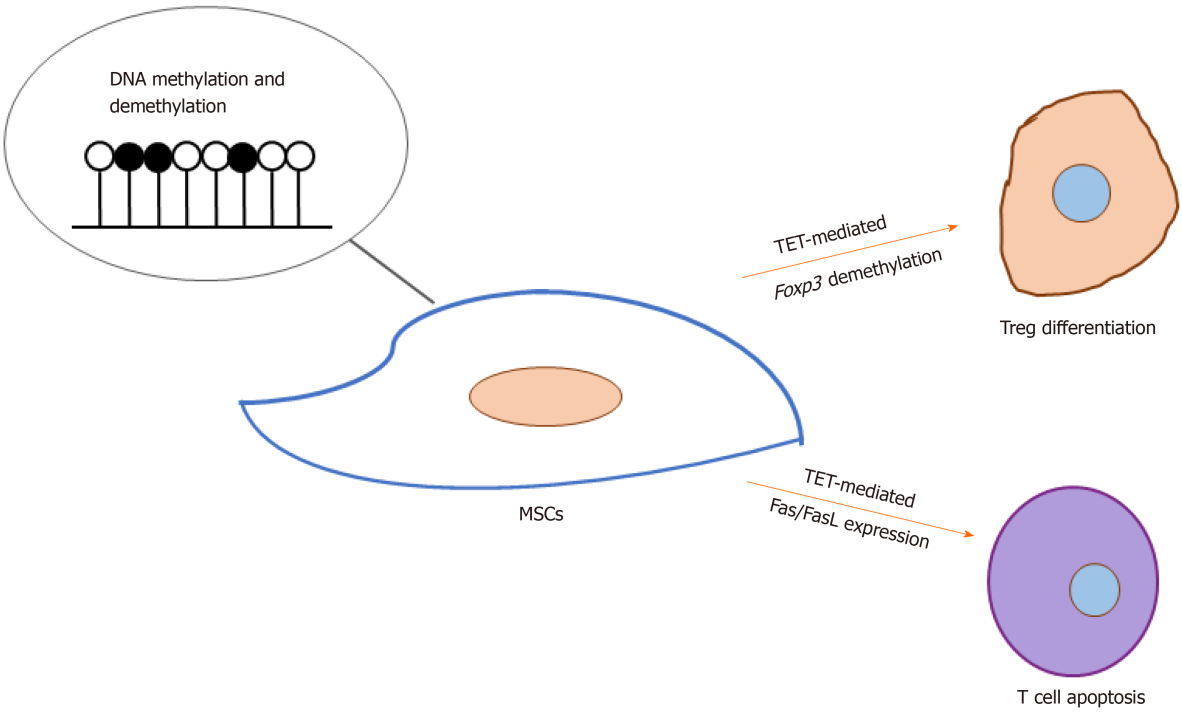

Figure 2 TET-mediated demethylation functions in regulatory T cell differentiation and mesenchymal stem cell-induced T cell apoptosis. MSCs: Mesenchymal stem cells; Treg: Regulatory T cells.

MSC-based clinical trials that have been administered, the mechanisms that underlie the multiple distinct MSC functions remain elusive. This review sheds light on the roles that DNA methylation and demethylation play in regulating MSC-based regeneration and immunomodulation, although it is possible that we overlooked a few studies due to our literature resource limitations. However, the precise function of DNA methylation and demethylation in different MSC types, as well as the associated underlying mechanisms, remain to be thoroughly investigated. This knowledge would inform the development of novel approaches for enhancing MSC-based tissue regenerative and immune therapies.

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ORIGINAL ARTICLE

Basic Study

How old is too old? *In vivo* engraftment of human peripheral blood stem cells cryopreserved for up to 18 years - implications for clinical transplantation and stability programs

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Abstract

BACKGROUND

Peripheral blood stem cells (PBSC) are commonly cryopreserved awaiting clinical use for hematopoietic stem cell transplant. Long term cryopreservation is commonly defined as five years or longer, and limited data exists regarding how long PBSC can be cryopreserved and retain the ability to successfully engraft. Clinical programs, stem cell banks, and regulatory and accrediting agencies interested in product stability would benefit from such data. Thus, we assessed recovery and colony forming ability of PBSC following long-term cryopreservation as well as their ability to engraft in NOD/SCID/IL-2Rγ^{null} (NSG) mice.

AIM

To investigate the in vivo engraftment potential of long-term cryopreserved PBSC units.

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METHODS
PBSC units which were collected and frozen using validated clinical protocols were obtained for research use from the Cellular Therapy Laboratory at Indiana University Health. These units were thawed in the Cellular Therapy Laboratory using clinical standards of practice, and the pre-freeze and post-thaw characteristics of the units were compared. Progenitor function was assessed using standard colony-forming assays. CD34-selected cells were transplanted into immunodeficient mice to assess stem cell function.

RESULTS
Ten PBSC units with mean of 17 years in cryopreservation (range 13.6-18.3 years) demonstrated a mean total cell recovery of 88% ± 12% (range 68%-110%) and post-thaw viability of 69% ± 17% (range 34%-86%). BFU-E growth was shown in 9 of 10 units and CFU-GM growth in 7 of 10 units post-thaw. Immunodeficient mice were transplanted with CD34-selected cells from four randomly chosen PBSC units. All mice demonstrated long-term engraftment at 12 wk with mean 34% ± 24% human CD45+ cells, and differentiation with presence of human CD19+, CD3+ and CD33+ cells. Harvested bone marrow from all mice demonstrated growth of erythroid and myeloid colonies.

CONCLUSION
We demonstrated engraftment of clinically-collected and thawed PBSC following cryopreservation up to 18 years in NSG mice, signifying likely successful clinical transplantation of PBSC following long-term cryopreservation.

Key words: Colony-forming units assay; Cryopreservation; Hematopoietic stem cells; Hematopoietic stem cell transplantation; *In vitro* techniques; Peripheral blood stem cell; Viability; Transplant; Long-term storage

Core tip: Peripheral blood stem cells (PBSC) are commonly cryopreserved awaiting clinical use for hematopoietic stem cell transplant. Long term cryopreservation is commonly defined as five years or longer, and limited data exists regarding how long PBSC can be cryopreserved and retain the ability to successfully engraft. We demonstrated engraftment of clinically-collected and thawed PBSC following cryopreservation up to 18 years in NSG mice, signifying likely successful clinical transplantation of PBSC following long-term cryopreservation.

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INTRODUCTION

Peripheral blood stem cells (PBSC) are the most common source of stem cells for hematopoietic stem cell transplantation (HSCT), being used for about two-thirds of all transplants[1]. PBSC are the standard of care for adult HSCT and are often used for pediatric autologous HSCT as well. PBSC are widely used due to the ease of collection from donors, the high numbers and quality of the hematopoietic stem cells (HSC), flexibility of timing for collection, and faster engraftment time compared to marrow. Another reason for this popularity is the relative ease of storage of PBSC. This allows for many centers to initially harvest enough PBSC for multiple transplants and cryopreserve them for future use in the setting of tandem/multiple transplants, or for use after relapse. The potential for long term storage is especially helpful due to the added difficulty of collecting adequate stem cells in a relapse setting after a first

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transplant[2].

Peripherally collected hematopoietic stem cell (HSC) units are held in cryopreservation around the world waiting to be used for the treatment of malignant and nonmalignant conditions in both children and adults. It is assumed that these cryopreserved cells can be stored for long periods and used with no negative impact on the patient receiving the cells for transplant[3,4]. Clinically, most PBSC units are used within a few months to a year from collection. It is currently not well delineated how long-term cryopreservation (commonly defined as > 60 mo) affects stem cell recovery, viability, and stem cell function during transplantation.

Whereas the ability to cryopreserve HSC for extended periods has been appreciated for over 25 years[5-8], data on PBSC viability after long-term cryopreservation is limited. Although decreased viability by trypan blue dye exclusion, decreased colony forming ability and/or decreased CD34+ cell content have been described for PBSC units cryopreserved for more than 10 years[9,10], other authors have reported that these in vitro parameters largely remain stable for up to 19 years, at least after initial losses due to freezing[11-13]. Furthermore, several studies report successful clinical engraftment of PBSC cryopreserved for at least 2 up to 11 years[4,12,14,15]. However, there is insufficient data about successful engraftment of PBSC units cryopreserved beyond 11 years.

Our aim in this study was to determine if long-term cryopreserved PBSC units could exhibit hematopoietic reconstitution after transplantation into immunodeficient mice. Such functional studies in mice document the engraftment capability of long term cryopreserved PBSC units and permit analysis of progenitor and mature cell subtypes present in engrafted mice. We hypothesized that long-term cryopreservation and thawing performed in a clinical Cellular Therapy Lab using validated protocols, would not negatively impact recovery of PBSC nor the ability for engraftment in NOD/SCID/IL-2Rγ^{null} (NSG) mice. Knowledge of successful engraftment of long term cryopreserved PBSC units is valuable information for cryopreservation facilities, as it demonstrates clinical use of stored PBSC units. Successful long-term cryopreservation of PBSC units also has implications for banking of HSC from other sources, and the banking of other cellular therapy products.

MATERIALS AND METHODS

PBSC collection, cryopreservation, thawing and separation

PBSC units used for this study were collected and cryopreserved for clinical use by the Indiana University Health Cellular Therapy Laboratory following standard operating procedures in place at the time of collection. These PBSC units were scheduled for discard once no further clinical needs were identified (reasons include death of patient or clinical practice no longer utilizing HSCT for the indicated disease). PBSC use for research purposes was included in patient consent obtained at the time of PBSC collection. The Institutional Review Board of the Indiana University School of Medicine approved this study.

Briefly, in a biological safety cabinet, a sample was removed from the apheresis collection bag for required clinical testing (*e.g.*, WBC count, ABO typing, sterility). The product volume was calculated by weighing the bag, and the total WBC/mL was calculated. The minimum freeze volume was next calculated, which is the minimum total volume of cells and freeze solution needed to ensure that the cell concentration was a minimum of 0.50×10^8/mL and a maximum of 5.0×10^8/mL. Plasma depletion was performed if the product volume was significantly greater than the minimum freeze volume. The product was then distributed into the desired number of freezing bags, and cell viability was determined by trypan blue staining prior to the addition of freeze solution. Final freeze volume was calculated by dividing actual volume by 0.8; freeze solution volume was calculated as 20% of the final freeze volume. Freeze solution consisting of a final concentration of donor plasma (if available) or 5% human serum albumin plus 10% DMSO was prepared in a 1:1 ratio, and chilled on ice for at least 15 min before adding to the freezing bags. Freezing mix was slowly added to the cells, and freezing bags were chilled if warming due to DMSO addition occurred. Following removal of samples for preparation of two cryovials for quality assurance and final sterility testing, freezing bags were placed into freezing canisters which were in turn placed into a controlled rate freezer and frozen to -80 °C over approximately 50 min. The final frozen product was transferred to the vapor phase of liquid nitrogen for storage.

Total nucleated cell count, viability and CD34+ cell content were determined at the time of PBSC collection, and these data were available for comparison at the time of thawing. Products were thawed using a standard clinical thaw and wash procedure.

A water bath filled with sterile normal saline was warmed to 39-41 °C. The product bag was removed from the freeze canister and, after identity was confirmed, the bag was submerged into the warmed saline and gently agitated until thawed. After thawing, the bag was transferred to a biological safety cabinet, the port covers removed and swabbed with 70% isopropanol, and the product was washed with a 1:1 ratio of 6% hetastarch and 5% human serum albumin to remove the DMSO prior to beginning experiments. HSC recovery was measured by the following: post-thaw cellular counts and viability as compared to pre-freeze, in vitro colony forming assays, and in vivo transplantation into NSG mice. Cellular measurements included: total nucleated cell counts, CD34+ cell count *via* flow cytometry, and percent viable cells by trypan blue dye exclusion.

In vitro colony assays

Thawed cells were plated for colony forming assays to assess burst-forming units-erythroid and colony forming units-granulocyte/macrophage (BFU-E/CFU-GM) utilizing standardized clinical assays. Normal donor PBSC previously validated in the Cellular Therapy Lab were used as a positive control for the BFU/CFU assay. Test cells were plated at concentrations to achieve approximately 100-150 total CFUs per 1mL dish (to yield high enough colony numbers to get accurate colony counts with minimal colony overlap). Cells used in this assay were free of DMSO. Colonies were scored after 14 d incubation in a humidified chamber.

In vivo NSG mouse assay

Most importantly, stem cell function was evaluated in vivo by transplantation into NSG mice. McDermott *et al*[12] established NSG mice as supporting greater engraftment of human hematopoietic stem cells than all other strains. Four randomly chosen thawed PBSC units, as a representative sample from the 10 PBSC units studied, were transplanted into NSG mice (n = 6-7 recipients for each thawed unit) to investigate engraftment potential. Prior to transplantation into immunodeficient mice, CD34+ cells were selected to deplete T cells and prevent recipient mice from developing graft-vs-host disease during the post-transplant period. CD34+ cells were isolated from the PBSC units by incubation with anti-CD34 antibody directly conjugated to magnetic microbeads (Miltenyi, Bergisch-Gladbach, Germany). Positive CD34+ cell selection was performed using the AutoMACS device (Miltenyi) according to the manufacturer's instructions, and yielded a purity of at least 93% CD34+ cells. NSG mice were conditioned with a single dose of sublethal 300-cGy total-body irradiation using a GammaCell 40 (Nordion International Inc., Ontario Canada). Transplantation was performed as we previously described[15] with 2×10^5 $CD34^+$ cells per mouse in 400 μL of IMDM, 0.1% BSA given by tail vein injection. Controls were age-matched NSG mice that received no irradiation or transplanted human cells. Peripheral blood evaluations at 4 wk represented short-term engraftment, and bone marrow evaluations at 12 wk represented long-term engraftment of human HSC. Evaluations at these time points included determination of the percentage of human CD45+, CD19+, CD3+, and CD33+ cells from the blood and/or bone marrow, and were performed as previously described[16]. All animal experiments were performed under supervision of the In Vivo Therapeutics Core using procedures approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

RESULTS

PBSC units were analyzed pre-freeze (*i.e.*, at the time of initial cryopreservation) and again following validated, standardized clinical thaw procedures (post-thaw). The total nucleated cell (TNC) count and percent viability were measured post-thaw and compared to pre-freeze values. TNC recovery (TNC post-thaw as a percentage of pre-freeze TNC) was calculated. BFU-E and CFU-GM were quantified using standardized colony assays. These data regarding the PBSC units are summarized in Table 1.

The PBSC units tested were cryopreserved for a mean of 17 years (range 13.6-18.3 years). The mean donor age at the time of collection was 47 years-old (range of 24-66 years). Diseases for which PBSC were clinically collected included glioblastoma multiforme, chronic myelogenous leukemia, multiple myeloma, and non-Hodgkin's lymphoma. The mean TNC recovery from the 10 units was 88% ± 12% (range 68%-110%). Variable post-thaw viability was evident with a mean of 69% ± 17% viability (range 34%-86%). Eight of the 10 units had a post-thaw viability of > 50%. Of the ten PBSC units, nine exhibited BFU-E growth and seven showed CFU-GM growth.

Analysis of PBSC post-CD34+ selection and engraftment data in NSG mice were obtained for four random units from patients with four different diagnoses. The four

Table 1 Cryopreserved peripheral blood stem cells unit characteristics

PBSC unit	Unit characteristics			Pre-freeze characteristics		Post-thaw characteristics			Colony forming ability	
	Donor age (yr)	Disease	Cryopreser-vation (yr)	TNC × 10^3/μL	%CD34	TNC × 10^3/μL	%TNC Recovery	%Viability	BFU-E/10^5	CFU-GM/10^6
A	25	GLIO	18.1	110	0.2	121	110	84	2	2
B	58	CML	17.7	120	0.5	111	103	86	6	10
C	46	MM	13.6	120	3.9	116	81	68	++	++
D	24	NHL	14.6	116	3.8	110	68	67	++	++
E	52	MM	17.9	127	0.2	164	90	86	0.3	0
F	66	MM	17	102	0.5	145	95	34	0	0
G	54	MM	18.3	134	0.5	114	79	81	4	6
H	52	MM	17.9	120	0.2	150	90	70	9	6
I	47	MM	17.2	114	1.5	120	80	62	0.3	0
J	47	MM	17.1	114	1.5	124	83	49	++	++

TNC: Total nucleated cells; BFU: Burst forming units; PBSC: Peripheral blood stem cells; CFU: Colony forming units; GLIO: Glioblastoma multiforme; CML: Chronic myelogenous leukemia; MM: Multiple myeloma; NHL: Non-Hodgkin's lymphoma; ++: Overgrowth too numerous to count.

units included the youngest (13.6 years) and second oldest (18.1 years) cryopreserved units. The CD34+ selected cell characteristics and engraftment findings are summarized in Table 2. CD34+ selected cells demonstrated vigorous growth of erythroid and myeloid colonies. Following transplantation, all mice demonstrated short- and long-term engraftment at 4 and 12 wk, respectively. Marrow from transplanted mice harvested at 12 wk post-transplant demonstrated a mean of 34% ± 24% human CD45+ cells, indicating substantial levels of long-term human cell engraftment. PBSC Unit "B" had the highest average human CD45 levels at 63.6%, despite being one of the older cryopreserved units with cryopreservation time of 17.7 years. All mice demonstrated multilineage differentiation on bone marrow at 12 wk with the presence of human CD19+ (B lymphocytes), CD3+ (T lymphocytes) and CD33+ (myeloid) cells by flow cytometry. This transplantation study demonstrates that PBSC can be cryopreserved for up to 18 years (*i.e.*, the age of the oldest cryopreserved unit) while retaining colony forming ability and the capability to engraft into NSG mice.

DISCUSSION

PBSC units are held in cryopreservation around the world awaiting clinical use. The ability to successfully store PBSC units is critical for patients who require multiple transplants or for storing PBSC units for future use if a patient relapses. Whereas frozen cord blood units are entirely infused for a transplant, sufficient PBSC for multiple transplants can be collected from a single donor and used over time. Indeed, tandem (or even triple) autologous transplants are currently used for patients with multiple myeloma, germ cell tumors, neuroblastoma, and pediatric brain tumors, among others. Most of these tandem transplants are performed within a year of PBSC collection; however, under certain circumstances, PBSC will be stored for later transplantation. Many institutions collect sufficient PBSC for at least two transplants for multiple myeloma patients, which may occur years apart. In addition, additional allogeneic PBSC may be collected and stored should the patient relapse and require a second transplant, need a stem cell boost for poor engraftment, or benefit from donor lymphocyte infusions for relapse or declining donor chimerism. Again, these infusions may occur years after the initial PBSC collection. The ability to successfully store and thaw long-term cryopreserved products is particularly important for autologous HSCT patients who may not successfully mobilize PBSC after relapse and salvage therapy and for allogeneic recipients whose donors may no longer be available for subsequent PBSC collections.

Regulatory agencies and accreditation bodies do not currently have specific guidelines or limitations on the duration of storage for cryopreserved cell therapies. The guidelines that are available state that cells used for hematopoietic or immunologic reconstitution must be preserved in a manner that is "appropriate for

Table 2 Post-CD34 selection characteristics and engraftment data

PBSC unit	Cryopreser-vation (yr)	Post-CD34 selection characteristics					Mouse bone marrow engraftment			
		TNC × 10^3/μL	%Viability	%Purity	BFU-E/10^5	CFU-GM/10^6	Mean %CD45	Mean %CD19	Mean %CD3	Mean %CD33
A	18.1	0.3	96	95	19	56	43.4	14.8	1.1	12.6
B	17.7	0.3	94	93	54	85	63.6	20.8	0.4	13.1
C	13.6	2.5	96	98	++	++	10.3	2.8	1	5.6
D	14.6	2.4	95	98	++	++	20.3	6.8	0.5	7.8

TNC: Total nucleated cells; PBSC: Peripheral blood stem cells; BFU: Burst forming units; CFU: Colony forming units; ++: Overgrowth too numerous to count.

long-term storage" and that caution should be used for cell units cryopreserved for longer than 5 years[17,18]. Furthermore, clinical transplantation programs as well as stem cell banking facilities are required to develop stability programs to demonstrate proficiency in processing, freezing, storing and thawing clinically-relevant cellular therapy products. At the present, programs must devise their own protocols for thawing frozen products, in part due to the lack of long-term data.

Several groups have demonstrated that viable CD34+ cells and/or colony-forming cells can be isolated in vitro from PBSC units cryopreserved for up to 19 years[3,9,11,12,19]. However, only a few reports exist demonstrating that long-term (defined in these studies as 2-11 years) cryopreserved PBSC can successfully engraft in vivo[2,4,7,8,14]. Two interesting paired studies in particular are enlightening, in which multiple myeloma patients had sufficient PBSC collected for multiple transplants. Patients had one transplant within months of collection, then had a second transplant years later. In this manner, the quality and function of a single PBSC collection could be compared over time. Pavlu *et al*[4] found no differences in the time to neutrophil and platelet engraftment in 50 myeloma patients who received a second autologous PBSC transplant 2-9 years after the first. Similarly, Liseth *et al*[14] reported a one day delay in both neutrophil and platelet recovery in 17 myeloma patients receiving a second transplant a mean of 3.5 years after the first; this finding was modest but significant in this small patient population. These studies indicate that PBSC can successfully engraft after cryopreservation for up to 11 years. Unfortunately, essentially no data exists for PBSC units stored beyond 11 years.

The paucity of data on long-term cryopreserved PBSC is somewhat surprising, especially since PBSC are by far the most common stem cell source for HSCT[1]. With limited data on long-term cryopreservation of PBSCs, investigations into the properties of human cord blood may be informative. More data on cryopreserved cord blood may exist since public cord blood banking is stringently regulated, in part because, unlike PBSC, cord blood units are typically used years after processing and freezing; thus, stability programs are necessary to monitor the quality of cord blood units between banks over time. Compounding this issue is the fact that, also unlike PBSC, cord blood units are usually processed and cryopreserved at a different institution, often in a different country, than where the transplant occurs. Efforts are underway to create international standards governing the processing, storage, and distribution of human cells and tissues, especially cord blood, harmonizing regulations from government agencies and adopting selected standards from accrediting bodies such as NetCord-FACT (Federation for the Accreditation of Cellular Therapies) and The Joint Accreditation Committee ISCT-Europe & EBMT (JACIE) to develop a set of minimal criteria for cord blood banking[20].

Yamamoto *et al*[21] evaluated 18 cord blood units in storage for over 10 years and found 84% viability, with CD34+ counts and in vitro colony forming unit activity (CFU) similar to controls. In the most extensive non-clinical studies to date, Broxmeyer *et al*[22-24], through a series of publications, evaluated cord blood units in cryopreservation for 9-10 years, 15 years and 21-23.5 years. In the 15-year study, this group found highly efficient cell recovery in 9 cord blood units, with post-thaw CD34+ counts similar to pre-cryopreservation counts. Furthermore, 3 of 4 units tested were successfully transplanted into NSG mice with evidence of engraftment. In the 21-year study, this group again found highly efficient cell recovery in 23 cord blood units with post-thaw CD34+ counts similar to pre-cryopreservation counts, cell recovery with colony formation, as well as successful engraftment into NSG mice with secondary repopulation.

Several groups examined engraftment of long-term cryopreserved cord blood in

patients[25-27]. Parmar *et al*[25] described engraftment of 15 cord blood units cryopreserved for 5-12 years. The only factors identified as significant for recipient survival were myeloablative conditioning and HLA mismatch, not the age of the cord blood unit. Two other groups transplanted 62 cord blood units cryopreserved from 5 to 11 years[27] and 22 cord blood units cryopreserved for 10-13.4 years[26], respectively, and found no difference in engraftment compared to younger units. In sum, these clinical studies indicate that cryopreserved cord blood retains its engraftment potential in vivo for at least 11 years.

Our study evaluated PBSC after an average of 17 years in cryopreservation. We focused upon PBSC units from patients which were collected, frozen and thawed using validated clinical protocols for human transplantation in place at the time of freezing and thawing, respectively; an evaluation of PBSC frozen using experimental procedures, such as uncontrolled freezing methods and/or storing frozen cells in mechanical freezers rather in vapor phase of liquid nitrogen[10,28] is beyond the scope of this study. We assessed the in vitro proliferative abilities of these cells, and, most importantly, assessed the in vivo capacity of these recovered cells to engraft and repopulate the hematopoietic system of sublethally irradiated NSG mice. The post-thaw viability of the 10 older units (mean 69 ± 17% viability, range 34%-86%) was similar to that obtained in the Cellular Therapy Lab for PBSC units frozen for < 7 wk and transplanted in quarter 4 of 2019 (73% ± 6% viability, range: 61%-84%, n = 55). Of the ten different PBSC units, nine exhibited BFU-E growth and seven showed CFU-GM growth. Interestingly, PBSC unit "F" that lacked both BFU-E and CFU-GM growth was the unit with the lowest post thaw percent viability of 34%, but this was not one of the oldest or youngest cryopreserved units. PBSC unit "F" was, however, obtained from the oldest patient in our cohort of samples. Moreover, the two units (units "E" and "I") which had no CFU-GM growth, and the unit which had no CFU-GM or BFU-E growth (unit "F"), were all from multiple myeloma patients. Of note, mobilization and collection of PBSC from patients with multiple myeloma often presents a challenge[29], and thawed PBSC products from myeloma patients may exhibit lower viability and TNC recovery than products from patients with other diagnoses[30]. Furthermore, colony formation is known to be reduced in myeloma patients relative to healthy donors[31]; however, PBSC from myeloma patients can still successfully engraft in immunodeficient mice[31] and patients[32] despite reduced or even absent colony-forming ability. Overall, these data indicate a highly efficient total cell recovery and viability, which is an important factor in determining the likelihood of engraftment.

Most importantly, both short- and long-term engraftment was demonstrated in NSG mice with differentiation into multilineage phenotypes. Although the ultimate success for PBSC following long-term cryopreservation would be long-term engraftment in humans, our data build upon the pre-clinical and clinical experiences with PBSC and cord blood detailed above, and imply that older PBSC units could also successfully be used for clinical applications. Based on these data, our institution increased the time of "safe storage" from 5 years to 12 years without requiring additional testing of the unit for viability (*e.g.*, thawing of a cryovial for testing), and will continue to increase the duration of safe storage based on ongoing stability data with longer cryopreservation times.

ARTICLE HIGHLIGHTS

Research background

Peripheral blood stem cells (PBSC) are commonly cryopreserved awaiting clinical use for hematopoietic stem cell transplant (HSCT). Long term cryopreservation is commonly defined as five years or longer, and limited data exists regarding how long PBSC can be cryopreserved and retain the ability to successfully engraft. Our study examines the engraftment potential of long-term cryopreserved PBSC units. This could allow for PBSC units to be stored for a longer time without repeated viability testing and for these units to be utilized in clinical HSCT.

Research motivation

We investigated the viability and colony-forming unit capacity in vitro, and the in vivo engraftment potential of long-term cryopreserved PBSC units. This was done to gain an understanding of the viability of long-term cryopreserved PBSC units so that these long-term cryopreserved units could be used for clinical HSCT.

Research objectives

Our intention was to investigate if long-term cryopreserved PBSC units, which are being preserved in stem cell banks for many years, can be utilized with successful in vivo engraftment. This will help with gaining insight to the potential use of long-term cryopreserved PBSC units.

Research methods
PBSC units were collected and frozen as per validated clinical protocols. The units were then thawed as per clinical standards of practice. Progenitor function was assessed with standard colony-forming assays. CD34-selected cells were transplanted into NOD/ SCID/IL-2Rγ^{null} (NSG) mice and stem cell function was assessed.

Research results
Ten long-term cryopreserved PBSC units (mean of 17 years) demonstrated appropriate post-thaw viability of which nine had BFU-E growth and seven showed CFU-GM growth. Immunodeficient NSG mice (6-7 recipient mice/PBSC unit) were transplanted with 4 randomly selected PBSC units that were cryopreserved for up to 18 years, and all mice showed short-term and long-term engraftment and reconstitution of human myeloid and lymphoid cells. Moving forward it will be important to analyze the engraftment of long-term cryopreserved PBSC units *in vivo* on a larger scale.

Research conclusions
This study demonstrates the appropriate long term engraftment of clinically collected and thawed PBSC units follow cryopreservation up to 17 years in immunodeficient mice. This is one of few studies that analyzes the *in vivo* engraftment potential of long-term cryopreserved PBSC units. This can allow institutions to safely increase the time of safe storage for PBSC units, without further viability testing of the units. These findings are beneficial for clinical programs, stem cell banks, and regulatory and accrediting agencies interested in product stability.

Research perspectives
In summary, this study demonstrates that long-term cryopreserved PBSC can exhibit short- and long-term engraftment in immunodeficient mice with differentiation into multilineage phenotypes. Future research would be to expand studies to look at *in vivo* engraftment on a larger scale and ultimately to apply this to clinical transplantation in humans.

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ORIGINAL ARTICLE

Basic Study

Safety of menstrual blood-derived stromal cell transplantation in treatment of intrauterine adhesion

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Abstract

BACKGROUND

Intrauterine adhesion (IUA) can cause serious damage to women's reproductive health, yet current treatment methods are difficult to achieve satisfactory results. In our previous studies, we demonstrated that menstrual-derived stromal stem cells (MenSCs), with high proliferative capacity and self-renewal ability, have a powerful therapeutic effect in patients with severe IUA. However, safety assessment of MenSCs transplantation is essential for its further application.

AIM

To evaluate the short-, medium-, and long-term biosafety of MenSCs *via* intrauterine transplantation in a rat model of IUA, with a focus on toxicity and tumorigenicity.

METHODS

MenSCs were injected into the sub-serosal layer of the uterus in an IUA rat model, for 3 d, 3 mo, and 6 mo separately, to monitor the corresponding acute, sub-chronic, and chronic effects. Healthy rats of the same age served as negative controls. Toxicity effects were evaluated by body weight, organ weight, histopathology, hematology, and biochemistry tests. Tumorigenicity of MenSCs was investigated in Balb/c-nu mice *in vivo* and by colony formation assays *in vitro*.

RESULTS

Compared with the same week-old control group, all of the IUA rats receiving MenSC transplantation demonstrated no obvious changes in body weight, main

organ weight, or blood cell composition during the acute, sub-chronic, and chronic observation periods. At the same time, serum biochemical tests showed no adverse effects on metabolism or liver and kidney function. After 4 wk of subcutaneous injection of MenSCs in Balb/c-nu nude mice, no tumor formation or cell metastasis was observed. Moreover, there was no tumor colony formation of MenSCs during soft agar culture *in vitro*.

CONCLUSION

There is no acute, sub-chronic, or chronic poisoning, infection, tumorigenesis, or endometriosis in rats with IUA after MenSC transplantation. The above results suggest that intrauterine transplantation of MenSCs is safe for endometrial treatment.

Key words: Menstrual blood-derived stromal cells; Endometrial treatment; Intrauterine adhesion; Stem cell transplantation; Biosafety; Toxicity

Core tip: Menstrual-derived stromal stem cells (MenSCs) with high proliferative capacity and self-renewal ability have a powerful therapeutic effect in patients with severe intrauterine adhesion. However, safety assessment of MenSC transplantation is essential for its further application. Here, we evaluated the short-, medium-, and long-term biosafety of MenSCs *via* intrauterine transplantation in an intrauterine adhesion rat model, with a special focus on toxicity and tumorigenicity. There was no acute, sub-chronic, or chronic poisoning, infection, tumor, or endometriosis in rats with intrauterine adhesions after MenSC transplantation, highlighting that intrauterine transplantation of MenSCs is safe for endometrial treatment.

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INTRODUCTION

Intrauterine adhesion (IUA) is a traumatic disease mostly associated with intrauterine surgery[1], mainly characterized by endometrial functional disorders, including abnormal menstruation (menstrual reduction or amenorrhea), thin endometrium, pelvic pain, implantation abnormality, infertility, and abortion[2]. With the increasing frequency of intrauterine operation, the incidence of IUA is gradually increasing. Approximately 2.8%-45.5% of secondary infertility cases are related to IUA[3]. However, for patients with severe IUA, conventional treatment methods, such as surgical isolation and hormone supplementation, cannot achieve the desired therapeutic effect. Especially, it is difficult to improve the fertility in patients with IUA.

Recent studies have shown that tissue and organ damage can be repaired effectively by stem cell transplantation. Mesenchymal stem cells (MSCs) are adult stem cells that can be easily collected and cultured from tissues and organs[4]. Due to the advantages of strong proliferative ability, high genetic stability, chemotactic properties, and low immunogenetic effects, MSCs play an important role in the field of regenerative medicine[5-7]. Recently, MSCs have been used in stem cell-based infertility treatment. Bone marrow-derived MSCs (BMSCs), umbilical cord-derived MSCs (UCMSCs), and endometrial-derived MSCs were all proved to be effective in recovering damaged endometrium[8-11].

Menstrual blood-derived stromal cells (MenSCs) are shedding endometrial stem cells that are obtained from menstrual blood, and were first reported by Meng *et al*[12]. These cells exhibit classic MSC characteristics, such as automatic cloning, high proliferation, and pluripotency[13]. Recent studies have shown that MenSCs improved a variety of diseases, including type 1 diabetes[14], liver disease[15,16], premature ovarian failure[17], osteochondral defects[18], heart disease[19], and cartilage damage[20]. It is worth

Institutional Animal Care of Shengjing Hospital Affiliated to China Medical University and were conducted in accordance with the AAALAC and IACUC guidelines.

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noting that MenSCs are easily obtained from abandoned menstrual blood in a non-invasive manner, which can be obtained periodically and autologously transplanted without trauma or ethical risk. Therefore, compared with BMSCs and adipose tissue-derived mesenchymal stem cells (ADSCs), MenSCs have greater clinical application potential on the premise of similar efficacy[21]. Our previous research confirmed that autologous MenSC transplantation can significantly promote endometrial morphology regeneration and functional recovery in seven patients with severe IUA, and achieved four positive pregnancies[22]. After MenSC transplantation, the endometrial pathology and uterine fertility of an IUA rat model were also improved[23]. Therefore, MenSC transplantation is a promising treatment for endometrial injury.

It is undeniable that MenSCs represent a new type of therapeutic stem cells. Many related treatment studies are still in the preclinical or phase 1 clinical trial phase. At present, the effectiveness of MenSCs in treating traumatic diseases has been confirmed. However, long-term observational data of clinical application of MenSCs is scarce, and systematic biosafety evaluation is still lacking[24].

In this study, we aimed to investigate the biosafety of intrauterine transplantation of MenSCs to treat endometrial injury over acute, sub-chronic, and chronic periods. Based on an IUA rat model, the safety of MenSC treatment was systematically evaluated for toxicity, tumorigenicity, and abnormal differentiation. Our results provide a theoretical basis for the clinical application of MenSCs in endometrial injury treatment.

MATERIALS AND METHODS

Culture and identification of MenSCs

MenSCs were cultured and identified as described previously[23]. In brief, sterile techniques were used to collect menstrual blood from three healthy volunteers, aged 25 to 30 years. After mixing with PBS, the samples of menstrual blood were lightly placed on the upper layer of an equal amount of Ficoll. The intermediate cell layer was separated and cultured in DMEM/F12 medium (1:1; HyClone, Logan, UT, United States) containing 10% fetal bovine serum (Gibco, Waltham, MA, United States) at 37 °C in a 5% atmosphere. MenSCs at passage 3 (P3) were collected and MSC surface markers were evaluated by flow cytometry (CD34, CD38, CD44, CD45, CD73, CD90, and CD105) (Supplemental Figures 1). Only well-grown and verified P3 MenSCs were used in this study.

Toxicology study

Establishment and treatment of a rat model of IUA: Forty-five eight-week-old female Sprague-Dawley rats were purchased from HFK Bioscience Co. (Beijing, China) and housed in a Specific-Pathogen-Free (SPF) laboratory (SYXK 2017-0004, China) at a temperature of 22 °C ± 1 °C, a relative humidity of 50% ± 1%, and a light/dark cycle of 12/12 h. Sterilized food and water were available *ad libitum*. All animal studies (including euthanasia procedures) were conducted in accordance with the regulations and guidelines of China Medical University institutional animal care and with the AAALAC and IACUC guidelines. A rat model of IUA was established according to the procedures outlined in our previous study. In brief, 30 female rats in estrus cycle were selected for surgery (n = 10 for each group). After anesthesia with 3% pentobarbital, the uterine horn was surgically exposed. The endometrium was then damaged mechanically using a 16 G syringe. After two estrus cycles, the abdominal wall was reopened and 5×10^5 MenSCs were injected into each uterine serosa. Five rats in each group received a placebo (PBS) and acted as controls.

Sample acquisition: The study involved three experimental groups according to observation time: Acute group (3 d), sub-chronic group (3 mo), and chronic group (6 mo). All rats were weighed prior to sacrifice and 5 mL of blood was collected from the abdominal aorta. Sodium citrate was added to 2 mL of peripheral blood to analyze the blood cell composition. The remaining peripheral blood was quickly centrifuged and the serum was separated for biochemical detection. After removing the surface adipose tissue, the brain, heart, liver, spleen, lungs, kidneys, thymus, adrenal glands, uterus, and ovaries of each rat were weighed and recorded. Subsequently, these organs were fixed in 4% paraformaldehyde, dehydrated, and then embedded in paraffin. Then, we prepared 5 μm serial sections of each tissue for staining.

Blood cell composition test and serum biochemical test: Freshly collected rat peripheral venous blood was immediately tested for blood cell composition (Procyte DX, IDEXX Laboratories, United States). Red blood cells, hematocritt, hemoglobin,

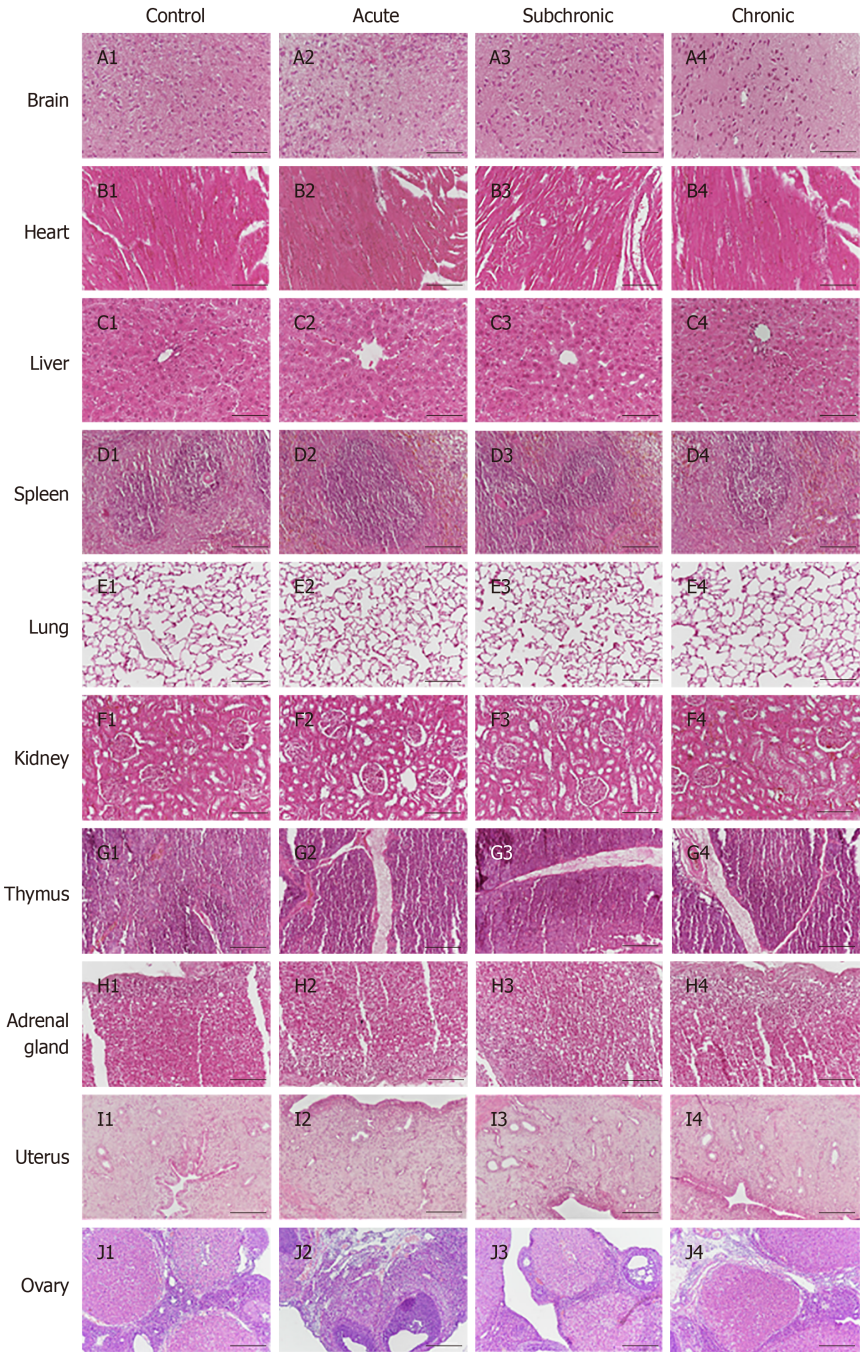

Figure 1 Histopathological analysis of intrauterine adhesion rats after menstrual-derived stromal stem cell treatment. Representative H&E staining of various organs (brain, heart, liver, spleen, lungs, kidneys, thymus, adrenal glands, uterus, and ovaries). No structural changes or injuries were detected in theses organs. Scale bar = 100 μm.

average red blood cell volume, average hemoglobin concentration, red blood cell distribution width, reticulocytes, white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, neutrophils, and platelet related concentrations or percentages were evaluated. Subsequently, blood glucose, urea, creatinine, total protein, albumin, globulin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and sodium, potassium, and chloride ion concentrations in the serum of each group of rats were detected (Catalyst One, IDEXX Laboratories, United States).

Histopathology

Paraffin sections of various organs were subjected to hematoxylin-eosin (HE) staining after dewaxing and dehydration. The tissue morphology of each tissue was then evaluated by light microscopy (NikonECLIPSE N80-i).

Tumorgenicity tests

***In vivo*:** P3 MenSCs were fluorescently labeled by transfection with green fluorescence protein (GFP)-labeled lentivirus (multiplicity of infection = 20). Subcutaneous injection in Balb/c-nu mice was applied to detect tumor-forming properties of MenSCs *in vivo*. Eight-week-old female Balb/c-nu mice weighing 20 g, were purchased from HFK Bioscience Co. (Beijing, China), and housed in a specific-pathogen-free (SPF) laboratory (SYXK 2017-0004, China). We then injected GFP-labeled 10^7 MenSCs into the epidermis on the groin of Balb/c-nu mice. The intensity and range of fluorescence were then evaluated every 7 d by 470-535 nm excitation. Each mouse was placed onto the scanning stage of the *in vivo* MS FX Pro system (Carestream, United States). Bioluminescence imaging was carried to identify the location of GFP-MenSCs. Images were acquired and analyzed with Carestream MI SE software.

***In vitro*:** The tumorigenicity of MenSCs was evaluated by soft-agar colony formation assays *in vitro*. HELA cells were used as a positive control. These cells were first suspended in complete culture medium with 0.35% low melting agarose, then the mixture was transferred onto solidified 0.6% agarose in a 6-well plate. Approximately 2. 5 × 10^3 and 5 × 10^3 cells were uniformly inoculated into the upper layer of each well and cultured in DMEM/F12 medium containing 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO_2. The monoclonal formation of MenSCs and HELA cells was observed under a microscope (Nikon ECLIPSE Hi) to investigate for malignant proliferation for a total period of 2 wk.

Statistical analysis

All data in this study are presented as the mean ± standard deviation (SD), and comparisons between groups were analyzed using one-way analysis of variance (ANOVA). Bonferroni post hoc tests were used to further investigate significant differences. Statistical analyses were carried out with Prism 8 software (GraphPad, San Diego, United States) and $P < 0.05$ was considered to represent a statistically significant difference.

RESULTS

Toxicity

Body weights: Over the entire experimental period, no deaths or adverse response were evident in either the control group or the MenSC transplantation groups. All rats represented with normal behavior without surgical complications. As shown in Table 1, there was no difference between each experimental group and the healthy controls in terms of body weight throughout the entire experimental period (P_{acute} = 0.207, $P_{sub\text{-}chronic}$ = 0.255, and $P_{chronic}$ = 0.696).

Organ weights: Next, we weighed the major organs of all rats. As shown in Table 2, there was no difference between the control groups and the treatment groups in terms of the relative weight of the brain, heart, liver, spleen, lung, kidney, thymus, adrenal glands, uterus, and ovaries, indicating that organ weights were within the normal range. No morphological change or color change was observed in any of the examined organs.

Hematology and biochemistry: Next, blood cell composition and serum biochemical and metabolic parameters were examined in two groups of rats (Tables 3 and 4). The blood cell compositions in the MenSC transplantation groups fluctuated slightly, but were all within the range of normal values. In addition, compared with controls of the same age, there were no changes in terms of serum biochemical or metabolic parameters in the MenSC transplantation groups.

Histopathology

Compared with controls of the same week-old age, there were no changes in the main organ morphology or size in the MenSC transplantation groups. In addition, we carefully examined the abdominal cavity of every rat, and no endometriosis or tumor formation was observed at 3 d, 3 mo or 6 mo after MenSC transplantation.

Next, histopathological examinations of the brain, heart, liver, spleen, lung, kidney thymus, thyroid, adrenal glands, uterus, and ovaries were carried out by HE staining. Representative histological images are shown in Figure 1. Compared with the control group of the same age, there were no obvious structural changes or tumor formation in the MenSC transplantation group.

Tumorigenicity studies

Table 1 Body weight changes of mice in toxicity study

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Body weight (g)	228.0 ± 18.18	228.8 ± 9.175	267.4 ± 26.16	265.8 ± 9.378	326.0 ± 22.75	303.7 ± 35.06

Measurements are given as the mean ± SD. Group 1: Control for acute group; Group 2: Acute group; Group 3: Control for subchronic group; Group 4: Subchronic group; Group 5: Control for chronic group; Group 6: Chronic group (n = 5 for each control group, n = 10 for each experimental group).

***In vivo* assay:** The tumorigenesis of MenSCs was examined *in vivo* (Figure 2) *via* hypodermic injection into Balb/c-nu mice. Four weeks post-injection, there was no tumor formation in the nude mice injected with MenSCs and the fluorescent-labeled MenSCs gradually disappeared over time. No fluorescent signals were detected in other parts of the body. In contrast, Balb/c-nu mice with HELA cell injection showed subcutaneous tumors and had surface gangrene (Supplementary Figure 2).

***In vitro* assay:** The tumorigenesis of MenSCs was also investigated *in vitro* using soft-agar assays. The tumorigenicity was negative for MenSCs *in vitro*, and no cell colonies were formed during 2 wk of culture (Figure 3A). In contrast, as for Hela cells, there were numerous colonies observed in the soft agar (Figure 3B). These tumor colonies grew in stacks and spread outward with strong tumorigenicity.

DISCUSSION

MSCs are the most commonly used stem cells in basic and clinical research. In addition to differentiation potential, MSCs also participate in the regulation of immune balance. Moreover, MSCs have the effect on microenvironment formation that is conducive to tissue regeneration[25]. Recent clinical studies have confirmed that BMSCs, ADMSCs, UCMSCs, and vascular endothelial MSCs provide therapeutic effects with regard to organ function improvement and tissue regeneration, and thus were widely used in clinical treatment research of cardiovascular disease, immune system disease, motor system injury, and digestive system disease. During the follow-up of these clinical studies, there were no tumor or serious complications associated with stem cell transplantation[26-30].

Up to now, only two clinical trials of MenSC transplantation have been reported. In 2009, Zhong's study indicated that transplantation of allogeneic MenSCs can effectively improve multiple sclerosis. During the one-year follow-up, there were no complications related to immune response and cell transplantation observed in all four patients[31]. Similarly, in our previous clinical trial, no transplant-related complication was found in all seven severe IUA patients after receiving autologous MenSC transplantation[22].

Currently, MenSC treatment studies were mostly in the preclinical or phase 1 clinical research stage[24]. Therefore, it is essential to acquire more clinical and basic research data in order to support the further clinical application of MenSCs. To transform MenSCs from an experimental product into a clinical treatment formulation, it is necessary to consider factors directly related to clinical application, such as indications, routes of administration, and dosage. Due to the high proliferative potential and multi-pluripotency, the toxicity and tumorigenicity of stem cells are the main concerns in clinical research[32]. Meanwhile, the biological safety should be assessed in appropriate *in vivo* and *in vitro* models. In this study, we transplanted MenSCs in an IUA rat model using a dose and method consistent with the clinical application, and evaluated the safety of MenSCs for acute, sub-chronic, and chronic observations. To our knowledge, it is the first comprehensive preclinical biosafety study of MenSCs.

In our study, 30 IUA rat models received 10^6 MenSCs *via* intrauterine sub-serosa injection. During the observation periods from 3 d to 6 mo, all these rats maintained normal body weight, without death, abnormal behavior, or transplant-related diseases. At the same time, the weight, shape, and appearance of the main organs remained normal. Moreover, compared with the control group, there were no differences in blood cell composition or ratio after MenSC transplantation. Serum biochemical results showed that the liver and kidney function in these rats was normal. The above results demonstrated that MenSCs were well tolerated, without initiation of abnormal immune response or organ dysfunction.

It is worth noting that the leukocytes and lymphocytes in all experimental groups were within the normal range, indicating that the preparation and transplantation process of MenSCs was sterile. Menstrual blood is usually obtained non-invasively

Table 2 Relative organ weights of rats in toxicity study

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Brain	1.314 ± 0.220	1.439 ± 0.176	1.480 ± 0.211	1.477 ± 0.197	1.441 ± 0.047	1.508 ± 0.176
Heart	0.718 ± 0.048	0.750 ± 0.087	0.744 ± 0.076	0.805 ± 0.064	0.826 ± 0.039	0.868 ± 0.039
Liver	8.383 ± 0.764	8.525 ± 1.012	8.563 ± 0.646	9.205 ± 1.385	9.982 ± 0.282	10.01 ± 0.872
Spleen	0.432 ± 0.101	0.490 ± 0.123	0.515 ± 0.051	0.506 ± 0.067	0.500 ± 0.018	0.501 ± 0.082
Lung	1.225 ± 1.104	1.269 ± 0.100	1.375 ± 0.129	1.374 ± 0.088	1.350 ± 0.084	1.374 ± 0.100
Kidney	0.900 ± 0.161	1.016 ± 0.267	1.028 ± 0.032	1.015 ± 0.181	1.012 ± 0.082	1.009 ± 0.144
Thymus	0.298 ± 0.021	0.335 ± 0.051	0.341 ± 0.072	0.339 ± 0.059	0.352 ± 0.032	0.338 ± 0.052
Adrenal gland	0.027 ± 0.003	0.025 ± 0.005	0.032 ± 0.002	0.029 ± 0.006	0.031 ± 0.002	0.030 ± 0.006
Uterus	0.512 ± 0.025	0.524 ± 0.017	0.511 ± 0.021	0.514 ± 0.020	0.536 ± 0.031	0.546 ± 0.045
Ovary	0.047 ± 0.004	0.049 ± 0.005	0.049 ± 0.004	0.051 ± 0.003	0.054 ± 0.005	0.053 ± 0.004

Measurements are given as the mean ± SD. Group 1: Control for acute group; Group 2: Acute group; Group 3: Control for subchronic group; Group 4: Subchronic group; Group 5: Control for chronic group; Group 6: Chronic group.

through the vagina, which is a unique advantage of MenSCs in regenerative medicine. However, this is also the main source of contamination risks during MenSC preparation. Therefore, the establishment of a quality control system is essential for MenSC clinical application.

Tumorigenicity is one of the most serious risk factors to be considered for the clinical application of MSCs[33], which is strictly related to genomic instability[34]. Only a few studies have investigated malignant lesions at MSC transplant sites, suggesting that the potential risk of tumor formation may still exist[35-38]. In contrast, some other studies have indicated that MSCs were not associated with tumorigenicity after intravenous or intramuscular application[39,40]. To date, only one article has reported spontaneous tumorigenic transformation due to long-term cultivation associated with genomic alterations in culture[41]. It is determined that MenSCs has no karyotype changes in long-term culture *in vitro*[14].

In this study, no tumor formation was observed in IUA rat models after 6 mo of intrauterine sub-serosa injection. After MenSC transplantation, no lump formed on the abdominal wall or organs. The physiological structure of all organs remained normal. Furthermore, we used Balb/c-nu mice to detect the tumorigenicity of MenSCs. The subcutaneous GFP fluorescence range gradually decreased without migration. These GFP-labled MenSCs completely disappeared at the fourth week, indicating that MenSCs are none-tumorigenic *in vivo*. In addition, soft agar assay demonstrated that MenSCs did not form any tumor-like cell population *in vitro*. These results provide good evidence that MenSCs is non-tumorigenic in clinical applications.

In conclusion, our current research confirms that intrauterine transplantation of MenSCs is safe, without toxicities or tumorigenicity. The results indicate that MenSCs are not only safe but also a promise source of cells for treating IUA and other types of endometrial damage. In addition, it is necessary to conduct longer follow-up studies on patients to fully ensure the safety of MenSC application.

Table 3 Selected hematology analyses of rats in toxicity study

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
RBC (× 10^{12}/L)	7.196 ± 0.090	7.225 ± 0.340	7.346 ± 0.418	7.462 ± 0.430	7.276 ± 0.421	7.300 ± 0.347
HCT (%)	39.32 ± 0.740	38.98 ± 1.992	39.08 ± 0.958	38.50 ± 2.253	38.90 ± 2.220	37.05 ± 0.576
MCV (fL)	54.64 ± 0.541	52.28 ± 0.784	53.28 ± 1.767	53.34 ± 0.845	53.00 ± 0.714	53.04 ± 1.681
MCH (pg)	18.66 ± 0.114	18.01 ± 0.470	18.10 ± 0.765	18.39 ± 0.341	17.82 ± 1.043	18.51 ± 0.905
MCHC (g/dL)	34.18 ± 0.523	34.42 ± 0.480	33.96 ± 0.493	34.50 ± 0.245	34.48 ± 0.590	35.12 ± 0.545
RDW (%)	17.42 ± 0.444	17.87 ± 0.912	17.78 ± 0.691	17.29 ± 1.152	17.30 ± 0.570	17.92 ± 0.651
RETIC (K/μL)	2.86 ± 0.434	3.78 ± 0.630	2.78 ± 0.691	2.93 ± 0.706	2.48 ± 0.798	2.81 ± 0.802
RETIC	214.7 ± 35.25	270.6 ± 39.98	202.2 ± 42.64	216.6 ± 49.50	162.3 ± 5.118	178.0 ± 19.04
WBC (×10^{12}/L)	7.644 ± 1.668	6.932 ± 2.50	7.592 ± 2.96	7.504 ± 2.121	6.440 ± 1.248	5.883 ± 2.897
NEU (%)	13.72 ± 4.217	17.50 ± 5.977	12.48 ± 2.104	15.40 ± 5.918	12.10 ± 2.351	14.66 ± 2.831
LYM (%)	75.06 ± 5.697	73.91 ± 6.311	80.36 ± 4.360	79.33 ± 7.194	78.88 ± 3.440	76.26 ± 2.858
MONO (%)	5.180 ± 0.832	6.800 ± 0.902	4.520 ± 1.117	5.540 ± 1.169	4.540 ± 0.963	6.160 ± 1.190
EOS (%)	0.60 ± 0.123	0.80 ± 0.340	0.80 ± 0.200	0.53 ± 0.200	0.62 ± 0.356	0.61 ± 0.166
BASO (%)	0.14 ± 0.114	0.25 ± 0.151	0.20 ± 0.141	0.18 ± 0.140	0.16 ± 0.114	0.19 ± 0.171
NEU (× 10^9/L)	1.024 ± 0.288	1.116 ± 0.495	0.976 ± 0.153	1.011 ± 0.468	1.096 ± 0.163	1.000 ± 0.096
LYM (× 10^9/L)	6.178 ± 1.593	4.849 ± 2.150	6.158 ± 2.684	6.002 ± 2.089	5.672 ± 0.854	5.398 ± 1.097
MONO (× 10^9/L)	0.386 ± 0.042	0.455 ± 0.161	0.426 ± 0.083	0.415 ± 0.120	0.406 ± 0.067	0.346 ± 0.111
EOS (× 10^9/L)	0.046 ± 0.011	0.05 ± 0.017	0.050 ± 0.029	0.037 ± 0.016	0.004 ± 0.012	0.034 ± 0.013
BASO (× 10^9/L)	0.01 ± 0.007	0.014 ± 0.010	0.001 ± 0.007	0.013 ± 0.07	0.016 ± 0.009	0.016 ± 0.012
PLT (K/μL)	1001 ± 193.2	1107 ± 178.6	1032 ± 142.8	974.4 ± 121.9	1169 ± 74.37	1075 ± 45.99
MPV (fL)	8.50 ± 0.100	8.43 ± 0.216	8.42 ± 0.148	8.63 ± 0.095	8.44 ± 0.089	8.47 ± 0.206
PDW (fL)	8.50 ± 0.158	8.62 ± 0.148	8.46 ± 0.422	8.30 ± 0.133	8.42 ± 0.303	8.71 ± 0.778
PCT (%)	1.037 ± 0.038	0.962 ± 0.137	0.870 ± 0.129	0.80 ± 0.151	0.844 ± 0.167	0.88 ± 0.107

Note: Measurements are given as the mean ± SD. RBC: Red blood cells; HCT: Hematocritt; MCV: Average red blood cell volume; MCH: Average hemoglobin concentration; RDW: Red blood cell distribution width; RETIC: Reticulocytes; WBC: White blood cells; NEU: Neutrophils; LYM: Lymphocytes; MONO: Monocytes; EOS: Eosinophils; BASO: Basophils; PLT: Platelet; MPV: Mean platelet volume; PDW: Platelet distribution width.

Table 4 Selected biochemistry analyses of rats in toxicity study

	Group 1	Group 2	Group3	Group 4	Group 5	Group 6
Urea	6.220 ± 0.512	6.260 ± 0.657	5.860 ± 0.493	6.19 ± 0.659	5.840 ± 0.270	6.380 ± 0.570
CREA	27.60 ± 5.128	32.30 ± 6.430	28.40 ± 4.722	26.40 ± 2.011	31.00 ± 6.557	31.10 ± 6.367
BUN/CREA	55.80 ± 11.71	48.90 ± 7.279	55.20 ± 8.899	58 ± 8.679	56.60 ± 5.771	57.80 ± 3.967
TP	60.8 ± 4.087	62.20 ± 5.673	61.80 ± 7.014	57.40 ± 2.914	60.20 ± 3.962	60.30 ± 5.165
ALB	31.80 ± 2.683	32.4 ± 4.060	36.80 ± 3.899	30.2 ± 3.584	30.00 ± 2.739	32.90 ± 3.695
GLOB	29.40 ± 1.817	29.8 ± 2.300	25.90 ± 3.194	27.4 ± 1.350	26.00 ± 3.674	27.80 ± 2.201
ALT	35.00 ± 6.042	34.7 ± 5.539	31.40 ± 8.355	38.30 ± 3.889	34.60 ± 7.162	36.30 ± 6.897
AST	73.40 ± 11.84	66.6 ± 14.010	70.20 ± 7.662	75.2 ± 15.050	64.20 ± 16.68	61.4 ± 13.16
ALKP	108 ± 25.03	121.7 ± 32.840	107.0 ± 37.36	100.3 ± 11.910	108.4 ± 23.39	95.20 ± 11.70
Na	142.6 ± 1.949	141.2 ± 2.348	143.2 ± 1.483	141.7 ± 2.627	142.2 ± 1.643	144.1 ± 2.234
K	5.180 ± 0.148	5.230 ± 0.457	4.820 ± 0.601	5.01 ± 0.213	4.700 ± 0.245	5.96 ± 0.390
CL	103.8 ± 2.387	106.2 ± 3.293	104.2 ± 2.387	106.6 ± 3.084	103.8 ± 2.775	105.4 ± 5.719

Note: Measurements are given as the mean ± SD. CREA: Creatinine; TP: Total protein; ALB: Albumin; GLOB: Globulin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALKP: Alkaline phosphatase; BUN: Blood urea nitrogen.

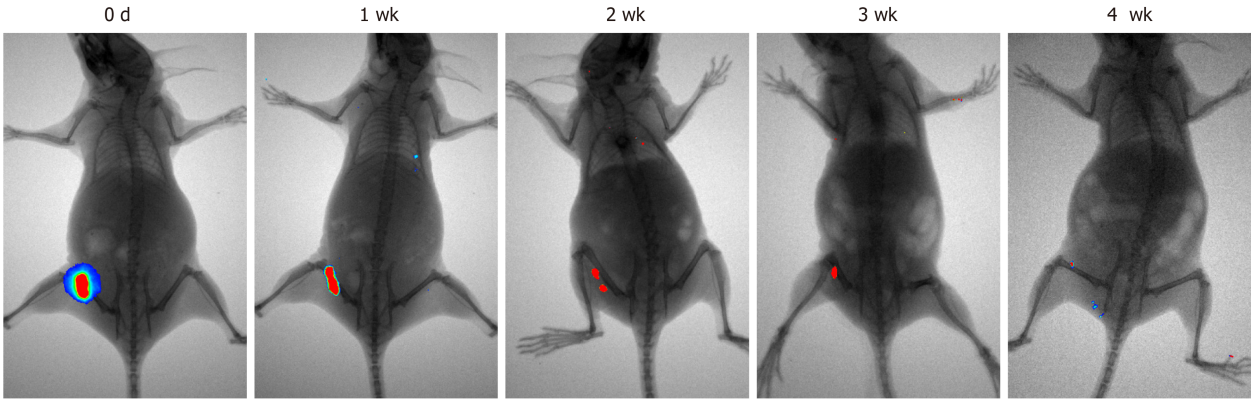

Figure 2 Tumorigenicity analysis of menstrual-derived stromal stem cells on nude mice 4 wk after subcutaneous injection. Representative photographs show the cell proliferation of menstrual-derived stromal stem cells (MenSCs) on nude mice 4 wk after subcutaneous injection. Fluorescent expression, which represented the MenSCs, gradually decreased over time. No metastatic or proliferative fluorescent signals were detected in other parts of the body.

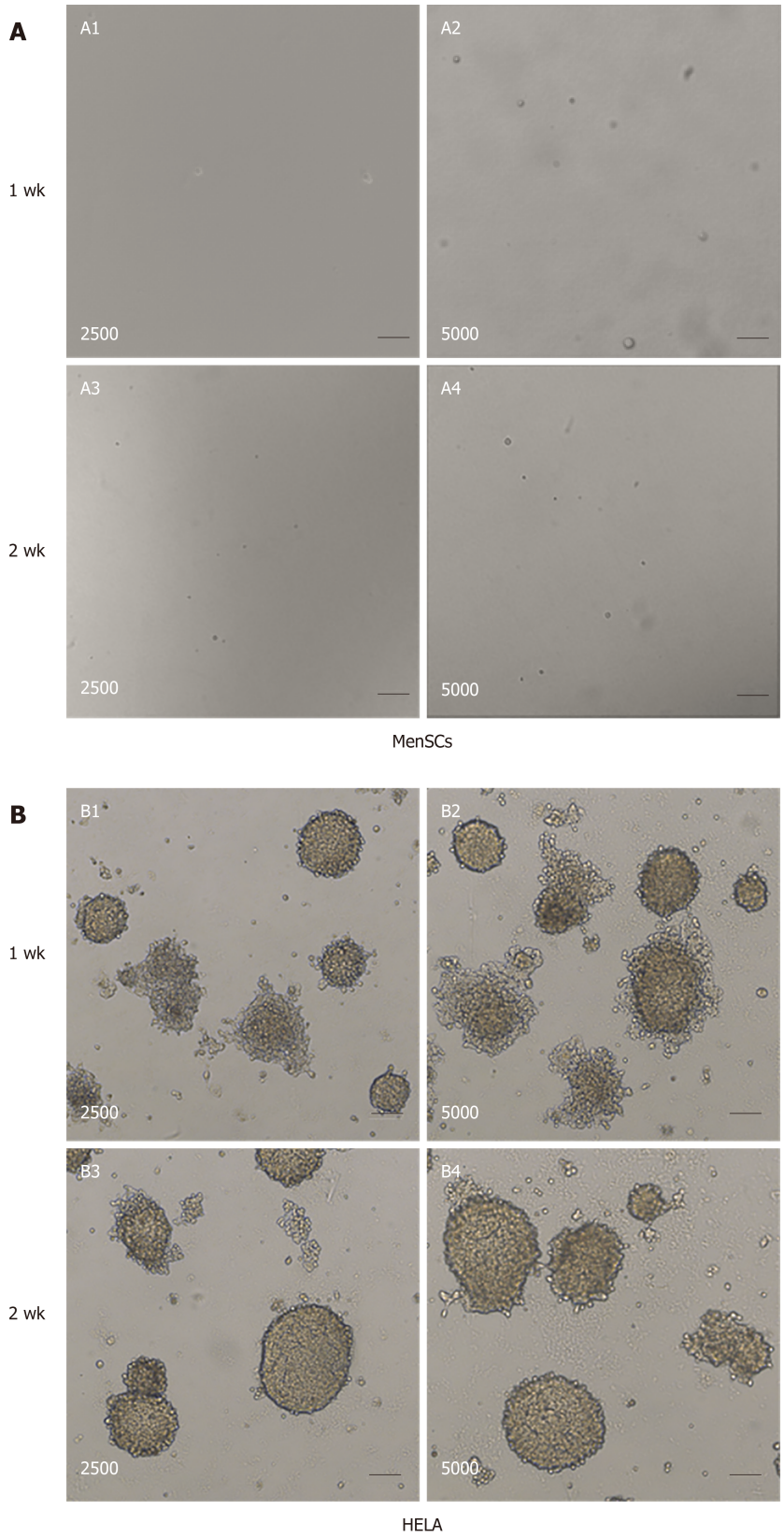

Figure 3 Tumorigenicity analyses of menstrual-derived stromal stem cells and Hela cells in soft-agar colony formation assay. A: Representative photomicrographs show the cell malignant proliferation of 2500 and 5000 menstrual-derived stromal stem cells (MenSCs) after 1 and 2 wk of culturing. No cell colony was formed in the MenSCs group. B: Representative photomicrographs show the cell malignant proliferation of 2500 and 5000 HELA cells after 1 and 2 wk of culturing. Scale bar = 100 μm. MenSCs: Menstrual-derived stromal stem cells.

ARTICLE HIGHLIGHTS

Research background

Intrauterine adhesion (IUA) can cause serious damage to women's reproductive health. In our previous studies, we demonstrated that menstrual-derived stromal stem cells (MenSCs), with high proliferative capacity and self-renewal ability, have a powerful therapeutic effect in patients with severe IUA.

Research motivation

Safety assessment of MenSCs transplantation is essential for its further application in patients with severe IUA.

Research objectives

The purpose of this study was to evaluate the short-, medium-, and long-term biosafety of MenSCs *via* intrauterine transplantation in a rat model of IUA, with a focus on toxicity and tumorigenicity.

Research methods

MenSCs were injected into the sub-serosal layer of the uterus in an IUA rat model, for 3 d, 3 mo, and 6 mo separately, to monitor the corresponding acute, sub-chronic, and chronic effects. Healthy rats of the same age served as negative controls. Toxicity effects were evaluated by body weight, organ weight, histopathology, hematology, and biochemistry tests. Tumorigenicity of MenSCs was investigated in Balb/c-nu mice *in vivo* and by colony formation assays *in vitro*.

Research results

Compared with the same week-old control group, all of the IUA rats receiving MenSC transplantation demonstrated no obvious changes in body weight, main organ weight, or blood cell composition during the acute, sub-chronic, and chronic observation periods. At the same time, serum biochemical tests showed no adverse effects on metabolism or liver and kidney function. After 4 wk of subcutaneous injection of MenSCs in Balb/c-nu nude mice, no tumor formation or cell metastasis was observed. Moreover, there was no tumor colony formation of MenSCs during soft agar culture *in vitro*.

Research conclusions

There was no acute, sub-chronic, or chronic poisoning, infection, tumorigenesis, or endometriosis in rats with intrauterine adhesions after MenSC transplantation. The above results suggested that intrauterine transplantation of MenSCs is safe for endometrial treatment.

Research perspectives

MenSCs are not only safe but also a promise source of cells for treating IUA and other types of endometrial damage. In addition, it is necessary to conduct longer follow-up studies on patients to fully ensure the safety of MenSC application.

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SYSTEMATIC REVIEWS

Stem cell homing, tracking and therapeutic efficiency evaluation for stroke treatment using nanoparticles: A systematic review

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Abstract

BACKGROUND

Stroke is the second leading cause of death worldwide. There is a real need to develop treatment strategies for reducing neurological deficits in stroke survivors, and stem cell (SC) therapeutics appear to be a promising alternative for stroke therapy that can be used in combination with approved thrombolytic or thrombectomy approaches. However, the efficacy of SC therapy depends on the SC homing ability and engraftment into the injury site over a long period of time. Nonetheless, tracking SCs from their niche to the target tissues is a complex process.

AIM

To evaluate SC migration homing, tracking and therapeutic efficacy in the treatment of stroke using nanoparticles

METHODS

A systematic literature search was performed to identify articles published prior to November 2019 that were indexed in PubMed and Scopus. The following inclusion criteria were used: (1) Studies that used *in vivo* models of stroke or ischemic brain lesions; (2) Studies of SCs labeled with some type of contrast agent for cell migration detection; and (3) Studies that involved *in vivo* cellular homing and tracking analysis.

RESULTS

A total of 82 articles were identified by indexing in Scopus and PubMed. After

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the inclusion criteria were applied, 35 studies were selected, and the articles were assessed for eligibility; ultimately, only 25 studies were included. Most of the selected studies used SCs from human and mouse bone marrow labeled with magnetic nanoparticles alone or combined with fluorophore dyes. These cells were administered in the stroke model (to treat middle cerebral artery occlusion in 74% of studies and for photothrombotic induction in 26% of studies). Fifty-three percent of studies used xenogeneic grafts for cell therapy, and the migration homing and tracking evaluation was performed by magnetic resonance imaging as well as other techniques, such as near-infrared fluorescence imaging (12%) or bioluminescence assays (12%).

CONCLUSION
Our systematic review provided an up-to-date evaluation of SC migration homing and the efficacy of cellular therapy for stroke treatment in terms of functional and structural improvements in the late stage.

Key words: Stem cell; Nanoparticles; Homing; Tracking; Near-infrared fluorescence image; Cellular therapy; Magnetic resonance image; Bioluminescence; Stroke

Core tip: The systematic review provided an up-to-date evaluation of stem cell (SC) migration homing, using nanoparticles based on the technical and scientific aspects and combined molecular images. Thus, the efficacy of SC therapy depends on the SC homing ability and engraftment into the injury site over a long period of time, providing functional and structural outcomes in preclinical studies, but limited evidence of outcomes in clinical studies.

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INTRODUCTION

Stroke is the second leading cause of death worldwide. Because of the increase in life expectancy and population growth, the total number of stroke cases was 104.2 million (UI 98.5-110.1) with considerably increased 3.1% worldwide in the last two decades. Furthermore, stroke patients may suffer from disabilities or incapacities requiring temporary or lifelong assistance, resulting in a substantial economic burden for poststroke care[1,2].

Thus, there is a real need to develop alternative treatment strategies for decreasing neurological deficits, and stem cell (SC) therapeutics appear to be an emerging paradigm in stroke therapy that represents a promising alternative for intervention[3,4].

SCs have the remarkable capability to differentiate into any cell of an organism while retaining the ability to self-replicate and keep the characteristics of their parental cells[5]. Preclinical research has already demonstrated the survival, functional integration, and behavioral effects of SC therapy in experimental stroke models[6-10], which provides a wide scientific basis for beginning small clinical trials of SC therapy in stroke patients. However, efforts to test the safety and efficacy of SCs and their derivatives [primarily mesenchymal SCs (MSCs) and mononuclear cells], not just as a stand-alone therapy but preferably in association with approved thrombolytic treatments or thrombectomy, may further increase the likelihood of the successful translation of SC therapy for stroke treatment clinical applications[11-16].

The efficacy of SC therapy depends on the SC homing ability and engraftment into the injury site over a long period of time, and tracking cells from their niche to the target tissues is a complex process[17,18]. The delivery process is affected by both chemical factors (such as chemokines, cytokines, and growth factors) and mechanical factors (for instance hemodynamic forces applied to the vessel walls in the form of

shear stress, vascular cyclic stretching, and extracellular matrix stiffness)[18]. Nevertheless, the monitoring of transplanted SC migration *in vivo* is usually achieved by labeling cells with a contrast agent and then scanning them *in vivo* through using molecular imaging[18].

Among the noninvasive molecular imaging modalities used for cell migration analysis, magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), near-infrared fluorescence (NIRF) imaging, and bioluminescence imaging (BLI) show specific characteristics with strengths and weaknesses of each imaging modalities regarding their technical peculiarities, tracking evaluation, translational stage, suitability to monitor SC transplantation[19-24], as shown in Table 1. MRI has a high spatial resolution between 0.02-0.1 mm and a temporal resolution on the order of minutes to hours. The advantages of MRI include a lack of a tissue penetration limit and the fact that it does not use radiation, but the disadvantages include the relatively low sensitivity, low contrast, high cost and long scanning time. As an alternative to improve sensitivity in the CTM traceability process, magnetic nanoparticles (such as magnetite and maghemite) are used, which exhibit biocompatibility, biodegradability, surface-to-volume ratio, and greater surface area. In addition, when its surface is modified with polymeric stabilizers and inorganic molecules (for example, silica, gold, gadolinium, fluorescent dyes) it not only increases sensitivity but also its specificity[25,26]. PET has a low spatial resolution between 1-2 mm and a temporal resolution on the order of seconds to minutes. The advantages include high sensitivity, excellent penetration depth, capability for whole-body imaging, while the disadvantages include the high cost of the cyclotron that is needed and radiation exposure. The SPECT spatial resolution is similar to that of PET, but the temporal resolution is on the order of minutes; the advantages include a high sensitivity and the lack of a tissue penetrating limit or a need for a cyclotron, and the disadvantages are due to radiation exposure and difficulties in quantifying the results. NIRF imaging and BLI have a low spatial resolution between 2-3 mm and 3-5 mm, respectively. The temporal resolution of both techniques is on the order of seconds to minutes; the advantages of NIRF imaging and BLI include high sensitivity, the lack of radiation exposure, low cost, and the fact that they are activatable. In addition, BLI has the advantages of simple equipment operation and non-damaging imaging; the disadvantages of both optical imaging techniques are the attenuation of sensitivity by overlying tissues and poor penetration depth. In addition, molecular imaging modalities shows a wide potentiality not only for in vitro studies and pre-clinical applications but also in the translation of some techniques in clinical studies, such as nuclear images (PET and SPECT) and MRI[19-24].

However, technological advances have led to the development of hybrid equipment that allows the use of different imaging modalities at the same time as well as the development of multifunctional probes that can be detected by different molecular imaging modalities, thus providing more information and the complementary evaluation of SC migration homing and tracking after implantation[20-22,25,26]. In addition, other techniques, such as BLI, that require the genetic modification of cells to express the signal, such as the luciferase enzyme signal, allow the evaluation of not only migration but also cellular viability after implantation[27-31].

Therefore, through a systematic review, the present study discusses studies of homing SC migration, tracking and therapy efficacy for stroke treatment using nanoparticles based on the technical and scientific aspects of (1) The characteristics of the SCs used in cell therapy; (2) The characteristics of the contrast agents used; (3) The processes of labeling SCs with nanoparticle-based contrast agents; (4) Preclinical models of stroke induction; and (5) Strategies for the administration of nanoparticle-labeled SCs and their use for studies of their subsequent homing, tracking and therapeutic efficacy for future clinical approaches.

MATERIALS AND METHODS

Search strategy

We searched publications published prior to November 2019 indexed in PubMed and Scopus. All procedures were performed according to the PRISMA guidelines[32]. The following selected criteria of interest, boolean operators (DecS/MeSH), and keyword sequences were used: (1) PubMed: (((((((“Cellular Therapy”[Title/Abstract]) OR “Stem cell”[Title/Abstract]) OR “stem cells”[Title/Abstract])) AND ((nanoparticle) OR nanoparticles)) AND (((“cerebral ischemia”[Title/Abstract]) OR “ischemic cerebrovascular accident”[Title/Abstract]) OR stroke[Title/Abstract]))) AND ((Homing) OR tracking); and (2) Scopus: ((TITLE-ABS-KEY (“Stem cell”) OR TITLE-

Table 1 Molecular imaging modalities

Image type	Tech-nique	Physical principle	Tracrer	*In vitro* imaging	Prec-lincal imaging	Clinical Imaging	Spatial reso-lution	Temporal reso-lution	Pene-tration depth	Sensi-tivity	Stren-gths	Limi-tations
Optical imaging	BLI	Visible light	Lumi-nescent proteins	Yes	Yes	No	3-5 mm	Seconds to minutes	1-2 cm	High (+++)	High sensi-tivity, non-radio-active, cell expansion	Low pene-tration depth, non-trans-lational
	FLI	Visible or NIRF light	Proteins or fluo-rescent dyes	Yes	Yes	No	2-3 mm	Seconds to minutes	< 1 cm	High (++)	High sensi-tivity, non-radio-active	Low pene-tration depth, autofluore scence
Nuclear imaging	PET	High-energy γ-rays	Radioisoto pes [89 Zr (78.4 h), 18 F (1.83 h), 11 C (0.34 h), 64 Cu (12.7 h), 68 GA (1.13 h)]	No	Yes	Yes	1-2 mm	Seconds to minutes	Limitless	High (++)	High pene-tration depth, high sensitivity	Radiation exposure, high cost
	SPECT	Low-energy γ-rays	Radioisoto pes [99 mTc (6.03 h), 123 I (13.2 h), 111 In (67.4 h)]	No	Yes	Yes	1-2 mm	Minutes	Limitless	High (++)	High pene-tration depth, high sensitivity	Radiation exposure, high cost
Magnetic imaging	MRI	Radio waves	Contrast agents	No	Yes	Yes	0.02-0.1 mm	Minutes to hours	Limitless	Low	High pene-tration depth, non-radio-active, high spatial resolution	High cost, low sensitivity and contrast

PET: Positron emission tomography; SPECT: Single-photon emission computed tomography; BLI: Bioluminescence; FLI: Fluorescence; MRI: Magnetic resonance imaging; NIRF: Near-infrared fluorescence.

ABS-KEY ("Cellular Therapy"))) AND ((TITLE-ABS-KEY (nanoparticle) OR TITLE-ABS-KEY (nanoparticles))) AND ((TITLE-ABS-KEY ("cerebral ischemia") OR TITLE-ABS-KEY ("ischemic cerebrovascular accident") OR TITLE-ABS-KEY (stroke))) AND ((TITLE-ABS-KEY (homing) OR TITLE-ABS-KEY (tracking))) AND (LIMIT-TO(DOCTYPE, "ar")) and (LIMIT-TO(LANGUAGE, "English")).

Inclusion and exclusion criteria

Only original articles written in the English language were considered for inclusion. The following inclusion criteria were used: (1) Studies that used *in vivo* models of stroke or ischemic brain lesions; (2) Studies that used SCs labeled with some type of contrast agent for cell migration detection; and (3) Studies that involved *in vivo* cellular homing and tracking analysis. Articles that were indexed in more than one database (duplicates), incomplete articles, abstracts, reviews, letters, communications, conference presentations, book chapters, editorials and expert opinions, as well as studies involving *ex vivo* analyses of cellular homing, were excluded.

Data compilation and review

In this review, five of the authors (Nucci MP, Filgueiras IS, Ferreira JM, Oliveira FA, Mamani JB, Rego GNA and Gamarra LF) (in pairs) independently and randomly selected data using the search strategy cited and verified the eligibility of the references. Discrepancies in study selection and data extraction between the two reviewers were discussed with a third reviewer and resolved. The reviewed papers were divided into four categories that addressed the following topics: (1) The characteristics of the nanoparticles used in the experiments and their interactions with cells (Nucci MP, Filgueiras IS, Rego GNA and Mamani JB); (2) The characteristics of

cells (type/source) and route of administration (Nucci MP, Filgueiras IS and Ferreira JM); (3) Stroke models (Nucci MP, Ferreira JM and Oliveira FA); and (4) The imaging techniques used for the evaluation of cell homing and tracking (Nucci MP, Oliveira FA and Gamarra LF).

Data analysis

All results were described and presented using the percentage distribution for all variables analyzed in the tables.

RESULTS

Overview of the reviewed literature

A total of 82 articles were identified by indexing in Scopus and PubMed. After the inclusion criteria were applied, 35 studies were selected, the articles were assessed for eligibility, and only 25 studies were included[28-30,33-54] (Figure 1). Of these, 22 articles (88%) had been published within the past 15 years (2009 to 2019). Most of the studies (76%) were conducted in Asia, mainly in China (48% of all articles), followed by South Korea (20% of all articles), the United States (8%), Canada (4%), and European countries (12%) (Table 2, Figure 1).

SC characteristics

The main characteristics of the SCs used in the studies (cell type, source and culture medium) are shown in Table 2. Regarding the type of SC, eleven[28-30,33,39,44,49-52,54,55] (44%) studies used SCs sourced from humans, nine[34,36-38,40,41,43,46,47,56] (36%) used SCs from rats (SCs from humans and rats were used most often), and only five[35,42,45,48,53] (20%) studies used SCs from mice. In terms of the cell source, ten[36-42,46,48,50] (40%) studies used SCs from bone marrow, four[30,34,45,47] (16%) studies used SCs from neonatal brain, three[28,29,44] (12%) studies used SCs from umbilical cord, the study by Lim *et al*[33] used SCs from adipose tissue, and three[35,45,49] (12%) studies used brain immortal lineage cells. Most of the studies [fifteen of 25 (60%)] used Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum during SC culture prior to cell application; two[29,42] (8%) studies used endothelial cell growth basal medium, the study by Argibay *et al*[38] used Iscove's modified Dulbecco's medium, and the study by Zhang *et al*[57] used StemPro NSCs. The major source of SCs is the bone marrow of rodents (rats and mice), followed by human neonatal brain, which is also widely used.

Contrast agent characteristics used in the SC labeling, homing and tracking analysis

Consecutively, the SCs were submitted to the labeling process with contrast agents for the evaluation of the SC homing and tracking process and the contrast agent physical-chemical properties were described in Table 3. In all studies, magnetic nanoparticles were used as the main contrast agent. Most studies (64%) used synthesized magnetic nanoparticles for the labeling process, and the other 7 (28%) studies used commercial nanoparticles and reported the companies supplying these nanoparticles as Feridex® (or Endorem®) by Advanced Magnetic, United States[49,51,52,54], and Guerbet, France[53]; the study by Janowski *et al*[44] used ferrite by BioPAL Inc., United States, and the study by Tan *et al*[41] used Resovist® by Fujifilm RI Pharma Co., Japan. In terms of the physical-chemical characteristics of the contrast agents, the concentration range was between 0.12 mg/mL[40] and 27.9 mg/mL[41], and the concentration of the contrast agent most commonly used was 11.2 mg/mL[49,51-54]. The nanoparticles had core sizes between 3.7 nm[38] and 30 nm[34,39] and hydrodynamic sizes ranging from 10.8 nm[40] to 900 nm[46]. In regard to the analysis of the process of cell labeling, the majority of studies have used nanoparticles coated with dextran[30,38,39,44,49,51-54]; the studies by Zhang *et al*[45], Wang *et al*[48] and Chen *et al*[28] used silica for coating, the study by Lim *et al*[33] used chitosan, the study by Duan *et al*[37,40] used poly(D, L-lactide), and the study by Tarulli *et al*[46] used divinyl benzene polymer. The zeta potential varied between -38 mV[39] and +32.8 mV[40]; eight studies[29,38,39,43,51-54,57] used nanoparticles with a negative zeta potential, and eight studies[29,33,35-37,40,43,44] used nanoparticles with a positive zeta potential. Of the studies, four[29,42-44] used rhodamine as the conjugated agent, the studies by Bai *et al*[42] and Lim *et al*[33] used Cy5.5, the study by Lu *et al*[35] used Nile red, the study by Zhang *et al*[45] used fluorescein isothiocyanate and the study by Tarulli *et al*[46] used Dragon green fluorophore. In the studies reporting R2 values, the nanoparticles exhibited the characteristics of a negative contrast agent, with R2 values ranging from 75.8 $mmol^{-1}s^{-1}$ (lower contrast power by T2) to 701 $mmol^{-1}s^{-1}$ (high contrast by T2).

The characteristics of the contrast agents allowed the detection of cells during

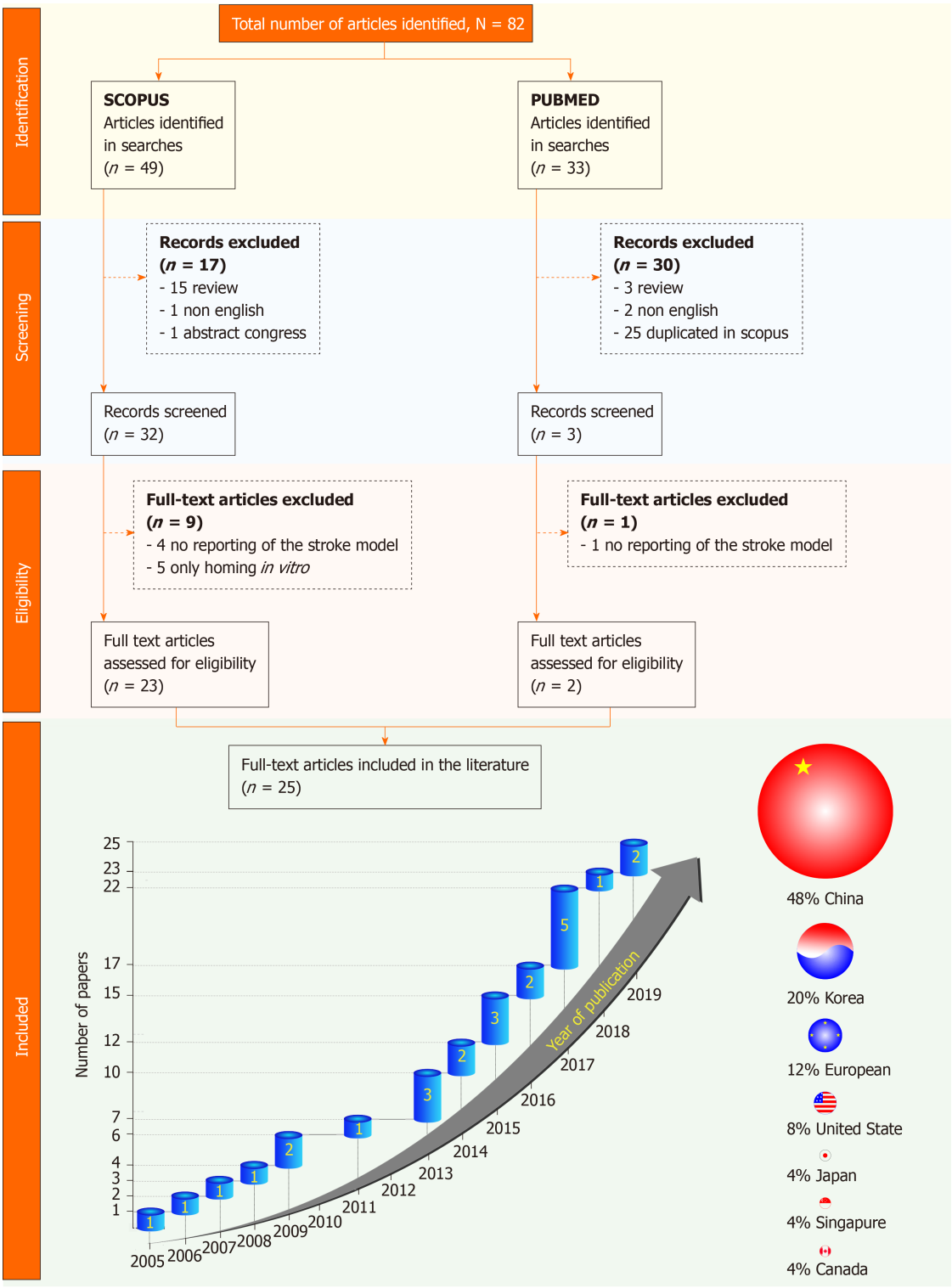

Figure 1 The PRISMA flow diagram provides more detailed information regarding the process of study selection. After the inclusion of studies, the first analysis focused on the publication year distribution; the graphic shows the number of studies per year and the distribution of the studies by the country in which the research was conducted.

homing by MRI in all studies, but 11 of the studies also used another agent contrast conjugated to iron oxide, allowing the bimodal detection of SCs; six (24%) studies[35,43-46,48] used visible field fluorescence, three (12%) studies[28-30] used BLI, three (12%) studies[33,34,42] used NIRF imaging and only one study[28] used photoacoustic imaging. Only one study[42] reported trimodal image detection using MRI, visible field fluorescence and NIRF technical assessments.

SC labeling process with the contrast agent

The cell labeling process is an important step where we have to balance two

Table 2 Characteristics of the studies and the stem cells used

Ref.	Yr	Country	Cell type	Source of cells	Medium culture - %FBS
Lim *et al*[33]	2019	South Korea	MSC	Human (adipose tissue)	DMEM - 10%FBS
Wang *et al*[29]	2019	China	MSC	Human (umbilical cord)	EBM-2 - 0%FBS
Yun *et al*[30]	2018	South Korea	NSC	Human (telencephalon)	NR
Argibay *et al*[38]	2017	Spain	MSC	Rat (bone marrow)	IMDM - 10%FBS
Duan *et al*[37]	2017	China	MSC	Rat (bone marrow)	DMEM - 10%FBS
Lu *et al*[35]	2017	China	NPC-Imm	Mice (C17.2)	DMEM - 10%FBS
Zhang *et al*[34]	2017	China	NSC	Rat (lateral ventricles)	StemPro NSC - 0%FBS
Lin *et al*[36]	2017	China	MSC	Rat (bone marrow)	DMEM -10%FBS
Zhang *et al*[39]	2016	China	NSC	Human (bone marrow)	NR
Duan *et al*[40]	2016	China	MSC	Rat (bone marrow)	DMEM - 10%FBS
Bai *et al*[42]	2015	China	MSC	Mice (bone marrow)	EBM-2
Chen *et al*[28]	2015	China	MSC	Human (umbilical cord)	DMEM-HG
Tan *et al*[41]	2015	Japan	MSC	Rat(bone marrow)	DMEM - 10%FBS,
Janowski *et al*[44]	2014	Poland	NSC	Human (umbilical cord)	DMEM-F12 - 2%FBS
Park *et al*[43]	2014	South Korea	MSC	Rat	DMEM - 0%FBS
Zhang *et al*[45]	2013	China	NPC-Imm	Mice (neonatal cerebellum)	DMEM - 10%FBS
Tarulli *et al*[46]	2013	Canada	MSC	Rat (bone marrow)	αMEM - 20%FBS
Liu *et al*[47]	2013	China	NSC	Rat (neonate)	DMEM-F12
Wang *et al*[48]	2011	China	MSC	Mice (bone marrow)	DMEM
Lee *et al*[50]	2009	Singapore	MSC	Human (fetal bone marrow)	DMEM - 10%FBS
Song *et al*[49]	2009	South Korea	NPC-Imm	Human (HB1.F3)	DMEM - 5%FBS
Kim *et al*[51]	2008	South Korea	MSC	Human	DMEM - 0%FBS
Guzman *et al*[52]	2007	United States	NSC	Human	HNCM
Syková *et al*[53]	2006	Czech Republic	MSC, rOEC	Mice; Human; Rat	NR
Zhu *et al*[54]	2005	United States	NSC	Human	NR

MSC: Mesenchymal stem cells; NSC: Neural stem cells; NPC-Imm: Neural progenitor cell - immortalised; ESC: Embryonic stem cell; rOEC: Rat olfactory ensheathing cells; C17.2: An immortalised mouse neural progenitor cell line; HB1.F3: An immortalized, clonal human NSC line; DMEM: Dulbecco's modified Eagle medium; DMEM-HG: Dulbecco's modified Eagle's medium high glucose; DMEM-F12: 50:50 mixture of DMEM and Ham's F12 medium; αMEM: Minimum essential medium Eagle: Alpha modification; EBM-2: Endothelial cell growth basal medium; IMDM: Iscove's modified Dulbecco's medium; FBS: Fetal bovine serum; StemPro NSC: Human neural stem cell culture medium; HNCN: Human neurosphere culture medium; NR: No reported.

important aspects, high internalization of contrast agents so that it has good detection sensitivity by molecular imaging techniques, but at the same time high cell viability after labeling, so it is necessary, the use of an adequate concentration of contrast agents, for a sufficient incubation time and choice of strategies that increase the internalization efficiency without causing damage to the cell.

By using the SC labeling process with SPION (Table 4), 32% of the selected studies showed that the cells used were from between passage 0 and 17[38], with the majority studies[33,36,40,50] using cells from the fifth passage. As described in the previous paragraph, magnetic nanoparticles were used as contrast agents for all studies, and 5 studies[49,51-54] used Feridex® (or Endorem®), a commercial nanoparticle manufactured by Advanced Magnetic, USA. In most studies[28-30,33-40,42,43,49,50], the iron oxide nanoparticles used were synthetized in-house by the labs. The concentration of contrast agent used during SC labeling ranged between 0.5[45] and 300 μg/mL[33], and the majority of studies (60%) used a concentration between 5 and 33 μg/mL. An incubation time of 24 h for the labeling process was the most frequent (36%) amount of time reported by the studies[38-42,46,47,50,52] and ranged between 0.5[45] and 72 h[49,53]. The main reagent used to induce internalization in 32% of the selected studies was poly L-lysine, which was combined with lipofectamine in the Lu study[35] and with an external magnetic field in the Park *et al*[43]'s study. Other studies[29,37,40] used poly-etherimide and protamine sulfate[51,52], and the Lim *et al*[33]'s study used tetraacetylated N-azidoacetyl-D-mannosamine. In fifteen of the 25 selected studies (60%), the efficiency of cell labeling was greater than 95%[28,29,33,35-38,41,46,48-50,52,54]; five of these studies used the ICP technique to quantify the iron load internalized into the cells[33,38,45,48,50,51], and five other studies[28,34,36,37,40,49] used the AAS technique for quantification, while the Guzman *et al*[52]'s study used semiquantitative analysis by MRI. The range for SPION

Table 3 Characteristics of the contrast agents used in the stem cell labeling, homing and tracking analysis by molecular imaging modalities

Ref.	Contrast agent	Concentration (mg/mL)	Core / Hydrodynamic size (nm)	Coating agent	Zeta Potential (mV)	Conjugated agent (Ex/Em: nm)	Image detection mode	R1 / R2 (mmol^{-1}. Sec^{-1})	Developer
Lim *et al*[33]	NP (BCN-Fe_3O_4)	NR	20/238.9	BCN, chitosan	+12.6	Cy5.5 (675/695)	Dual (Mgt, NIRF)	NR/526.1	Synthesized
Wang *et al*[29]	Alkyl-SPIO	NR	NR/80-120	Alkyl-PEI	Approximately +21.0	NA	Dual (Mgt, BLI)	NR/549.7	Synthesized
Yun *et al*[30]	$Zn_{0.4}Fe_{2.6}O_4$ (ZnMNP)[12]	NR	NR	Dextran	NR	NA	Dual (Mgt, BLI)	NR	Synthesized
Argibay *et al*[38]	Fe_3O_4[1]	NR	3.7/94	Dextran	-11.0	NA	Mono (Mgt)	NR/701	Synthesized
Duan *et al*[37]	Fe_3O_4-LCP	0.12	6/136	PDLLA	+18.0	NA	Mono (Mgt)	NR/500.2	Synthesized
Lu *et al*[35]	PAsp(DMA)-Lys-CA_2 (C-NP)[2]	NR	NR/64.1	NR	+15.32	Nile red (552/636)	Dual (Mgt, VFL)	NR/460.5	Synthesized
	PEG-Lys-CA_2 (N-NP)[2]		NR/69.4		+0.10			NR/462.9	
Zhang *et al*[34]	Ferritin[2]	NA	NA	NA	NA	NA	Dual (Mgt, NIRF)	NR	Synthesized
Lin *et al*[36]	SPION	0.25	NR/128	ASP	+21.6	NA	Mono (Mgt)	NR/296	Synthesized
Zhang *et al*[39]	SPION	NR	30/50	Dextran	NR	NA	Mono (Mgt)	NR/300	Synthesized
Duan *et al*[40]	Fe_3O_4-LCP[2]	0.12	6/136	PDLLA	+18.0	NA	Mono (Mgt)	NR/500.2	Synthesized
	Fe_3O_4[2]	1.00	6/10.8	PLL	+32.8			NR/457.2	
Bai *et al*[42]	bCD-Gd	NR	NA/24.4	NA	NR	Cy5.5 (675/695) Rhod (565/620)	Tri (Mgt, NIRF, VFL)	8.6/NR	Synthesized
Chen *et al*[28]	GRMNB[1]	NR	NA/130	Silica	NR	NA	Dual (Mgt, BLI)	1.21/127.89	Synthesized
Tan *et al*[41]	γ-Fe_2O_3 (ferucarbotran)	27.90	4/60	Carboxydextran	NR	NA	Mono (Mgt)	NR	Resovist®, Fujifilm RI Pharma Co. Ltd., Tokyo, Japan
Janowski *et al*[44]	Fe_3O_4	2.00	8/35	Dextran	+31.0	Rhod (565/620)	Dual (Mgt, VFL)	30.4/75.8	BioPAL Inc, Worcester, MA, USA
Park *et al*[43]	PCION	NR	11/371.6	PEG	+28.6	Rhod (565/620)	Dual (Mgt, VFL)	NR	Synthesized
Zhang *et al*[45]	fmSiO_4@SPIONs	NR	30/151 30/148	Silica	-22.5	FITC (490/525)	Dual (Mgt, VFL)	NR/309.53 NR/231.74	Synthesized
	fdSiO_4@SPIONs				-38.0				
Tarulli *et al*[46]	Fe_3O_4 (MPIO)	NR	NR/900	DBP	< 0	DGF (480/520)	Dual (Mgt, VFL)	NR	NR
Liu *et al*[47]	SPION	NR	NR	NR	NR	NR	Mono (Mgt)	NR	NR
Wang *et al*[48]	Fe_3O_4 (PMNC)	NR	8/120	Silica	-38.0	Rhod (565/620)	Dual (Mgt, VFL)	3.81/435	Synthesized
Lee *et al*[50]	MGIO	NR	5/602	PMG	NR	NA	Mono (Mgt)	NR	Synthesized
Song *et al*[49]	$FeO_{1.44}$ (Feridex)	11.20	5-6/50-180	Dextran	-12.0	NA	Mono (Mgt)	23.9/98.3	Advanced Magnetic, Cambridge, MA, United States
Kim *et al*[51]	$FeO_{1.44}$ (Feridex)	11.2	5-6/50-180	Dextran	-12	NA	Mono (Mgt)	23.9/98.3	Advanced Magnetic, Cambridge, MA, United States

Guzman *et al*[52]	$FeO_{1.44}$ (Feridex)	11.2	5-6/50-180	Dextran	-12	NA	Mono (Mgt)	23.9/98.3	Berlex Laboratories, Wayne, NJ, United States
Syková *et al*[53]	Fe_3O_4 (Endorem)	15.8	4.3-5.6/150	Dextran	-12	NA	Mono (Mgt)	40/160	Guerbet, Roissy, France
Zhu *et al*[54]	$FeO_{1.44}$ (Feridex)	11.2	5-6/50-180	Dextran	-12	NA	Mono (Mgt)	23.9/98.3	Advanced Magnetic, Cambridge, MA, United States

[1]transduced with the luciferase protein (Vector Type - Lentiviral FUGW-Luc2).
[2]transduced with the GFP protein (Vector type - eGPF/FTH). Ex/Em: Excitation/Emission; NP: Nanoparticle; BCN: Bicyclo[6.1.0]nonyne; Fe_3O_4: Iron oxide; SPIO: Superparamagnetic iron oxide; LCP: Loaded cationic polymersomes; ZnMNP: Zinc-doped ferrite magnetic nanoparticle; PAsp(DMA): Poly(aspartic acid-dimethylethanediamine); Lys-CA: Lysine-cholic acid; C-NP: Cationic nanoparticle; PEG: Polyethylene glycol; N-NP: Neutral nanoparticle; SPION: Superparamagnetic iron oxide nanoparticles; bCD-Gd: Bacterial cytosine deaminase-gadolinium; GRMNBs: Gold nanorods crystal-seeded magnetic mesoporous silica nanobeads; PCION: Poly-(ethylene glycol)-coated cross-linked iron oxide nanoparticles; $fmSiO_4$@SPIONs: Fluorescent mesoporous silica-coated SPIONs; $fdSiO_4$@SPIONs: Fluorescent dense silica-coated SPIONs; PMNC: Polystyrene magnetite nanocluster; MGIO: Microgel iron oxide; MPIO: Micron-sized superparamagnetic iron oxide particles; NR: Not reported; NA: Not applicable; Alkyl-PEI: Amphiphilic low molecular weight polyethylenimine; PDLLA: Poly(D,L-lactide); PEI: Polyetherimide; ASP: Spermine-modified amylose; PLL: Poly-L-lysine; DBP: Divinyl benzene polymer; PMG: Precursor microgel; Cy5.5: Cyanine5.5; siRNA: Small interfering RNA; Rhod: Rhodamine B; pDNA: Plasmid DNA; FTH: Ferritin heavy chain; FITC: Fluorescein isothiocyanate; DGF: Dragon green fluorophore; Mgt: Magnetic; NIRF: Near infrared fluorescence; BLI: Bioluminescence imaging; VFL: Visible field fluorescence.

quantification was between 0.2 pgFe/cell[49] and 33.3 pgFe/cell[50], and 40% of the selected studies did not mention this information. In terms of cellular viability analysis after the labeling process, 56% of studies reported this analysis, of which 36% of studies[29,33-37,40,45,48] used the CCK-8 assay to reveal that more than 90% of cells were viable; the other 10% of studies used different techniques for the cellular viability analysis, such as LDH assays[38], MTT assays[28], flow cytometry[46], and cell counting[52], and these studies also reported high cellular viability. Other *in vitro* analyses of the labeling process were used in the selected studies, such as confocal imaging[33,46], MRI[29,33-37,40,43,48,49], BLI[28-30], electron microscopy[29,30,34,36-38,40,43,48,50,53] and microarrays[50].

Stroke model and brain injury evaluation, the target of SC migration

Stroke was studied mainly with two models, which used either an intraluminal filament to occlude the passage of blood flow to the brain or the photothrombosis technique. Brain damage caused by stroke induction attracts SCs to the target region due to chemotactic signals released by compromised tissue. The first model was reported in 68% of the selected studies (Table 5), and stroke was modeled *via* middle cerebral artery occlusion[28,30,34-40,43,45,47-49,51,52], with the exception of the Tan *et al*[41]'s study, which used lacunar infarction. This model was performed in rodents (72% rats), and when rats were used, the majority of studies used Sprague-Dawley males (85%)[30,34-37,40,43,47,49,51,52], followed by Wistar male rats[38,41]. Mice were used in 5 studies: two of the 5 studies[28,39] used C57 black male mice, two studies[45,48] used CD1 female mice, and only the Guzman *et al*[52]'s study used nonobese diabetic/severe combined immunodeficiency male mice. In terms of the weights and ages of the animals used in the studies, the rats used were adults[28,30,34,36,37,40,52] that weighed 250 g in the majority of studies[30,36,37,41,43,49,51] the weights ranged from 240 g[30,41] to 300 g[38,49,51], with the exception of the Liu *et al*[47]'s study, in which the rats weighed between 160 and 180 g. Mice had a weight ranging from 20[39] to 30 g[28]. The total number of animals used in the studies ranged from 6[49] to 133[38]. The type of ischemia used in the stroke models was transient in most studies, with an average of 120 minutes of ischemia time[28,34,36,43,49]; the ischemia time ranged from 10[52] to 180 min[48]. Most studies used inhaled anesthetics, such as sevoflurane[38], halothane[35], and isoflurane[41,49,52], followed by injected anesthetics, such as pentobarbital[34,39] and chloral hydrate[28,47], and agent anesthetics were also used[43,51]. In all animals, a midline neck incision was performed to access the medial cerebral artery, and only two studies[38,51] controlled blood flow during the procedure. Brain injury was detected by MRI in all studies.

The photothrombotic stroke model (Table 6) was performed more often in mice (approximately 67%) than in rats; the mouse strain used in two studies was Balb/c nude (male/female), and the Bai study used a diabetic mouse model and wildtype mice (male). The rat strain used in two studies was Wistar (male/female), and the Tarulli *et al*[46]'s study used Long Evans (male). The animal ages ranged from 8 to 12 wk, and the mouse weight was between 20 and 25 g in two studies. The number of animals used in the selected studies ranged from 8 to 39. This stroke model used Rose Bengal administered at a dosage of 100 mg/kg for intraperitoneal administration and

Table 4 Stem cell labeling process

Ref.	Cells	Passage	Contrast agent	Concentration μg/mL)	Incubation time (h)	Strategy of internalization	Efficiency	Quantification		Cellular viability		Others analysis
								(pgFe/cell)	Technique	Method	Results	
Lim *et al*[33]	MSC	P5-P7	NP(BCN-Fe_3O_4)	300	2	Ac4ManNAz	98.7%	15.3	ICP-MS	CCK-8 assay	> 95%	CF, SEM, CEM, MRI
Wang *et al*[29]	MSC	P2-P7	Alkyl-SPIO	an appropriate amount of Alkyl-PEI/SPIO (N/P = 20)	6	PEI	High Eff.	NA	NA	CCK-8 assay	> 90%	BLI, MRI
Yun *et al*[30]	NSC	NR	ZnMNP	50	NR	PLL: 1.5 g/mL	NR	4.6	NR	NA	NA	TEM, BLI
Argibay *et al*[38]	MSC	P0-P2, P9, P17	Fe_3O_4	100	24	PLL: 1.5 μg/mL	High Eff.	0.9-7.7	ICP-OES	LDH assay	NSD	TEM
Duan *et al*[37]	MSC	P3-P5	Fe_3O_4-LCP	15	1.5	PEI	Approximately 100%	Approximately 9	AAS	CCK-8 assay	> 90%	TEM, MRI
Lu *et al*[35]	NPC	NR	C-NP	10	4	PLL and Lipofectamin	Approximately 99.3%	NA	NA	CCK-8 assay	> 95%	MRI, VFL
			N-NP				Approximately 8.7%					
Zhang *et al*[34]	NSC	P2-P3	Ferritin	MOI: 10	24	PLL	Approximately 63%	3.5	AAS	CCK-8 assay	NSD	TEM, MRI, PB
Lin *et al*[36]	MSC	P5-P9	ASP-SPION	30	1	NA	Approximately 100%	2.68	AAS	CCK-8 assay	> 90%	MRI, TEM
Zhang *et al*[39]	NSC	NR	Anti-CD15-SPION	NR	NR	NA	NR	NA	NA	NA	NA	NA
Duan *et al*[40]	MSC	P3-P5	Fe_3O_4-LCP	15	1.5	PEI	LCP > PLL	8.373	AAS	CCK-8 assay	> 90%	TEM, *in vitro* MRI
			Fe_3O_4	25	24	PLL		9.214				
Bai *et al*[42]	MSC	NR	bCD-Gd	2 μmol	24	PLL	NR	NA	NA	NA	NA	NA
Chen *et al*[28]	MSC	NR	GRMNB	10	2	NR	High Eff.	33.62	AAS	MTT	87.6	BLI
Tan *et al*[41]	MSC	NR	Feru-carbotran	NR	24	NA	Approximately 95%	NA	NA	NA	NA	NA
Janowski *et al*[44]	NSC	NR	Fe_3O_4	25	48	PLL: 375 ng/mL	NR	NA	NA	NI	NI	NA
Park *et al*[43]	MSC	NR	PCION	1	0.25	PLL, EMF	NR	NA	NA	NA	NA	TEM, MRI
Zhang *et al*[45]	NPC	NA	fmNP	5, 10, 20, 33	0.5, 1, 2, 3	NA	fmNP > fdNP	5-30	ICP-AES	CCK-8 assay	90%-98%	TB
			fdNP					1-2.5		NA	NA	NA
Tarulli *et al*[46]	MSC	NR	MPIO	18.8	24	NA	95%	54	Flow cytometry	Flow cytometry	Approximately 94%	CF
Liu *et al*[47]	NSC	NR	SPION	14	24	NA	NR	NR	NR	NI	NI	NA
Wang *et al*[48]	MSC	NR	PMNC	0.5 mmol	1	NA	Approximately 100%	16-20	ICP-OES	CCK-8 assay	> 95%	TEM, CF, MRI
Lee *et al*[50]	MSC	P5, P6	MGIO	50	24	NA	Approximately 97%	33.3	ICP-OES	NI	> 95%	TEM, microarray

Song *et al*[49]	NPC	NR	Feridex	112.4	72	NA	Approximately 100%	0.2	AAS	TB	Unaffected	MRI
Kim *et al*[51]	MSC	NR	Feridex	1	12-16	PS	NR	2.6	ICP/MS	NI	NI	NA
Guzman *et al*[52]	NSC	NR	Feridex	5	24	PS: 2.5 μg/mL	98%	Halved every 3 d (%)	Semiquantitative (MRI)	Cell counting	Approximately 92%	NA
Syková *et al*[53]	MSC, rOEC	NR	Endorem	112.4	48-72	NA	NR	NA	NA	NA	NA	TEM
Zhu *et al*[54]	NSC	NR	Feridex	NR	1	Effectene	High Eff.	NA	NA	NR	NI	NA

MSC: Mesenchymal stem cells; NSC: Neural stem cells; ESC: Embryonic stem cell; rOEC: Rat olfactory ensheathing cells; P: Passage; NR: No reported; NA: Not applicable; NP: Nanoparticle; BCN: Bicyclo[6.1.0]nonyne; Fe_3O_4: Magnetite; SPIO: Superparamagnetic iron oxide; ZnMNPs: Zinc-doped ferrite magnetic nanoparticles; LCP: Loaded cationic polymersomes; C-NP: Cationic nanoparticle; N-NP: Neutral nanoparticle; ASP: Spermine-modified amylose; SPION: Superparamagnetic iron oxide nanoparticle; bCD-Gd: Bacterial cytosine deaminase-gadolinium; GRMNB: Gold nanorods crystal-seeded magnetic mesoporous silica nanobeads; MOI: Multiplicities of infection; PCION: Poly-(ethylene glycol)-coated cross-linked iron oxide nanoparticles; fmNP: FmSiO4@SPIONs; fdNP: FdSiO4@SPIONs; MPIO: Micron-sized superparamagnetic iron oxide particles; PMNC: Polystyrene magnetite nanocluster; MGIO: Microgel iron oxide; Alkyl-PEI: Amphiphilic low molecular weight polyethylenimine; MOI: Multiplicities of infection; Ac4ManNAz: Tetraacetylated N-azidoacetyl-D-mannosamine; PEI: Polyethylenimine; PLL: Poly-L-Lysine; EMF: External magnetic field; PS: Protamine sulfate; High Eff.: Hight efficiency; LCP: Loaded cationic polymersomes; AAS: Atomic absorption spectrophotometer; CCK-8: Cell counting kit-8; LDH: Lactate dehydrogenase; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; TB: Turnbull blue; NSD: No significant differences; CF: Confocal fluorescence; SEM: Scanning electron microscope; CEM: Cryoelectron microscope; MRI: Magnetic resonance image; BLI: Bioluminescence image; TEM: Transmission electron microscopy; VFL: Visible field fluorescence; PB: Prussian blue.

at a lower dosage intravenously. The photosensitizer most commonly used was Rose Bengal, which was administered at a dosage of 100 mg/kg intraperitoneally, but the Lee *et al*[50]'s study used 7.5 mg/kg administered by the tail vein; the Lim *et al*[33]'s study used 10 mg/kg given by the penile vein, and most studies[29,33,42] performed 15 min of laser application after the administration of the photosensitizer. The Lee *et al*[50]'s study performed 10 min of laser application, and the laser parameters used in the Lee *et al*[50]'s study were 60 W (power), 603 nm (wavelength) and 3 mm (diameter). The selected studies do not have a similar laser incidence (brain induction) area, and most studies[29,33,42] used the left temporal region (+2.0 ML to Bregma point). All the selected studies used MRI for injury (ischemia) evaluation, 2 studies[33,42] used NIRF, and the other 2 studies[33,50] used histological analysis with triphenyltetrazolium chloride.

Two studies of clinical evaluation were included in the systematic review; one involved case reports of global cerebral ischemia in children at 18 mo, in which the injury evaluation was performed by MRI[44], while the other involved approximately 16 cases of open brain trauma caused by a mixture of focal and global ischemic processes, which were evaluated by comparing the cellular therapy effect *vs* that of the control group using fMRI and PET[54].

Imaging techniques used to detect SC migration

The main imaging technique used by all the selected studies for the tracking and homing analysis of SCs labeled with SPIONs was magnetic resonance (Table 7). The maximum time of the homing evaluation used by the selected studies was 160 d or 4 mo (Janowski *et al*[44]'s study). All selected studies used acute tracking analysis (first 48 h after cell implantation); 3 studies[35,38,43] analyzed immediate homing (less than 24 h), while the other 22 of the 25 (88%) selected studies used a homing evaluation time between 3 and 7 d. Thirteen studies[30,34,36,37,40,41,44,47-49,51-53] used a maximum time of 14 d. Ten of 25 (40%) studies[28-30,33,38,39,41,42,52,56,58] used a MR preclinical equipment system, and of these, seven of 10 studies[28,29,38,39,42,51,56] used MR equipment obtained from the Bruker Company. Regarding the MR clinical equipment used by 60% of all the selected studies, this equipment was most often obtained from General Electric (50%) and the Phillips Medical System (45%); four studies[35,37,43,47] used an animal coil, and three studies[44,45,50] used a human coil. Most studies used ImageJ with MRI software. The largest magnetic field used by the selected studies was 9.4 T[33,38]; the magnetic field ranged between 1.5[44,45,50] to 9.4 T[33,38], and most studies used 3.0 T[30,34-37,39,40,43,46,47]. The main weighted image type used by the selected studies was T2, and only the Bai *et al*[42] and Kim *et al*[58] studies also used T1 images. The most used sequence (mode) was Fast Spin Echo - FSE[30,35,37,39,40,42,45,46,48], the other MRI parameters are given in Table 7.

The NIRF imaging technique was also used by three of the selected studies[33,34,42] for the tracking and homing analysis of SCs (Table 8); these studies analyzed immediate (less than 24 h) and acute homing (first 48 h), and the maximal time of the homing evaluation used by the selected FT studies was 42 d or 6 wk (Zhang *et al*[34]'s study); 2

Table 5 Stroke models induced by filament intraluminal middle cerebral artery, brain injury evaluation and animal features

Ref.	Animals						n / N	Ischemia type	Ischemia time (min)	Filament type	Anesthesia	Brain induction area (AP; ML to bregma in mm)	Blood flow analysis	Injury evaluation
	Ischemia mechanism	Specie	Type	Sex	Weight (g)	Age (wk)								
Yun *et al*[30]	MCAo	Rat	SD	M	240-260	Adult[1]	3-8/50	T	30	3-0 nylon suture	NR	MNI	NI	TTC, MRI
Argibay *et al*[38]	MCAo	Rat	Wistar	M	280-300	NR	6/133	T	45	silicon rubber-	3%-4% sevoflurane	MNI	Laser-Doppler	MRI
Duan *et al*[37]	MCAo	Rat	SD	M	250-280	Adult[1]	6/54	NR	NR	NR	NR	MNI	NI	MRI
Lu *et al*[35]	MCAo	Rat	SD	NR	NR	NR	6/12	T	90	4-0 nylon suture, silicone coated tip	1% halothane	MNI	NI	MRI
Zhang *et al*[34]	MCAo	Rat	SD	NR	250-280	Adult[1]	NR/30	T	120	NR	PB (40 mg/kg)	MNI	NI	MRI
Lin *et al*[36]	MCAo	Rat	SD	M	250-280	Adult[1]	6/18	T	120	NR	NR	MNI	NI	MRI
Zhang *et al*[39]	MCAo	Mice	C57BL / 6J	NR	20-25	8	NR/45	T	20	Nylon poly-1-lysineco ated	PB (6 mL/kg)	MNI	NI	MRI
Duan *et al*[40]	MCAo	Rat	SD	M	NR	Adult[1]	NR/24	NR	NR	NR	NR	MNI	NI	MRI
Chen *et al*[28]	MCAo	Mice	C57BL / 6J	M	25-30	Adult[1]	NR/NR	T	120	square knot using a 10 suture	CH (0.4 g/kg)	zygoma /squamosal bone	NI	MRI
Tan *et al*[41]	Lacunar infarction	Rat	Wistar	M	240-260	NR	NR/22	P	NA	NA	2%-4% ISO	0; 3	NI	MRI
Zhang *et al*[45]	MCAo	Mice	CD1	F	NR	4	NR/NR	P	NA	6-0 rounded tip nylon	NR	MNI	NI	MRI
Park *et al*[43]	MCAo	Rat	SD	NR	250-280	NR	8/16	T	120	Micro clip 24 mm	Rompum (10 mg/kg) + Zoletil (30 mg/kg)	MNI	NI	MRI
Liu *et al*[47]	MCAo	Rat	SD	M	160-180	NR	6-8/48	NR	NR	Nylon	10% CH (300 mg/kg)	MNI	NI	MRI
Wang *et al*[48]	MCAo	Mice	CD1	F	NR	4	7/21	T	180	6-0 rounded tip nylon	NR	NI	NI	MRI
Song *et al*[49]	MCAo	Rat	SD	M	250-300	NR	3/6	T	120	NR	4% ISO	MNI	NI	MRI
Kim *et al*[51]	MCAo	Rat	SD	M	250-300	NR	2-6/13	P	NA	NR	ket. (80-100 mg/kg) + AM (5 mg/kg)	MNI	EEG	MRI
Guzman *et al*[52]	MCAo	Rat	SD	M	NR	Adult[1]	5/10	P	NA	NA	ISO	MNI + rhinal fissure	NI	MRI

	Global	Mice	NOD-SCID	NR	NR	0-1PN	12-16/28	T	5-10	NA	Cryoane-strhe-tized	NA	NI	MRI

[1]Adult: Rat with 8-16 wk and mice with 6 to 20 wk. MCAo: Middle cerebral artery occlusion; SD: Sprague-Dawley; CD1: An outbred mice derived from a group of outbred Swiss mice; NOD/SCID: Nonobese diabetic/severe combined immunodeficiency; M: Male; F: Female; NR: No reported; n/N: Number of animals per group/total number of animals; T: Transient; P: Permanent; PN: Postnatal; NA: Not applicable; Ket: Ketamine; Xyl.: Xylamine; ISO: Isoflurane; AM: Aceprozazine maleate; CH: Chloral hydrate; PB: Pentobarbital; MNI: Midline neck incision; EEG: Electroencephalogram; TTC: Triphenyltetrazolium chloride; MRI: Magnetic resonance imaging.

studies[33,34,42] used Cy5.5 as the fluorescence agent, and the other parameters are given in Table 8. The BLI technique was used by 3 of the selected studies[28-30] for the tracking and homing analysis of SCs (Table 9), and all studies analyzed immediate (less than 24 h) and acute homing (first 48 h). The maximal time of the homing evaluation used by the selected BLI studies was 21 d or 3 wk (Yun *et al*[30]'s study). All studies used luciferase with eGFP as a lentiviral vector and D-luciferin as a fluorescence agent. The dose, time of acquisition and other parameters are given in Table 9.

SC administration strategies after stroke induction, their migration analysis, and the therapeutic effect

After the brain injury induction, SCs are administered by different routes, systemic or local, with their particularities as to the time after stroke induction, number and volume of cells administered. The parameters adopted in the administration of the cells can interfere with the successful migration and the therapeutic effect. The main characteristics of SCs and SC tracking, homing and therapeutic efficacy in the selected studies are described in Table 10. Fifteen (60%) studies[28,29,33,34,36-38,40-43,46,48,50,51] used mesenchymal SCs as the cell type, and the main source was human bone marrow (Table 2) *via* a xenogeneic graft in 53% of the studies[28,29,33,42,43,46,48,50], *via* an allogeneic graft in 33% of the studies and *via* an autologous graft in one study[51]; of the 40% studies that used neural SCs, 60% used a xenogeneic graft, 30% used an allogeneic graft and 10% used an autologous graft. Only the Sykova *et al*[55]'s study used both xenogeneic and allogeneic grafting. The time of SC implantation after stroke was commonly reported by the selected studies[29,30,37,39,42,49]; the time of implantation after the acute stage of stroke (24 h) ranged from 30 min[28] to 14 d[43]. Regarding cell administration, the main route used by the selected studies was intracerebral (64%), in which 13 (81%) studies administered the cells in the contralateral side of the stroke injury (IC-CTL), one (6%) study administered the cells in the ipsilateral side of the injury, and one study (6%) did not report the specific area of the brain in which the cells were implanted; *via* this route, the maximum volume of implanted cells was 10 μL, which commonly contained 5×10^5 cells. Another cell administration route reported in six (24%) studies[28,38,45,47,49,53] was the intravenous route (tail and jugular), and the intraarterial (intracarotid) route was used in three (12%) studies[30,38,42]; the intracardial route was used by the Wang *et al*[29]'s study. These systemic routes allowed the administration of a greater volume, ranging from 100 to 700μL, with a similar quantity of cells (approximately 5×10^5 cells). The range in the number of cells used in the selected studies was between 2.0×10^4 (Janowski *et al*[44], 2014; Lee *et al*[50], 2009) and 4.0×10^6 (Song *et al*[49], 2009); most studies[28,29,35-37,40,41,45] used 5.0×10^5, since the most commonly used SC implantation volume used by the selected studies[36,43,45,47-50] was 5 μL, which ranged between 2[58] and 700 μL[46]. All of the selected studies observed the positive presence of SCs labeled with SPION in the ischemic area. After the homing analysis, these cells were monitored for 21 d by different imaging techniques. The outcome of cellular therapy was analyzed by different approaches, including functional behavioral assessment, structural morphometric analysis of the decrease in the ischemic lesion volume and the evaluation of cellular differentiation using various types of immunohistochemical analysis. To assess the functional outcome of cellular therapy, 8 studies reported behavioral assessment by different tools, for which 6 studies showed positive improvement in the functional analysis mainly after 14 d of cell implantation (ranging from 7 to 21 d). The structural outcome of the infarct volume was reported in 14 studies, in which 11 showed effective improvements as a decrease in the infarct volume in the late stage (14 d after cells implantation). Cellular differentiation was analyzed by measuring different molecular proteins such as Ki67, NeuN, GFAP, TuJ1, MAP2, BrdU, Nestin, TUNNEL, CD31, CD11, CD15, GFP, and MAPK, as well as by using reverse transcription polymerase chain reaction and tyrosine hydroxylase assays, which reveal positive markers of cellular differentiation mainly 7 d after cell implantation.

The systematic review outcomes are schematically illustrated in Figure 2, which shows each aspect analyzed for the SC homing, tracking and therapeutic efficacy

Table 6 Stroke models induced by the photothrombosis of middle cerebral artery, brain injury evaluation and animal features

Ref.	Animals						Photo-sensitizer - rose bengal (dose; *via*)	Laser application parameters				Anes-thesia	Brain induc-tion area (AP; ML to Bre-gma in mm)	Injury evalu-ation
	Ische-mia mecha-nism	Specie	Type	Sex	Weight (g)	Age (wk)	*n* / *N*		Time (min)	Dia-meter (mm)	Wave-lengh (nm)	Power (W)			
Lim *et al*[33]	PT	Mice	Balb / c nude	M	20-25	10	3-5/19	10 mg / mL; penile vein	16	NR	561	NR	Zoletil (50-30 mg/kg i.p.)	0.5; 2.5	MRI, NIRF, TTC
Wang *et al*[29]	PT	Mice	Balb / c nude	F	20-23	8	4-6/39	100 mg/kg	15	4	NR	NR	PB (50 mg/kg i.p)	-2.0; 2.0	MRI
Bai *et al*[42]	PT	Mice	Db/Db	M	NR	8	4/8	100 mg/kg; i.p.	15	NR	NR	NR	1% ISO	0.0; 2.0	MRI, NIRF
		Mice	Wild type	M	NR	8	10/20	100 mg/kg; i.p.	15	NR	NR	NR	1% ISO	0.0; 2.0	MRI, NIRF
Tarulli *et al*[46]	Focal devascu la-rization	Rat	Long Evans	M	NR	8-12	3/9	NA	NA	NA	NA	NA	ISO + Ketop-rofen	3.0/-4.0; 1.5/4.5	MRI
Lee *et al*[50]	PT	Rat	Wistar	F	NR	NR	NR/22	7.5 mg / mL; tail vein	10	3	603	60	Ket. (7.5 mg/100 g) + Xyl. (1 mg/100 g)	-2.0; -3.0	MRI, TTC
Syko-vá *et al*[53]	Photoch emical	Rat	Wistar	M	NR	8-12	NR/NR	NR	NA	NA	NA	NA	NR	NI	MRI

Blood flow analysis was not reported in any of the selected studies that used stroke models induced by photothrombosis; due to the model induction, all studies showed permanent ischemia after occlusion induction in the specific brain region. *n/N*: Number of animals per group/total number of animals; W: Watts; AP: Anterior-posterior; ML: Medial-lateral; PT: Photothrombosis; Db/Db: Diabetic mice model; M: Male; F: Female; NR: No reported; i.p.: Intraperitoneal; NA: Not applicable; ISO: Isoflurane; PB: Pentobarbital; Ket: Ketamine; Xyl.: Xylamine; MRI: Magnetic resonance imaging; NIRF: Near-infrared fluorescence; TTC: Triphenyltetrazolium chloride.

evaluation for stroke treatment using nanoparticles.

DISCUSSION

The current systematic review examined preclinical studies of the homing and tracking of MSCs with SPION used for the treatment of ischemic stroke and found that this cellular therapy improves outcomes overall. The effects were robust regardless of the species, delivery route, time of administration in relation to stroke, MSC immunogenicity, and MSC dose. These results support further translational studies of MSCs in the treatment of ischemic stroke in humans.

The results described above corroborate the recent systematic review of Boncoraglio[59], which reported the exponential growth of the use of this therapeutic method in Eastern countries, mainly in China (Figure 1), by utilizing human cells extracted from bone marrow. It was observed that 15 studies (60%) used mesenchymal cells and 10 (40%) used neural cells, this characteristic or cellular pattern, evidenced by the studies selected in this review, corroborates the current literature and the review[59] cited. The MSC have strong immunomodulatory potential into ischemic or damage area[60], mainly autologous and allogeneic source. The most selected studies used bone marrow as source of SCs, but the human (40%), the review cited[59], showed in these studies, stronger functional effects in the meta-analysis, the most studies of this study used too human SCs of bone marrow.

The selected studies have demonstrated the presence of SCs labeled with

Table 7 Magnetic resonance imaging features for stem cell homing evaluation

Ref.	Equipment system	Analysis software	MF (Tesla)	Sequence	Weighted images (TR/TE; ms)	FOV; MT; ST (mm)	Homing evaluation time
Lim *et al*[33]	PC - Agilent Technologies	ImageJ (NIH)	9.4	T2	T2: 4000/32.5	NA; NA; 1.0	1, 3, 7, 10, 14 d
Wang *et al*[29]	PC - PharmaScan - Bruker	ImageJ (NIH)	7.0	TSE FLASH GRE	T2: 3000/NA	20 × 20; 256 × 256; 1.0	1, 3, 7 d
		ParaVision (Bruker)			T2*: 159.4/5	55 × 55; 256 × 256; 1.0	
Yun *et al*[30]	Philips Medical Systems; an animal coil	NA	3.0	FSE	T2: 4000/80	50; 256 × 256; 0.5	1 d, 3 w
Argibay *et al*[38]	PC - Bio Spec - Bruker; surface coil array	ImageJ (NIH)	9.4	MGE	T2*: 2.9/1.5	19.2 × 19.2; 192 × 192; 1.0	4 h
Duan *et al*[37]	Achieva - Philips Medical Systems; 4-channel rat coil	ImageJ (NIH)	3.0	FSE	T2: 800/60	60; 256 × 256; 1.0	1-4, 6-8 wk
				FFE	T2*: 500/18		
Lu *et al*[35]	Achieva - Philips Medical Systems; 4-channel rat coil	NA	3.0	FSE	T2: 200/31	60 × 60; 267 × 268; 1.0	1, 3, 7, 14 d
				FFE	T2*: 500/18		
Zhang *et al*[34]	Achieva - Philips Medical Systems	ImageJ (NIH)	3.0	FSE	T2: 800/60	60 × 60; 256 × 256; 1.0	1-6 wk
				PDW	PDW: 3000/20		
				FFE	T2*: 500/18		
Lin *et al*[36]	Intera - Philips Medical Systems	ImageJ (NIH)	3.0	Multi SE	T2: 2000/20-80	80 × 80; 160 × 266; 2.0	1-6 wk
Zhang *et al*[39]	PC - PharmaScan - Bruker	ImageJ (NIH)	7.0	Turbo RARE	T2: 6000/ 60	30; 256 × 256; 0.5	2 d, 8 d
				FLASH GRE	T2*: 400/3.5		
Duan *et al*[40]	Achieva - Philips Medical Systems	ImageJ (NIH)	3.0	FSE	T2: 800/60	60; 256 × 256; 1.0	1, 2, 3, 4, 6 wk
				PDW	PDW: 3000/20		
				FFE	T2*: 500/18		
Bai *et al*[42]	PC - PharmaScan - Bruker	ImageJ (NIH)	7.0	SE	T1: 500/15	20 × 20; 256 × 256; 1.0	1, 3, 5, 7, 10, 14 d
				FSE	T2: 2000/50		
Chen *et al*[28]	PC - Bio Spec - Bruker	ImageJ (NIH)	7.0	RARE SE	T2: 3000/50	25.6; 256 × 256; 0.7	3 d, 7 d, 14 d
Tan *et al*[41]	PC - Unity INOVA, Varian	NR	7.0	SE	T2: 2500/60	30 × 30; 512 × 512; NR	1-42 d
Janowski *et al*[44]	Sonata Maestro Class - Siemens; 8-channel head coil	Osirix (Pixmeo) Amira (Visage Imaging)	1.5	SWI	T2*: 49/40	230; 168 × 256; 1.6	1 d, 1 wk, 1 mo, 2 mo, 4 mo
Park *et al*[43]	Achieva - Philips Medical Systems; animal coil	NA	3.0	SE	T2: 11000/125	NA; 284 × 286; 0.7	0 h, 2 d
Zhang *et al*[45]	Sigma - GE Healthcare; a human head coil	NA	3.0	FSE	T2: 5840/104	45 × 45; 256 × 256; 1.5-2.0	1 d, 3 d
				Map MSME SE	T2: 3500/20-160		
Tarulli *et al*[46]	Sigma - GE Healthcare	NA	3.0	FSE	T2: 4500/35-75	40 × 40 × 17; 256 × 256; 1.0	1 d, 7 d, 14 d
				3D-SPGR	T2*: 25/7	40 × 40 × 20; 256 × 256; 1.0	
Liu *et al*[47]	Sigma - GE Healthcare; a rat coil		3.0	T2*	T2*: 2560/6.8	6.0; NR; 1.6	1, 7, 21 d
Wang *et al*[48]	Sigma - GE Healthcare	NA	3.0	FSE	T2: 5840/104	45 × 45; 256 × 256; 1.5	1, 7, 30 d
Lee *et al*[50]	Sigma - GE Healthcare; a clinical coil	NA	1.5	TSE	T2: 2000/81	90; 192 × 192; 1.5	0, 1, 5, 12 d
				GRE	280/20	20; 160 × 160; 1.5	
Song *et al*[49]	Sigma - GE Healthcare	NA	1.5	T2	T2: 3500/80	60 × 60; 256 × 160; 2.0	1d, 3d, 1-4 wk
				3D GRE	T2*: 50/20	80 × 80; 256 × 160; 2.0	

Kim *et al*[51]	PC - Bio Spec - Bruker	NA	4.7	SE RARE FLASH	T1: 600/14 T2: 5000/90 T2*: 758 × 30	40 × 30; 256 × 192; 1.0	2 d, 1 w, 2 w...10 wk
Guzman *et al*[52]	PC - Varian Medical Systems	NA	4.7	SE 3D GRE	T2: 2500/45 T2*: 600/5	40; 256 × 256; 1.0 30 × 30 × 30; 128× 128 × 128	2 d, 7 d, 35 d
Syková *et al*[53]	PC - Bio Spec-Bruker	NA	4.7	FGE	T2: NA	NA	1 d, 1-7 wk
Zhu *et al*[54]	Sigma - GE Healthcare	NA	3.0	SE	T2: 200/20	NA	1 d, 7 d

MRI: Magnetic resonance imaging; PC: Preclinical MRI scanner; NIH: National Institutes of Health; NA: Not applicable; MF: Magnetic field; T2: Transverse relaxation time; FSE/TSE: Fast or turbo spin echo; FFE: Fast field echo; PDW: Proton density-weighted; GRE: Gradient echo; MGE: Multiple gradient echo; SPGR: Spoiled gradient recalled echo; SE: Spin echo; FGE: Fast gradient echo; FLASH: Fast low angle shot; PDW: Proton density-weighted; RARE: Rapid acquisition with refocused echoes; SWI: Susceptibility weighted imaging; MSME: Multi-spin-multi-echo; SPGR: Spoiled gradient recalled echo; TR: Time repetition ; TE: Echo time ; FOV: Field-of-view; MT: Matrix; ST: Slice thickness.

superparamagnetic iron oxide nanoparticles in the ischemia area from a few minutes to several days after preclinical stroke induction. However, during the last 15 years, the understanding of the mechanisms of action has significantly advanced; rather than cell replacement, the benefit of SC treatments in stroke seems to result from indirect mechanisms, such as immunomodulation, which are intended to suppress the postischemic inflammatory response and enhance endogenous repair[60].

The meta-analysis study[61] examined the quality of the preclinical MSC studies, given the important bearing this has on translation potential. Over the past 10 years, our group has been improving the evidence finding process for developing treatments for neurological recovery through SCs labeled with iron oxide nanoparticles; in this study, we used the PRISM method, and the median quality score was the same as that in the Boncoraglio *et al*[59]'s study, which is the most recent and comprehensive meta-analysis of studies of SC transplantation for ischemic stroke. The quality of the twenty-five selected studies in this review was also found to be poor, and the majority of studies reported by Boncoraglio *et al*[59] showed an unclear risk of bias due to poor methodological reporting. This recent review showed that there are two major trial paradigms or approaches reflected in the translated results that were used to improve bedside stroke care: Neuroprotection in the acute phase and neurorestoration in the chronic phase[59]. The massive, early and fast delivery of SCs into the ischemic area reduces acute tissue injury and benefits from the paracrine effect of SCs, suppressing oxidative stress, inflammation, and mitochondrial impairment to suppress the apoptosis process[62,63]. During late SC delivery (more than 36 h after ischemic damage), the same studies[62,63] suggest that the chemokine signaling of SCs near the damaged/ischemic areas has already waned, and engraftment is intended to initiate brain remodeling by stimulating quiescent SCs to begin reparative processes, as long as they remain in damaged areas. Even so, SC administration results in enhanced recovery of sensorimotor function, promotion of synaptogenesis, stimulation of nerve regeneration, and suppression of tissue plasminogen activator-induced brain damage[64]. Therefore, the analysis of the homing and tracking SC processes is a pivotal strategy for utilizing preclinical results to increase translational knowledge to improve stroke care at the bedside.

In addition, Sohni *et al*[65]'s review suggests that MSC homing is inefficient and that many MSCs are trapped in the lungs following systemic administration. Therefore, it is imperative to trace the fate of the injected cells to truly achieve clinical translation aims. The same study cited several molecular imaging techniques to track the injected cells *in vivo*, such as BLI, SPECT, PET, and MRI. In this review, the maximum time of the homing evaluation used by all selected studies was 160 d or 4 mo (Janowski *et al*[44]'s study) by MRI; two studies[43,50] reported an immediate homing analysis after SC implantation at 0 h by MRI, 3 studies[35,38,43] analyzed homing fairly quickly (less than 24 h), and most of the selected studies (88%) used homing evaluation times ranging from 1 to 7 d. Late homing evaluation occurred in 13 studies[30,34,36,37,40,41,44,47-49,51-53] at least 14 d after implantation, and this was the most common scenario in the recent literature. Only 3 of the 25 selected studies[33,34,42] performed tracking and homing analysis of SCs by using retroviral vectors to express fluorescent proteins, and the maximum time of homing measured by NIRF was 6 wk, which is nearly 1.5 mo[34]. The maximum time of the BLI homing analysis was reported as three weeks[30]. Sohni *et al*[65]'s review proposed that the use of multifunctional (dual-labeled cells) nanoparticles or molecular imaging techniques increased the efficacy of determining the SC dose and route of inoculation owing to the time window after stroke and phase

Table 8 Near-infrared fluorescence imaging features for stem cell homing evaluation

Ref.	Agent	Equipment	Software	Excitation / Emission wavelength (nm)	Time of exposition	Follow-up
Lim *et al*[33]	DBCO-Cy5.5	IVIS Lumina Series III (PerkinElmer)	Living Image (PerkinElmer)	670/NA	1 min	*In vivo* at 1, 3, 7, 10, 14 d; *ex vivo* at 2, 27, 30, 33, 36 h
Zhang *et al*[34]	LV-FTH-EGFP	small animal *in vivo* FLI system (*in vivo* FxPro; Carestream)	MI (Carestream)	487/509	NA	1, 2, 3, 4, 5, 6 wk
Bai *et al*[42]	Cy5-5	Maestro *in vivo* imaging system (CRi, Woburn)	Maestro v. 2.10.0	675/695	NA	1, 3, 5, 7, 10, 14 d

DBCO: Dibenzylcyclooctyne; Cy5.5: Cyanine 5.5; LV-FTH-EGFP: Lentiviral vector-encoding ferritin heavy chain and enhanced green fluorescent protein; NA: Not applicable; MI: Molecular imaging software; Cri: Cambridge research and instrumentation.

effects (early or late) in SCs in the damaged area. Many important aspects were not addressed in most selected studies included in this review.

However, our group showed in a previous study the first standardized methodological approach for triple modal imaging of SCs after stroke in a rodent model, demonstrating SC homing, tracking and therapeutic efficiency using a low dose and a systemic route[25]. In this review, only 6 of the 25 selected studies used bimodal imaging, while three used NIRF[33,34,42] and three used BLI[28-30] combined with MRI. In our previous study, in which fluorescence was combined with resonance imaging techniques, our results showed that correlation analysis of the MNP load internalized into MSCLuc determined *via* MRI, ICP-MS and NIRF techniques resulted in the same correlation coefficient of 0.99. Evaluation of the BLI, NIRF, and MRI signals *in vivo* and *ex vivo* after labeled MSCLuc were implanted into animals showed differences in the contrast images according to the different MNP concentrations, and the physical signals were associated with different techniques (MRI and NIRF; 5 and 20 μg Fe/mL, respectively). Therefore, the temporal analysis showed the acute and late effects of SCs implanted in the sham groups (at 4 h and 6 d) and in the lesion due to the chemical receptors involved in brain damage by comparing the sham group and stroke group, improving the imaging techniques that assist systemic SC administration/dose assessment.

Furthermore, other questions (limitations) are also relevant regarding clinical translation of the results, such as culture conditions, the number of passages, donor age, the toxicity of the contrast agent used in the SC labeling process, and host factors (aging), among others, due to the absence of a reasonable understanding of the pharmacokinetics of the administered cells, which in itself would be an overall nonnegligible adverse effect. In this review, most of the selected studies reported a low cell passage (no later than the fifth passage), and the literature highlighted that a higher passage was associated with decreased telomerase activity, paracrine function, and renewal potential, which reduced cell differentiation and the immunomodulatory impact[66-68]. In terms of the toxicity of the contrast agent used in the SC labeling process, which was usually iron[69], all selected studies used iron oxide nanoparticles as the contrast agent, and the highest SPION concentration was 300 μg/mL[33]; however, the cell viability after the labeling process remained high (more than 95%) according to the CCK-8 assay, and the other selected studies also showed high cell viability when using low SPION concentrations. In our previous study[70], we showed that a high SPION concentration (100 μg/mL) maintained cell differentiation and the absence of cytotoxicity. The most recent selected studies used equipment that generated a high magnetic field (9.4 T), which was developed for preclinical imaging with rodent-specific coils, such as that used in the Lim *et al*[33] and Argibay *et al*[38] studies; this increased the detection sensitivity of the nanoparticles and generated greater opportunities for broader temporal analyses as well as the use of labeled SCs with lower SPION concentrations.

Although there were limitations/biases in all the selected studies included in this review, the studies that used behavioral or structural analysis/outcomes showed success in terms of neurological improvement using some sensitive motor tests as well as the reduction of the penumbra or ischemic brain area. Four decades of preclinical research demonstrating the survival, functional integration, and behavioral effects of transplanted SCs in experimental/preclinical stroke models have provided an ample scientific basis to facilitate the translation of clinical trials of SC therapy into

Table 9 Bioluminescence imaging features for stem cell homing evaluation

Ref.	Lentiviral vector	Equipment	Software	Substrate	Dose	Image acquisition	Follow-up
Wang *et al*[29]	Luc2/eGFP	IVIS Lumina Series III (Perkin-Elmer)	NR	D-luciferin (Promega, United States)	100 mL (30 mg/mL)	10 min after injection	1 d, 3 d, 7 d
Yun *et al*[30]	Fluc/eGFP	IVIS® Spectrum imaging system (Perkin Elmer)	NR	D-luciferin (Promega, United States)	150 mg/kg	NR	1 d, 1 wk, 3 wk
Chen *et al*[28]	Luc/GFP	IVIS Imaging System 200 Series (Caliper)	Living Image 3.0 (Xenogen Corp.)	D-luciferin (Caliper)	270 mg/g	15 min after injection	0, 14 d

All substrates were administered intraperitoneally. Luc: Luciferase; Fluc: Firefly luciferase; GFP: Green fluorescent protein; eGFP: Enhanced GRP; NR: Not reported.

treatments for stroke patients[62]. Although therapeutic efficacy has been demonstrated by the functional and structural outcomes of preclinical studies, there have been no relevant outcomes in clinical studies[11]. The best time window for cellular therapy for ischemic stroke has not yet been defined, and a recent clinical trial[71] and Cochrane review[59] suggested a time window between 24 and 36 h after the stroke event. However, a long clinical follow-up is necessary in combination with the use of the homing imaging technique as the gold standard to address the gap between the clinical application and the preclinical cellular therapy outcome. Thus, the prescription of SCs labeled with SPION according to this review may help improve future clinical trials.

Table 10 Stem cell administration, homing and cellular therapeutic efficiency

Ref.	Cell Type	Immuno-genicity	Time from stroke (h)	Cell administration			Groups	Follow-up	Outcome			
				Route	Number	Volume (μL)			Behavior	Infarct volume	Molecular proteins/ others	Cells migration
Lim *et al*[33]	MSC	XNG	NR	IC-CTL	1×10^6	5	Stroke + cells *vs* Stroke-cells	1, 3, 7, 10, 14 d	NR	(+)	NR	(+)
Wang *et al*[29]	MSC	XNG	24	ITC[1]	5×10^5	100	Alkyl-SPIO/siPHD2 > Alkyl-SPIO/si	1, 3, 7 d	(+) mNSS; FFT at 14 d	(+) 7 d	(+) Ki67; CD31 -7 d; (+) NeuN -14 d	(+)
	MSC	XNG	24	ITC[1]	5×10^5	100	Alkyl-SPIO/si *vs* saline	1, 3, 7 d	(+) mNSS; FFT at 14 d	(-) 7 d	(+) Ki67; CD31 -7 d; (+) NeuN -14 d	(+)
Yun *et al*[30]	NSC	XNG	24	IA-IC	3×10^6	100	Mag-Cells > UL-Cells/saline	0, 3, 5, 7, 21 d	(+) Cilinder at 21d	NR	(+) MAP2; Nestin; GFAP; TuJ1 -7d	(+)
Argibay *et al*[38]	MSC	ALG	8	IA; IV-jugular	2×10^5; 1×10^6	300	D-MNP-labeled MSC (IA × IV)	4, 24, 72 h	(-) Cilinder	(-) at 14d	(-) CD31; Ki67; DCX	(+)
Duan *et al*[37]	MSC	ALG	48	IC-CTL	5×10^5	3	Labeled cell > UL-cells	1, 2, 3, 4, 6, 8 wk	(-) mNSS	(-)	(-) TUNNEL (-) GFP	(+)
							Labeled/UL *vs* control	1, 2, 3, 4, 6, 8 wk	(+) mNSS at 3, 4, 6, 8 wk	(+) at 4, 6, 8 wk	(+) TUNNEL 7-21 d, (+) GFP 7-21 d	(+)
Lu *et al*[35]	NPC	ALG	NR	IC-IPS	5×10^5	2.5	labeling with N-NPS	0, 3, 7, 14 d	NR	(+)	(+) Nestin	(+) low
							labeling with C-NP	0, 3, 7, 14 d	NR	(+)	(+) Nestin	(+)
Zhang *et al*[34]	NSC	XNG	48	IC-CTL	5×10^5	3	FTH-EGFP-NSC > non transducec NSC	1, 2, 3, 4, 5, 6 wk	(+) mNSS at 1-6 wk	(+) at 1-6 wk	(+) GFAP; Nestin; CD11b at 6 wk	(+)
Lin *et al*[36]	MSC	ALG	48	IC-CTL	5×10^5	NR	ASP-SPION *vs* UL *vs* PBS	1, 2, 3, 4, 5, 6 wk	(-) mNSS	(-)	(-) GFAP; NeuN; CD11	(+)
Zhang *et al*[39]	NSC	XNG	7d	IC-CTL	NR	7	Stroke pure > Stroke + Ara-C	0, 2, 8 d	NR	(+) at 8 d	(+) CD15+; Nestin at 8 d	(+)
Duan *et al*[40]	MSC	ALG	48	IC-CTL	5×10^5	3	PLL-SPION or PM > UL	1, 2, 3, 4, 5, 6 wk	NR	(+) at 4, 6 wk	(-) GFP	(+)
Bai *et al*[42]	MSC	XNG	24	IA - IC	1×10^6	100	DM + RWJ + cell > DM + cells	1, 3, 5, 7, 10, 14 d	NR	(+)	(+) p38 MAPK at 7 d	(+)[2]
Chen *et al*[28]	MSC	XNG	30 min	IV-femoral	5×10^5		Mag-cells > UL-cells	0, 3, 7, 14 d	(+) VM at 14, 28 d	(+) at 14 d	(+) TuJ1; NeuN; GFAP at 28 d; (+) RT-PCR[1] at 28 d	(+)

Tan *et al*[41]	MSC	ALG	7 d	IC-CTL	5×10^5	10	Stroke + cells over time	0, 1, 7, 14, 21, 42 d	NR	NR	(-) GFP and NeuN at 7 d; (+) GFP and NeuN at 6 wk	(+)
Janowski *et al*[44]	NSC	AuTL	NR	IC	2×10^4	10	Case over time	0, 1, 7, 60, 120 d, 33 mo	NR	NR	NR	(+)
Park *et al*[43]	MSC	XNG	14d	IC-CTL	6×10^5	5	Pcion/pDNA MSC *vs* control	1, 2 d	NR	(-)	NR	(+)
Zhang *et al*[45]	NPC	ALG	24	IC-CTL	5×10^5	5	fsiSPION-NPC *vs* control	1, 3 d	NR	(+)	(+) Nestin	(+)[2]
	NPC	ALG	24	IV-tail	1×10^6	300	fsiSPION-NPC *vs* control	1, 3 d	NR	NR	(+) Nestin	(+)
Tarulli *et al*[46]	MSC	XNG	72	IV-tail	3×10^6	700	MPIO-BMSC *vs* UL-BMSC	1, 7, 14 d	NR	NR	NR	(+)
Liu *et al*[47]	NSC	XNG	NR	IC-CTL	3×10^4	5	Stroke + NSC_FA > Stroke + NSC	1, 7 d	NR	NR	(+) Sox-2 BrdU at 21 d	(+)
Wang *et al*[48]	MSC	XNG	7d	IC-CTL	1×10^5	5	FMNC-MSC > UL-MSC *vs* control (FMNC)	0, 1, 7, 30 d	NR	NR	(+) TuJ1	(+)
Lee *et al*[50]	MSC	XNG	48	IC-CTL	2×10^4	5	M600-MSC *vs* FC-MSC	1, 5, 12 d	NR	NR	NR	(+)
	MSC	XNG	48	IV-tail	2×10^6	500	M600-MSC *vs* control	5, 12 d	NR	NR	NR	(+)
Song *et al*[49]	NPC	XNG	24	IC-IPS	4×10^5	5	FO-NPC *vs* control	1, 3, 7, 14, 21, 28 d	NR	NR	(+) BrdU; GFAP at 28 d	(+)
	NPC	XNG	24	IV-tail	4×10^6	500	FO-NPC *vs* control	1, 3, 7, 14, 21, 28 d	NR	NR	(+) BrdU; GFAP at 28 d	(+)
Kim *et al*[51]	MSC	AuTL	7d	IC-IPS/CTL	1×10^5	2	Feridex®-labeled hMSC over time for both vias	2d, 1, 2, 4, 6, 8, 10 wk	NR	NR	(-) GFAP; TH; MAP2; TuJ1; Nestin at 10 wk	(+)
Guzman *et al*[52]	NSC	XNG	7d	IC-CTL	3×10^5/5 $\times 10^4$		NSC-SCns-SPION	3, 9, 12,18 wk	NR	NR	(+) SC121 or SC101; TuJ1; GFAP; MAP2 at 18 wk	(+)
Syková *et al*[53]	rOEC	ALG	NR	IC-CTL	NR	NR	OEC-SPION over time	3-7 wk	NR	NR	(+) NeuN; GFAP at 28 d	(+)
	MSC	XNG	NR	IV-femoral	NR	NR	MSC over time	6-30 d	NR	NR	(+) NeuN; GFAP at 28 d	(+)
Zhu *et al*[54]	NSC	AuTL	NR	IC	NR	NR	Patients treat with NSC and no treat	2 yr	(+) SEP and DRS at 6, 9 mo	(+) cells uptake by PET at 3, 6 mo	NI	(+)

[1]left ventricle.
[2]In addition to cell migration analysis, studies reported biodistribution analysis after stem cell administration. The Bay study[42] reported biodistribution in the liver, spleen, heart, lungs, and kidneys; Zhang *et al*[45] reported that the SPION-labelled cells IV > IA at 3 d after injection were detected in spleen, liver, heart, kidney, and lung. MSC: Mesenchymal stem cells; NSC: Neural stem cells; ESC: Embryonic stem cell; rOEC: Rat olfactory ensheathing cells; NR: No reported; XNG: Xenogeneic; ALG: Allogeneic; AuTL: Autologous; IC: Intracerebral; IC-CTL: IC contralateral; ITC: Intracardially; IC-IPS: IC ipsilateral; IV: intravenous; IA-IC: Intraarterial through internal carotid artery; Alkyl-SPIO: Amphiphilic low molecular weight superparamagnetic iron oxide; Mag: External magnet; UL: Unlabeled; siPHD2: siRNA against PHD2; C-NP: Cationic nanoparticle; N-NP: Neutral nanoparticle; FTH-eGFP: ferritin heavy chain:

Enhanced green fluorescent protein; ASP-SPION: Spermine-modified amylose superparamagnetic iron oxide nanoparticle; FBS: Fetal bovine serum; Ara-C: Cytosine arabinosine; PLL: Poly-L-Lysine; PM: Polymersone; PCION: Poly-(ethylene glycol)-coated cross-linked iron oxide nanoparticles; MPIO: Micron-sized superparamagnetic iron oxide particles; DM: Diabetes mellitus; RWJ: RWJ67657; fsiSPION: fmSiO4@SPION; FC: Ferucarbotran; FA: Folic acid; FO: Ferumoxide; FMNC: Fluorescent-magnetite-nanocluster; mNSS: Modified neurological severity score; FFT: Foot-faults test; VM: Vertical movement; SEP: Somatosensory evoked potential; DRS: Disability rating scale; MAP2: Microtubule-associated protein 2; GFAP: Glial fibrillary acidic protein; TuJ1: Neuron-specific class III beta-tubulin; GFP: Green fluorescent protein; MAPK: Mitogen-activated protein kinase; RT-PCR: Reverse transcription polymerase chain reaction; BrdU: 5′-Bromo-2′-deoxyuridine; TH: Tyrosine hydroxylase.

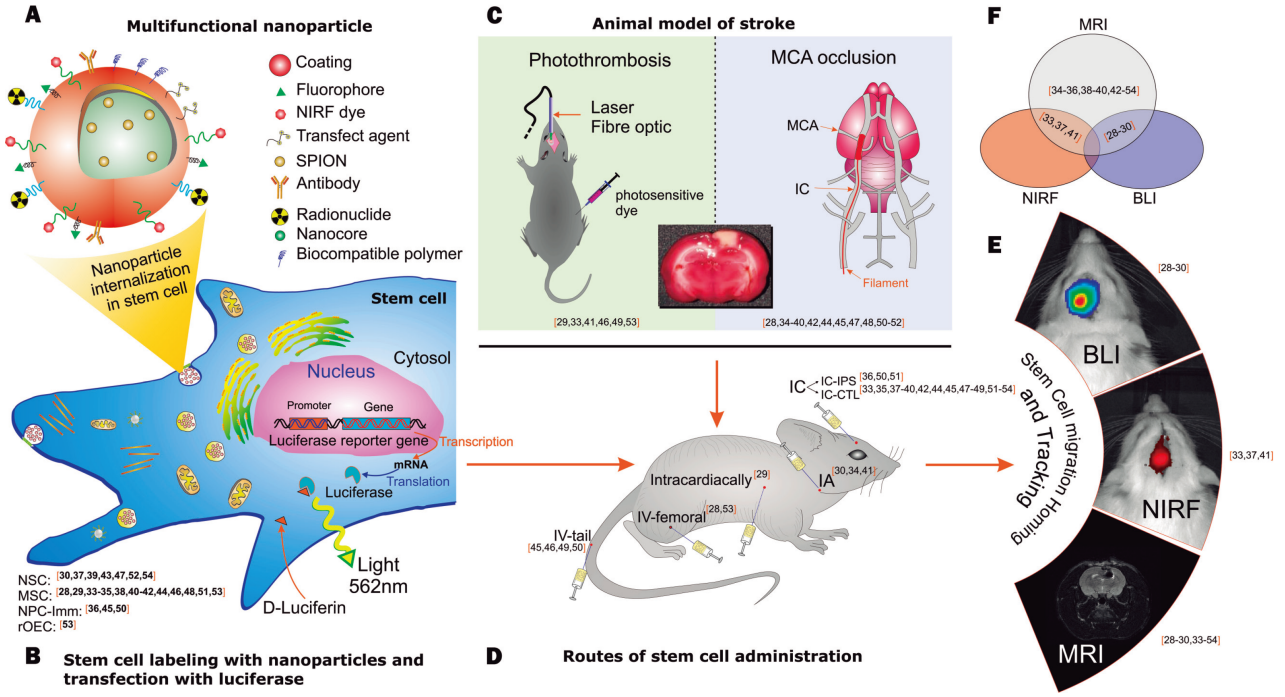

Figure 2 Schematic illustration of the aspects of stem cell homing, tracking and therapeutic efficacy evaluated in stroke using nanoparticles in the selected studies included in this review. A: The multifunctional nanoparticle characteristics; B: Characteristics of stem cells labeled with nanoparticles/contrast agents transfected with luciferase; C: Characteristics of the induction of the animal models of stroke; D: Routes of stem cell administration; E: Molecular imaging techniques of stem cell migration homing and tracking; F: The combined imaging techniques used in the stem cell homing analysis. MSC: Mesenchymal stem cells; NSC: Neural stem cells; NPC-Imm: Neural progenitor cell - immortalized; ESC: Embryonic stem cell; rOEC: Rat olfactory ensheathing cells; IV: Intravenous by tail and femoral veins; IA: Intra-arterial by intracarotid; IC: Intracerebral; CTL/IPS: Contralateral or ipsilateral of brain injury; BLI: Bioluminescence; NIRF: Near-infrared fluorescence; MRI: Magnetic resonance imaging; MCA: Middle cerebral artery.

ARTICLE HIGHLIGHTS

Research background

Stroke survivors commonly suffer from disabilities requiring temporary or lifelong assistance, resulting in a substantial economic burden for poststroke care and stem cell (SC) therapeutics appear to be a promising alternative for intervention in stroke therapy. However, the efficacy of SC therapy depends on the SC homing ability and engraftment into the injury site over a long period of time.

Research motivation

The analysis of the homing and tracking SC processes is a pivotal strategy for utilizing preclinical results to increase translational knowledge to improve stroke care at the bedside.

Research objectives

In this systematic review, we aim to evaluate SC migration homing, tracking and therapeutic efficacy in the treatment of stroke using nanoparticles.

Research methods

A systematic literature search was performed to identify articles published prior to November 2019 that were indexed in PubMed and Scopus. The following inclusion criteria were used: (1) Studies that used *in vivo* models of stroke or ischemic brain lesions; (2) Studies of SCs labeled with some type of contrast agent for cell migration detection; and (3) Studies that involved *in vivo* cellular homing and tracking analysis.

Research results

A total of 82 articles were identified by indexing in Scopus and PubMed. After the inclusion

criteria were applied, 35 studies were selected, and the articles were assessed for eligibility; ultimately, only 25 studies were included. Most of the selected studies used SCs from human and mouse bone marrow labeled with magnetic nanoparticles alone or combined with fluorophore dyes. These cells were administered in the stroke model (to treat middle cerebral artery occlusion in 74% of studies and for photothrombotic induction in 26% of studies). Fifty-three percent of studies used xenogeneic grafts for cell therapy, and the migration homing and tracking evaluation was performed by magnetic resonance imaging as well as other techniques, such as near-infrared fluorescence imaging (12%) or bioluminescence assays (12%).

Research conclusions

Our systematic review provides a comprehensive, up-to-date evaluation of the SC migration and efficacy of cellular therapy for brain injury. Cellular therapy demonstrated considerable efficacy with regard to the functional and structural evaluation, as well as the differentiation of the cells in the late stage of evaluation (after 7 d of cell implantation), using protein molecular and other tests.

Research perspectives

In summary, a long clinical follow-up is necessary in combination with the use of the homing imaging technique as the gold standard to address the gap between the clinical application and the preclinical cellular therapy outcome. Thus, the prescription of SCs labeled with SPION according to this review may help improve future clinical trials.

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