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Review Article

Two novel mechanisms for maintenance of stemness in mesenchymal stem cells: SCRG1/BST1 axis and cell—cell adhesion through N-cadherin☆

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Summary Mesenchymal stem cells (MSCs) retain the ability to self-renew and differentiate into mesenchymal cells. Therefore, human MSCs are suitable candidates for use in regenerative medicine and cell therapies. Upon activation by tissue damage, MSCs contribute to tissue repair through a multitude of processes such as self-renewal, migration, and differentiation. However, loss of self-renewal and multi-lineage differentiation potential occurs at a high rate during cell doubling. Effective MSC therapies require the establishment of new techniques that preserve MSC multipotency after lengthy cell expansions. Here, two novel mechanisms are described for maintenance of stemness in MSCs *via* scrapie responsive gene 1 (SCRG1)/bone marrow stromal cell antigen-1 (BST1) ligand—receptor combination and cell—cell adhesion through N-cadherin. These two mechanisms findings provide a valuable tool for regenerative medicine and cell therapeutic methods that require the *ex vivo* expansion of human MSCs while maintaining native stem cell potential.

☆ Scientific field of dental science: Stem cell biology.

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Contents

Introduction

Regenerative medicine and cell therapies have garnered attention as innovative methods for the treatment of serious diseases resulting from malfunctions or defects of the body. Cells used in these regenerative therapies include multipotent cells such as adult stem cells and embryonic stem (ES) cells. Recently, the advent of induced pluripotent stem (iPS) cells has contributed further to the development of regenerative medicine by removing ethical and immune barriers.

Mesenchymal stem cells (MSCs) are adult stem cells with the ability to differentiate into mesenchymal cells such as osteoblasts, adipocytes, chondrocytes, and fibroblasts, while retaining self-renewal and migration abilities [1]. In stem cell therapy, human MSCs are expanded *in vitro* and subsequently auto-implanted, eliminating the risk of immune rejection. Upon activation by tissue damage *in vivo*, MSCs contribute to tissue-repair through a multitude of processes such as self-renewal, migration, and differentiation. Human MSCs, first derived from bone marrow, have now been isolated from various tissues such as subcutaneous adipose, articular cartilage, and synovial membrane tissues [2—5]. Isolation of MSCs from a population of various cell types requires the use of cell surface markers. MSCs have been established that fulfill the following criteria: (i) adhering to plastic culture dishes; (ii) being positive for the markers CD73 (ecto-5′-nucleotidase, *NT5E*), CD90 (Thy-1 cell surface antigen, *THY1*), and CD105 (endoglin, ENG); (iii) being negative for the markers CD34, CD45 (leukocyte common antigen, *LCA*), HLA-DR, CD14, CD11b (integrin αM, *ITGAM*), CD79a (immunoglobulin-associated α), and CD19; and (iv) differentiating into osteoblasts, adipocytes, and chondrocytes *in vitro* [6]. In addition to these previously known cell surface markers, useful markers for isolating MSCs and maintaining multipotency have been identified. Recently, Mabuchi et al. reported that human MSCs that are positive for CD271 (low-affinity nerve growth factor receptor, *LNGFR* or p75 neurotrophin receptor, p75NTR) and CD90 and that are highly positive for CD106 (vascular cell adhesion molecule-1, *VCAM1*) retain high propensities for both of self-renewal and multipotency [7]. Thus, the combination marker LNGFR^{+}THY1^{+}VCAM1^{high+} (LTV) can be used to isolate potent human MSCs.

Because of their rarity *in vivo*, MSCs could be used after expansion for therapies in regenerative medicine [8]. *Ex vivo* expanded MSCs have been used for bone regeneration [9]. However, long-term *in vitro* culture of MSCs attenuates their ability for self-renewal and multipotency [10]. After a long period of *ex vivo* expansion, MSCs become large and flat and lose their ability to divide. *In vitro* expansion of MSCs is associated with gradual accumulation of senescent cells [11], telomere erosion [12], and changing phenotypes [13,14]. Thus, *ex vivo* expansion of MSCs seems to degrade multipotency; it is thus important to establish novel MSC expansion techniques that do not sacrifice multipotency even after long-term culture. For regenerative medicine and cell therapeutic purposes, this issue must be addressed. In this review, expression of the cell surface markers CD271 and CD106 and report novel trigger mechanisms for activation of the intracellular signaling pathway that regulates the maintenance of stem-like features, such as migration, self-renewal, and multipotency.

Maintenance of stemness by a novel ligand—receptor combination

Identification of SCRG1 as a novel ligand in MSCs

The gene expression of a cysteine-rich cytokine-like peptide, scrapie responsive gene 1 (*SCRG1*, also known as stimulator of chondrogenesis 1), was found to decrease during osteoblastic differentiation of human bone marrow-derived MSCs by using DNA microarray analysis [15]. Therefore, SCRG1 is expressed only in undifferentiated MSCs. While the function of SCRG1 is unknown, its expression was found to increase in the brains of mice infected with scrapie [16]. Recent studies by Dron et al. have reported that SCRG1 is involved in neurodegeneration and autophagy associated with transmissible spongiform encephalopathy due to scrapie infection [17,18]. SCRG1 is highly conserved in vertebrates and it consists of 98 amino acid residues,

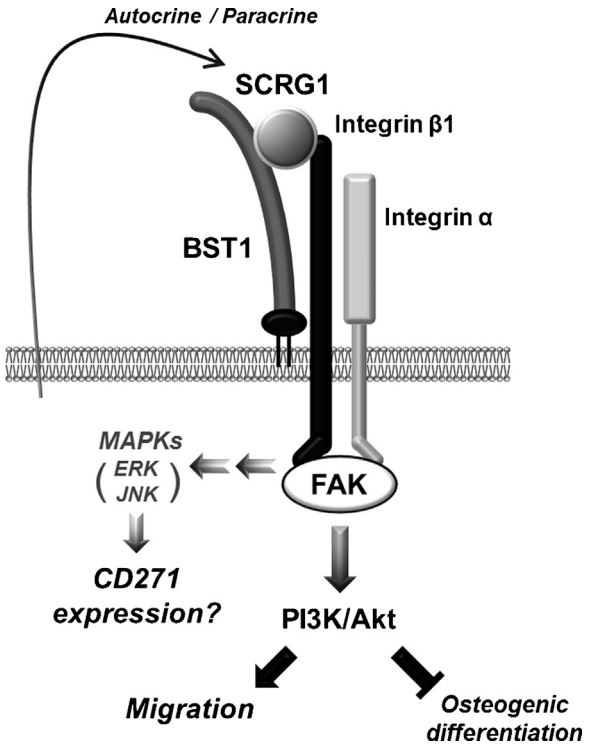

Figure 1 Proposed molecular mechanism for maintenance of stemness by the novel ligand—receptor combination SCRG1/BST1. SCRG1 is hypothesized to be secreted into the extracellular space by MSCs and to have autocrine/paracrine activity. The putative receptor for SCRG1 is a complex of BST1 and integrin β1 on the MSC cell surface. The SCRG1/BST1 axis promotes MSC migration and suppresses osteogenic differentiation through the FAK/PI3K/Akt signaling pathway. SCRG1/BST1 maintains the stemness of MSCs and CD271 expression.

including a 20-residue signal peptide at the N-terminus [19—21]. Therefore, SCRG1 protein is secreted into the extracellular space by bone marrow-derived MSCs, resulting in exhibition of autocrine/paracrine activities.

Identification of BST1 as a novel SCRG1 receptor

The SCRG1 receptor, which was predicted to be localized on the MSC membrane surface, consists of a complex composed of bone marrow stromal cell antigen-1 (BST1, CD157) and integrin β1 by co-immunoprecipitation analysis with a cross-linker (Fig. 1) [15]. BST1 is an ectoenzyme with a glycosyl phosphatidylinositol (GPI) anchor and NADase/ADP-ribosyl cyclase activity in the CD38 family [22,23]. It has been found on the cell surface of stromal [24] and bone marrow-derived cells [25], and it facilitates pre-B-cell growth and induces cell migration [26]. In a complex with integrin β1 or β2, BST1 has been shown to promote the phosphorylation of focal adhesion kinase (FAK) in studies using an agonistic monoclonal antibody [27—29]. Furthermore, it has also been reported that BST-1 regulate the adhesion and migration of leukocytes *via* phosphorylation of phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases (MAPKs) [30,31]. However, the BST1 ligand has not yet been demonstrated *in vivo*, and SCRG1 represents the first putative ligand for this protein.

Migration induced by FAK/PI3K/Akt activation *via* the SCRG1/BST1 axis

Stimulation with human recombinant SCRG1 was enhance the phosphorylation of FAK in human bone marrow-derived MSCs; this effect was completely eliminated, however, by BST1 knockdown [15]. Generally, the phosphorylation of FAK *via* integrin β is important for migration during cytoskeletal reorganization [32]. In fact, the SCRG1/BST1 axis promoted the migration of human bone marrow-derived MSCs through the activation of the FAK/PI3K/Akt signaling pathway *via* autocrine/paracrine activity (Fig. 1) [15]. These phenomenon results suggest that the SCRG1/BST1 axis promotes MSC tissue-forming ability by stimulating and maintaining migratory activity.

Cell migration is closely related to stem cell homing. Stem cell therapy relies on the appropriate homing and engraftment capacity of stem cells. Chemokines such as monocyte chemotactic protein-1 (MCP-1/CCL2) and/or stromal cell-derived factor-1 (SDF-1/CXCL12), and their receptors such as CCR2 and CXCR4 promote the effective homing of MSCs. The activation of signal transduction pathways *via* chemokine stimulation, cell adhesion in the extracellular matrix by integrin plays an important role in migration [33]. In addition, MSCs migrate and adhere to fibroblasts *via* MCP-1 secreted from the fibroblasts [34]. MSC—fibroblast adhesion promotes the secretion of anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β1. Interestingly, SCRG1 suppresses LPS-induced CC-chemokine ligand 22 (CCL22; also known as MDC) production through ERK1/2 activation in mouse macrophage-like cells [35]. Therefore, the migration ability of MSCs *via* SCRG1 and/or MCP-1 may be associated with immunosuppressive effects.

Expression of CD271 *via* the SCRG1/BST1 axis

As described above, LTV-positive human MSCs are characterized by a high capacity for self-renewal and multipotency [7]. Maintenance of these capabilities is a prerequisite for proliferation of MSCs *in vitro*. The addition of SCRG1 led to the maintenance of CD271 expression and self-renewal ability in a long-term primary culture of human bone marrow-derived MSCs [15]. The SCRG1/BST1 axis did not affect the expression of other important MSC markers, such as CD90 and CD106. Surprisingly, stimulation of SCRG1 preserved expression of CD271 and octamer-binding transcription factor-4 (OCT-4/*POU5F1*) in human bone marrow-derived MSCs, even after long-term passages. In addition, SCRG1 preserved osteogenic activity in human primary MSCs, even after long-term passages. These results suggest that SCRG1 maintains self-renewal and multipotency in MSCs, similar to the effect previously reported for the ES cell marker OCT-4 and the homeobox protein NANOG. OCT-4 and NANOG act as transcription factors by binding to the promoter region of the DNA (cytosine-5)-methyltransferase 1 (*DNMT1*) gene [36]. DNMT1 suppresses differentiation-inducing and cell proliferation-

inhibiting factors p21 (Cip/*Waf1*) and p16 (*INK4A*), thereby promoting cell proliferation [36]. On the other hand, the Wnt signaling pathway is enhanced in CD271$^+$ MSCs, inducing expression of OCT-4 and NANOG [37,38]. In experiments associated with long-term culture of primary human MSCs, the maintenance of OCT-4 expression was demonstrated by stimulation with SCRG1 [15]. Therefore, it appears that SCRG1 regulates the expression of not only CD271 but also OCT-4 in human bone marrow-derived MSCs. This strongly suggests that the SCRG1/BST1 axis maintains the stemness of MSCs. Nevertheless, the specific intracellular signaling pathways responsible for regulating the expressions of CD271 and OCT-4 are still unknown (Fig. 1).

Maintenance of MSC stemness by the SCRG1/BST1 axis

While SCRG1 maintains the osteogenic differentiation potential of MSCs, it suppresses the induction of osteoblastic differentiation (Fig. 1) [15]. This result indicates that it is the role of the SCRG1/BST1 axis to maintain an undifferentiated state in human bone marrow-derived MSCs by suppressing osteoblastic differentiation. Osteoblastic differentiation of MSCs involves the expression of cell proliferation inhibitory factor p21, which is regulated by the hypoxia inducible factor-1α (HIF-1α)-TWIST pathway [39—42]. Proliferation and differentiation are conflicting phenomena. Thus, activation of the TWIST pathway by HIF-1α promotes osteoblastic differentiation and suppresses cell proliferation of MSCs [43]. As described above, SCRG1 maintains the expression of OCT-4 in primary cultured human bone marrow-derived MSCs [15]. Thus, SCRG1 will be involved in the maintenance of osteogenic differentiation potential by suppressing the expression of p21 through OCT-4. However, growth inhibitory mechanism *via* the SCRG1/BST1 axis in MSCs was not yet confirmed.

Ex vivo expansion of MSCs seems to degrade multipotency; it is thus important to establish novel MSC expansion techniques that do not sacrifice multipotency, even after long-term culture periods. Effective expansion of MSCs *ex vivo* may be achieved by utilizing recombinant SCRG1. However, SCRG1 also induces the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and c-jun N-terminal kinase (JNK) in MSCs [15]. As yet, the role of MAPK cascade activation by SCRG1/BST1 is still unclear. Among various possibilities, activation of MAPKs may be involved in CD271 expression and the ability to differentiate into other cell types. In order to take advantage of SCRG1 in regenerative medicine and cell therapy, further investigation onto the intracellular signaling pathway activated by SCRG1/BST1 is required.

Maintenance of stemness by cell—cell adhesion through cadherin

Cell density-dependent CD106 expression

After homing of MSCs into on damaged tissue, cell—cell adhesion between MSCs is essential for MSC-dependent tissue regeneration *in vivo*. However, the adhesion molecules responsible for cell—cell adhesion and communication between MSCs under various culture conditions remain to be clarified. The expression of cell adhesion molecule CD106, an MSC marker, is dependent on the status of cell density of human bone marrow-derived MSCs. The expression level of CD106 was clearly up-regulated in MSCs grown under at high cell densities [44]. CD106 is primarily expressed in vascular endothelial cells and is involved in adhesion of lymphocytes onto vascular endothelium by binding to integrin $\alpha4\beta1$ (very late antigen-4, VLA-4) and integrin $\alpha4\beta7$ (lymphocyte Peyer's patch adhesion molecule, LPAM) on the cell surface of the lymphocytes [45,46]. CD106 expression is also induced by stimulation with inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1β. In particular, induction of CD106 expression in MSCs promotes homing towards wounds and damaged tissues [47,48]. Intriguingly, high cell density-dependent CD106 expression inhibited the migratory ability of MSCs [44]. In addition, integrin VLA-4 was expressed on the cell surface of MSCs. Therefore, the CD106-induced inhibition of MSC migration may be dependent on cell—cell adhesion caused by the binding of CD106 to VLA-4.

NF-κB activation by cell—cell adhesion through N-cadherin

The high cell density-induced expression of CD106 in human bone marrow-derived MSCs was clearly suppressed by IκB kinase (IKK) inhibitors, suggesting that the cell density-induced signal was dependent on the nuclear factor-κB (NF-κB) signaling pathway [44]. In addition, the expression of CD106 increases *via* activation of the NF-κB pathway induced by cell—cell adhesion with N-cadherin in cultures with high cell densities [44,49]. The overexpression of full-length N-cadherin in MSCs markedly increased high cell density-induced CD106 expression, whereas the overexpression of truncated N-cadherin lacking its intracellular domain suppressed high cell density-induced CD106 expression [49]. These results indicate that, in MSCs, high cell density-inducted expression of CD106 is mediated by cell—cell adhesion through N-cadherin (Fig. 2).

Cadherin is a major factor in adherens junctions (AJs) belonging to the calcium-dependent transmembrane protein family. E-cadherin is the main cadherin in the AJs of epithelial cells, while other cadherins, including N-cadherin, P-cadherin, R-cadherin, and VE-cadherin, form AJs in other cell types [50,51]. MSCs predominantly express N-cadherin, which is also observed in other cell types [52,53]. N-cadherin-mediated AJs are important in connective tissue physiology and are critical for the regulation of cell attachment and migration [54], wound healing [55], metastatic potential [56], and embryonic development [57,58], as well as the differentiation and formation of numerous specialized tissues, including fibrous connective tissues [59,60].

The phosphorylation status of proto-oncogene tyrosine—protein kinase Src in MSCs and found that phosphorylation of Src kinase was enhanced at high cell densities [49]. The phosphorylation of Src kinase in MSCs was markedly upregulated by the overexpression of full-length N-cadherin. In contrast, overexpression of a truncated version of N-cadherin lacking the intracellular domain did not affect Src kinase phosphorylation. In addition, the high cell

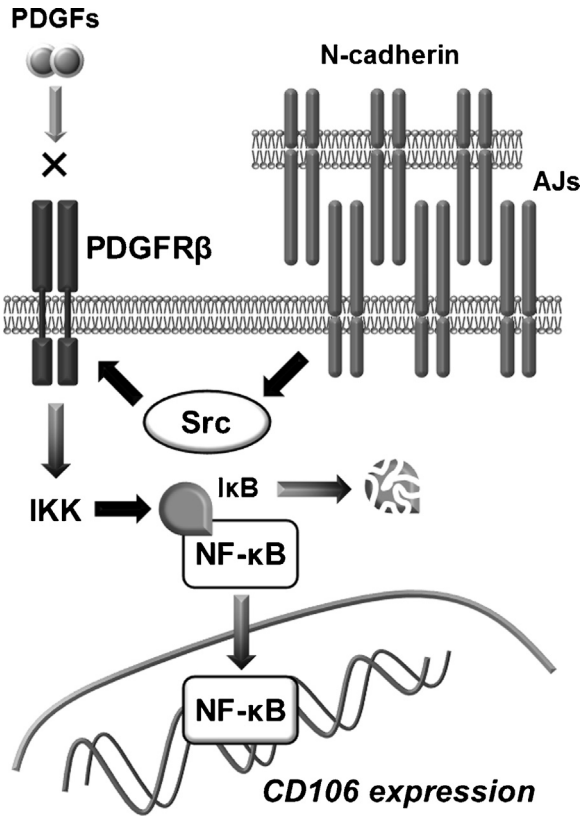

Figure 2 Proposed molecular mechanism for maintenance of MSC stemness by cell—cell adhesion through cadherin. The expression of CD106 in MSCs is dependent on cell density and increases *via* activation of the NF-κB pathway by cell—cell adhesion with N-cadherin in cultures with high cell densities. The phosphorylation of Src kinase through the intracellular domain of N-cadherin plays an important role in the upregulation of CD106. In the MSC intracellular signaling pathway, Src tyrosine kinase and PDGFRβ are ligand-independently activated by N-cadherin through cell—cell adhesion.

density-induced CD106 expression was reduced by an Src kinase inhibitor [44]. These results indicate that the phosphorylation of Src kinase through the intracellular domain of N-cadherin plays an important role in the upregulation of CD106 by cell—cell adhesion (Fig. 2).

Ligand-independent PDGFR activation by cell—cell adhesion

The CD106 expression in MSCs is significantly suppressed by treatment with an inhibitor of the platelet-derived growth factor (PDGF) receptor (PDGF receptor, *PDGFR*) [49]. In general, PDGFR is activated by its ligand, PDGF. There are four PDGF isoforms (A—D), which form homo- or heterodimers (*e.g.*, PDGF-AA, PDGF-AB, PDGF-BB, etc.) [61]. Of these, PDGF-BB exhibits the strongest activity [54] and has been approved by the Food and Drug Administration for the treatment of patients with bone defects in the oral and maxillofacial regions [62—65]. PDGF-BB is mainly produced by platelets and has been implicated in tissue repair [61]. There are two isoforms of PDGFR (α and β), which also form homo- or heterodimers (PDGFRα/α, α/β, and β/β) [61]. PDGFRα is reportedly expressed in MSCs and osteoblast progenitor cells, and PDGFRα-positive cells exhibit a high osteoblastic differentiation capacity [66]. In MSCs, there is evidence that PDGF-BB promotes PDGFα-positive cell migration into artificial bones without inhibiting osteoblastic differentiation [67]. In addition, TGF-β1-induced osteoblastic differentiation of MSCs is synergistically enhanced by treatment with PDGF-BB [68]. Despite this, PDGF-BB did not affect the expression of CD106 in MSCs [49]. The intracellular signaling pathways in MSCs, Src tyrosine kinase and PDGFRβ are ligand-independently activated by cell—cell adhesion through N-cadherin [44,49]. Furthermore, activation of PDGFRβ induces the expression of CD106 through the NF-κB pathway in MSCs [44,49]. Therefore, cell—cell adhesion induced by high cell densities enhances the expression of CD106 through N-cadherin and the ligand-independent activation of PDGFRβ in human bone marrow-derived MSCs (Fig. 2).

Concluding remarks

In recent years, tissue engineering and regenerative medicine have been widely explored [69]. The dental pulp and periodontal ligament have attracted attention as a source of MSCs — which can differentiate into osteoblasts, cement blasts, fibroblasts, and vascular endothelial cells — independent from the bone marrow and adipose tissues [70]. Differentiation of MSCs from periodontal tissue into odontoblasts has also been reported [71]. In periodontal tissue regeneration, repair of the alveolar bone and cementum is an important goal. Recently, we reported the establishment of MSC lines from salivary glands [72].

Cytokines and chemokines secreted from MSCs have been implicated in the immunosuppression and repair of damaged tissues [73—76]. In addition, the direction of MSC differentiation is regulated by stimulation with various growth factors, cytokines, and chemokines, as demonstrated in the differentiation of bone marrow-derived MSCs [77,78, and our unpublished data]. Interestingly, MSCs enhance anti-inflammatory effects by increasing the secretion of anti-inflammatory cytokines and simultaneously maintaining differentiation potential through cell—cell adhesion with fibroblasts.

MSCs contribute to tissue repair processes through cell proliferation, migration, and differentiation. The clinical use of adult organ-derived MSCs depends on obtaining sufficient cell populations for transplantation through *ex vivo* expansion. Our recent study suggested that the SCRG1/BST1 axis maintains stemness and the expression of CD271 as an MSC marker in human bone marrow-derived MSCs [15]. In addition, we have demonstrated that the expression of CD106 as an MSC marker is dependent on cell density [44,49]. In long-term culture of MSCs *in vitro*, the stemness of MSCs can be maintained by the addition and/or overexpression of SCRG1 while maintaining cell—cell adhesion (Fig. 3). We have additionally established fluorescently tagged immortalized MSC lines derived from different tissues of GFP- and tdTomato-transgenic mice [72,77]. These cell lines can be used for *in vivo* proliferation and differentiation of MSCs, as well as *in vivo* imaging studies to test cell therapies and regenerative medicine techniques, providing insight into

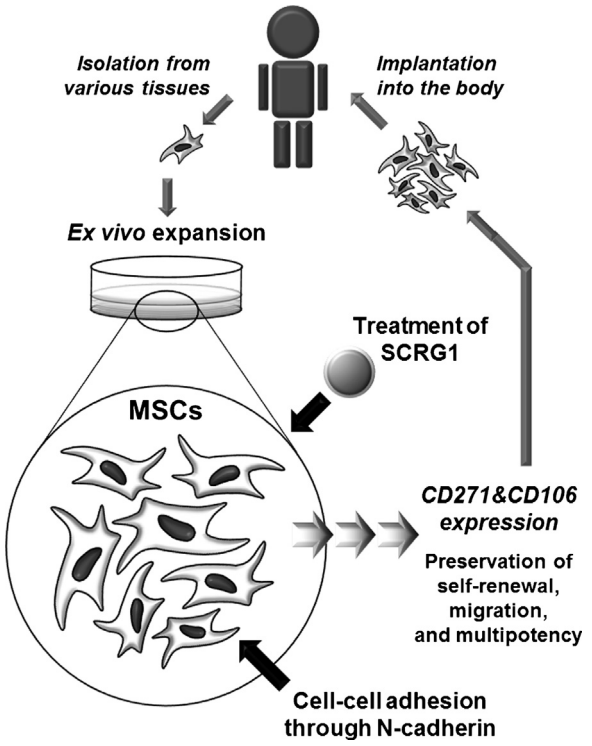

Figure 3 The clinical use of MSCs derived from various tissues depends on obtaining sufficient cell populations for transplantation through *ex vivo* expansion. During long-term *in vitro* culture of MSCs, stemness can be maintained by the addition and/or overexpression of SCRG1 and the promotion of cell—cell adhesion.

diseases such as bone and immune disorders, fibrosis, and cancer progression or metastasis. These findings provide new insights into the molecular mechanisms of MSCs, as well as a novel perspective for methods of *ex vivo* expansion to maintain native stem cell potential for regenerative medicine and cell therapy.

Conflicts of interest

The authors declare no competing financial interests.

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