

Recent advances of single-cell RNA sequencing technology in mesenchymal stem cell research

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Abstract

Mesenchymal stem cells (MSCs) are multipotent stromal cells with great potential for clinical applications. However, little is known about their cell heterogeneity at a single-cell resolution, which severely impedes the development of MSC therapy. In this review, we focus on advances in the identification of novel surface markers and functional subpopulations of MSCs made by single-cell RNA sequencing and discuss their participation in the pathophysiology of stem cells and related diseases. The challenges and future directions of single-cell RNA sequencing in MSCs are also addressed in this review.

Key words: Mesenchymal stem cells; Single-cell RNA sequencing; Pathophysiology; Novel surface markers

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Core tip: Mesenchymal stem cells (MSCs) are an important kind of multipotent stroma cells *in vivo*. Previous studies have focused on large groups of cells. In this review, we focus on advances in the identification of novel surface markers and functional subpopulations of MSCs made by single-cell RNA sequencing and discuss their participation in the pathophysiology of stem cells and related diseases.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells with great potential for clinical applications^[1-3]. However, there are many problems that need to be solved before the wide application of MSC therapy. First, MSCs exhibit heterogeneity at multiple levels, for example, among donors and tissue sources and within cell populations^[4]. Although the criteria for MSCs were established in 2006 by the International Society of Cell Therapy^[5], they need to be improved on the basis of new knowledge. Surface markers for identifying specific groups of MSCs or predicting their potential for cell differentiation remain to be elucidated. MSCs are produced and expanded *in vitro* to assure the availability of sufficient cell numbers for clinical therapy. Cell morphology changes, proliferation ability decreases, along with other alterations in cell function, and how culture conditions influence MSCs remains unclear. Finally, the efficacy of MSC therapy varies among different clinical studies, and more data are needed to explore the mechanism of immunoregulation and tissue repair^[6]. Single-cell sequencing is a powerful tool for characterizing heterogeneous cell populations and identifying novel stem cell types^[7-13]. The aims of this review are to emphasize the advances in the identification of novel surface markers and functional subpopulations of MSCs by single-cell RNA sequencing (scRNA-seq) and discuss their participation in the pathophysiology of stem cells and related diseases.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells are defined as multipotent mesenchymal stromal cells that can be isolated from many adult organs. They were first reported in 1974 by Friedenstein^[14] and were described as colony-forming unit fibroblasts. These cells have the capacity to differentiate into mesodermal tissues, such as bone, cartilage, and fat cells^[15,16], as well as other tissues, such as myocytes and neural cells^[17]. Moreover, the trophic function of MSCs in supporting hematopoietic stem cells (HSCs) is well studied^[17]. In preclinical studies, the advantages of suppressing the inflammation and immunoregulation of MSCs have attracted great interest^[18,19]. On the basis of these properties, many clinical trials are using MSCs to treat orthopedic diseases, degenerative diseases, and autoimmune diseases affecting single or multiple organs.

CELL HETEROGENEITY OF MSCS

According to the minimal criteria developed by the International Society of Cell Therapy in 2006 for defining MSCs, they must be adherent cells with a spindle-shaped morphology in standard culture conditions; they must express CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules; and they must be capable of differentiating into osteoblasts, adipocytes, and chondroblasts *in vitro*^[5]. However, cell-to-cell variation exists at multiple levels (Figure 1). First, MSCs can be found in various kinds of connective tissues, even when cultured under standard conditions. MSCs from adipose tissue and bone marrow show different surface markers and differentiation capacities because of distinct biological, chemical, and mechanical stresses in stem cell niches^[20]. For MSCs originating from the same tissue, those from different donors present distinct functions due to the influence of age, health condition, and other individual differences^[4]. Moreover, MSCs form clones, and cell heterogeneity exists both interclonally and intracolonally. Kuznetsov *et al*^[21] reported that only half of clonal MSC implants differentiated into bone in mice. Extracellular matrix genes and osteogenic transcription factor-related genes show increases in highly osteogenic clones compared to poorly osteogenic clones^[22]. MSCs within a clone also present differences in cell morphology and differentiation ability. For instance, cells located at the outer periphery of clones express higher levels of genes (*MKI67* and *PODXL*) related to cell proliferation, while extracellular matrix genes (*VCAM1*) tend to exhibit higher expression in interior MSCs^[23].

To identify cell subsets with specific functions in heterogeneous MSCs, cell surface markers and global molecular signatures have been continuously explored^[24]. Single-cell-derived colonies with small, rapidly dividing cells show a high colony-forming efficiency. STRO-1, CD146, and CD271 have been identified as cell surface markers^[25,26]. However, markers of proliferation cannot predict the differentiation potential of MSCs, and cell subsets sharing similar surface markers exhibit different chondrogenic differentiation abilities under the same culture conditions^[27]. RNA sequencing and microarray analysis have helped to elucidate transcriptional

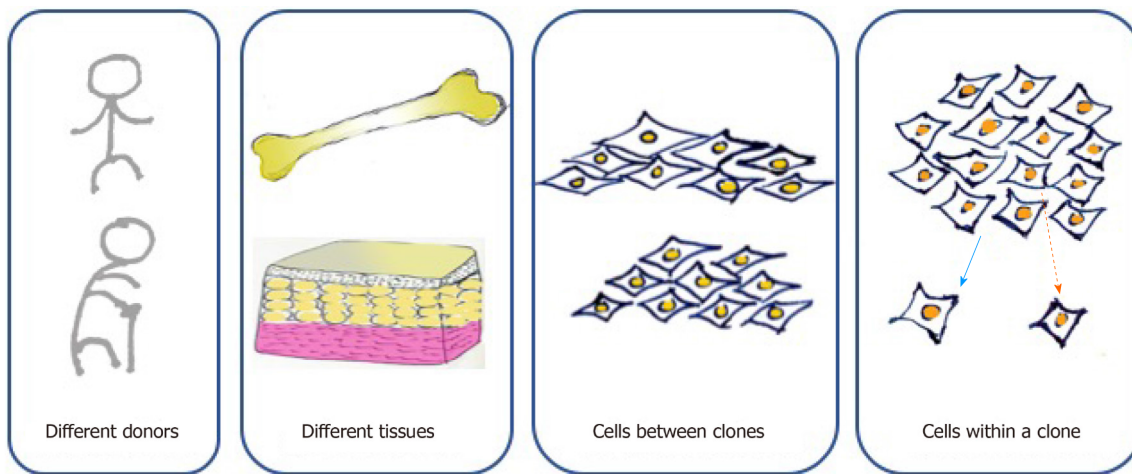


Figure 1 Cell heterogeneity of mesenchymal stem cells at multiple levels. Mesenchymal stem cells (MSCs) originating from different donors present distinct functions due to the influence of age, health condition, and other individual differences. MSCs originating from the same donor but different tissues, such as adipose tissue and bone marrow, show different surface markers and differentiation capacities because of distinct biological, chemical, and mechanical stresses in stem cell niches. Moreover, MSCs form clones, and cell heterogeneity exists both interclonally and intraclonally.

signatures predicting differentiation potential. Osterix and distal-less homeobox5 are the main transcription factors involved in osteoblast differentiation^[28], while peroxisome proliferator-activated receptor gamma and CCAAT/enhancer-binding protein alpha are a signature of adipogenic potential^[29]. In addition, MSCs with specific surface markers of differentiation potential may present various physiological functions. Studies have proved that CD105+ MSCs exhibit myogenic potential, which could help to repair the infarcted myocardium^[30]; however, the differentiation incidence is low *in vivo*. Other studies have shown that an enhanced paracrine effect could be more important in the myocardial repair of CD105+ MSCs, and the subpopulations need to be further identified^[31,32]. CD106 (VCAM-1) is a cell surface protein related to the adhesion of leukocytes to the vascular endothelium^[33]. CD106+ MSCs show enhanced multipotency, while their protein expression decreases with extended passaging or differentiation. Moreover, CD106+ MSCs display an immunosuppressive ability by mediating cell-to-cell contact with immune cells, as reported in human placenta^[34]. Increasing evidence shows that MSCs contain multiple subsets with specific surface markers. More work is needed to identify these subpopulations and clarify their biological functions.

SINGLE-CELL SEQUENCING

Cells are the smallest functional unit of organisms. Cellular heterogeneity is a universally acknowledged characteristic of biological tissues, including MSCs. Even in almost pure cell groups, gene expression differs among individual cells because of the variability of the intrinsic regulatory system and extrinsic microenvironment. Single-cell sequencing, which includes single-cell transcriptome-, epigenome-, and genome-sequencing technologies, provides powerful tools for characterizing heterogeneous cell populations^[10,13,35]. As scRNA-seq is one of the most widely used of these approaches, this review will mainly discuss the advances of scRNA-seq applications.

Tang *et al.*^[36] first reported the methodology for scRNA-seq in 2009, which provides an unbiased view of the transcriptome at a single-cell resolution. Since that time, scRNA-seq strategies have been constantly updated, including methods for cell capture, RNA capture, cDNA amplification, and library establishment and bioinformatics tools for bulk data analyses^[37,38]. For example, by using a combination of the one-bead-one-cell droplet approach and a special barcoding strategy, Drop-seq and inDrop systems were developed, which are high-throughput systems that can handle thousands or even tens of thousands of individual cells in a single experimental run^[39]. Defining subpopulations within a dataset of MSCs can be achieved by unbiased clustering and differential gene expression analysis^[40-42]. More recently, a “pseudotime” trajectory was produced in a reduced-dimension data space, which helps to record the spatial or lineage information of MSCs^[35,43]. To date, scRNA-seq has been widely applied in the fields of early embryo development, neuroscience, cancer, and stem cell research.

SINGLE-CELL SEQUENCING TO ASSESS THE MULTIPOTENCY FUNCTION OF MSCS

MSCs can differentiate into osteoblasts, adipocytes, and chondrocytes. Specific surface markers and global molecular signatures have been reported in studies based on bulk cells or tissues^[4,44]. Novel functional subsets of MSCs have been identified at a single-cell resolution in recent years^[7,9,13,45]. Worthley *et al.*^[45] identified a subpopulation of skeletal stem cells in mice that express gremlin 1 as osteochondroreticular stem cells in the bone marrow. This subset of cells can generate osteoblasts, chondrocytes, and reticular marrow stromal cells but not adipocytes. Chan *et al.*^[46] reported skeletal stem cells (mSSCs) with a similar differentiation potential in mice. Subsequently, using scRNA-seq, Chan *et al.*^[47] identified novel surface markers in human bone-derived cells that were similar to mSSCs. PDPN+CD146-CD73+CD164+ cells are likely enriched in human skeletal stem cells (hSSCs) in the bone. The self-renewal ability and multipotency of the cells are maintained, and injury-induced hSSCs (PDPN+CD146-CD73+CD164+) represent a regenerative response to skeletal injury in a human bone xenograft mouse model. In addition, hSSCs and hSSC-derived subsets express a series of potential hematopoiesis-supportive cytokines, including ANGPT1, CSF1, SDF, IL27, IL7, and SCF. Gene expression was also compared among hSSCs, mSSCs, and iPSC-derived hSSCs, and hSSCs isolated from any source were shown to cluster together. These cells are capable of forming the whole skeletal lineage hierarchy, which suggests great potential for use in regenerative medicine. Endosteal and outer periosteal compartments are both important to bone physiology, and each compartment maintains separate pools of cells separated by the bone cortex. Debnath *et al.*^[48] identified periosteal stem cells (PSCs) in mice that form bone in an intramembranous manner, which differ from other skeletal MSCs that mediate endochondral ossification. In addition, the discrete existence of PSCs suggests that bone is composed of separate pools of stem cell progenitors that provide a special derived microenvironment for each type of stem cells (Table 1). To some extent, PSCs are specialized for intramembranous bone formation. The identified subsets contribute to fracture healing as well as the modeling of the bone cortex, providing a promising novel clinical target for bone fracture regeneration.

Tissue-resident mesenchymal progenitors (MPs) are responsible for tissue maintenance and regeneration. Scott *et al.*^[9] revealed hypermethylated in cancer 1 (Hic1) to be a marker for MPs. scRNA-seq and ATAC-seq analysis demonstrated that Hic1+ MPs present distinct functions and lineage potential in skeletal muscle regeneration by providing stage-specific immunomodulation and trophic and mechanical support. Moreover, Giordani *et al.*^[49] mapped ten mononuclear cell types in mouse muscle by scRNA-seq and illustrated gene signatures and key discriminating markers in each group, which can help to understand more about muscle-resident cell type identities and muscle diseases.

Regarding adipose tissues, Liu *et al.*^[50] performed an analysis of adipose-derived mesenchymal stem cells (ADSCs) from three donors by scRNA-seq, and the resulting high-quality dataset is valuable for distinguishing the heterogeneity of cultured ADSCs at a single-cell resolution. Merrick *et al.*^[51] found that dipeptidyl peptidase-4/CD26+ mesenchymal cells are highly proliferative, multipotent progenitors that give rise to ICAM1+ and CD142+ committed preadipocytes that can differentiate into mature adipocytes. The *in vivo* origin of adipose stem cells is currently poorly understood. Schwalie *et al.*^[52] identified distinct subsets of adipose stem cells in the stromal vascular fraction of subcutaneous adipose tissue. The CD142+ group was shown to suppress adipocyte formation in a paracrine manner. The potentially key role of adipogenesis-regulatory cells in regulating adipose tissue plasticity is related to metabolic diseases such as type 2 diabetes.

Other studies have identified subpopulations of Col2a1-creER-marked neonatal chondrocytes that behave as transient mesenchymal precursor cells at the growth plate borderline^[53]. With the application of scRNA-seq technology, more subsets and specific surface markers of MSCs have been revealed, which helps not only to predict differentiation potential but also to explain the regulatory network under physiological and pathological conditions.

SINGLE-CELL SEQUENCING TO INVESTIGATE THE IMMUNOREGULATORY AND TROPHIC FUNCTIONS OF MSCS

MSCs can modulate both the innate and adaptive immune systems, including effects

Table 1 Characterization and function of stem cells

Ref.	Marker	Cell	Function	Species
Worthley <i>et al</i> ^[45]	Gremlin 1+	Osteochondroreticular stem cell	Generate osteoblasts, chondrocytes and reticular marrow stromal cells but not adipocytes	Mouse
Chan <i>et al</i> ^[46]	CD45-Ter119-Tie2-AlphaV+Thy-6C3-CD105-CD200+	Skeletal stem cells (mSSCs)	Bone cartilage and stromal progenitor	Mouse
Chan <i>et al</i> ^[47]	PDPN+CD146-CD73+CD164+	Skeletal stem cells (hSSCs)	Response to skeletal injury; express potential hematopoiesis-supportive cytokines	Human
Debnath <i>et al</i> ^[48]	CTSK-mGFP+CD200+CD105-	Periosteal stem cells (PSCs)	Form bone in an intramembranous manner	Mouse
Scott <i>et al</i> ^[9]	Hypermethylated in cancer 1 (Hic1) +	Mesenchymal progenitors (MPs)	Provide stage-specific immunomodulation and trophic and mechanical support	Mouse
Merrick <i>et al</i> ^[51]	Dipeptidyl peptidase-4 (DPP4)/CD26+	Mesenchymal cells	Give rise to ICAM1+ and CD142+ committed preadipocytes that can differentiate into mature adipocytes	Mouse
Schwalie <i>et al</i> ^[52]	CD142+	Adipose stem cells	Suppress adipocyte formation in a paracrine manner	Mouse

on neutrophils, macrophages, dendritic cells, natural killer cells, B lymphocytes, and T lymphocytes^[19]. For example, MSCs impede B lymphocytes from differentiating into plasma cells as well as secreting immunoglobulins. They can promote the generation of regulatory T cells while inhibiting the differentiation of helper T cells^[19]. The immunosuppression function can be executed *via* direct cell-cell interactions and paracrine actions. Many molecules secreted by MSCs are responsible for immunosuppression, including TGF- β , IL-10, PGE2, IDO, and NO. Although MSCs have been applied to treat several autoimmune diseases, such as Crohn's disease, rheumatoid arthritis, and systemic lupus erythematosus, the mechanism underlying the immunosuppressive ability of MSCs *in vivo* is not clear^[1,18].

In addition, MSCs are capable of supporting the maintenance, expansion, and differentiation of HSCs by producing growth factors, chemokines, interleukins, and extracellular matrix molecules. HSCs cotransplanted with MSCs *in vivo* ameliorated HSC engraftment and improved hematopoietic function recovery. In addition, MSCs secrete chemokines such as Ang-1 and CXCL12 to promote angiogenesis by recruiting endothelial progenitor cells. They can also produce neurotrophic factors that are important in neurogenesis and neurodegenerative diseases, such as amyotrophic lateral sclerosis and multiple sclerosis. The multipotency of MSCs is considered an important function for tissue regeneration and the treatment of degenerative diseases. However, less than 1% of transplanted MSCs could be found in the host bone of a patient who suffered from severe osteogenesis imperfecta. Similar observations were made in patients with eye diseases who were receiving MSC therapy, and no clear evidence showed MSC engraftment into the retina. Other functions, such as the roles of trophic factors, should also be considered in MSC therapy.

Although the importance of MSCs in bone marrow in supporting HSCs has been recognized since 1974^[14], the molecular complexity of this relationship and its response to stress are unclear. Tikhonova *et al*^[54] mapped the transcriptional signatures of bone marrow vascular, perivascular, and osteoblast cells in mice at single-cell resolution and revealed novel cellular subsets and cellular sources of pro-hematopoietic factors *in vivo*. The vascular notch delta-like ligands (Dll1,4) play critical roles in HSC differentiation and lineage commitment and are downregulated under stress-induced changes. These authors elucidated the cellular architecture of the bone marrow niche and revealed a heterogeneous molecular landscape that responds to stress. Severe *et al*^[24] reported that CD73+ BMSCs contribute to hematopoietic stem and progenitor cell engraftment and acute hematopoietic recovery.

Currently, effective cell surface markers are available to identify hematopoietic cells, and it is still difficult to elucidate the interactions with classical myeloid and

lymphoid cells *in vivo*. Jaitin *et al*^[55] analyzed spleen tissues using scRNA-seq, and single-cell transcriptional states in dendritic cells and hematopoietic cells were considered together to reveal gene-module activity and cell-type heterogeneity in both steady-state conditions and pathogen activation states.

SINGLE-CELL SEQUENCING TO INVESTIGATE CELL PROLIFERATION AND THE RESPONSE TO CULTURE CONDITIONS

Large numbers of MSCs are needed for clinical therapy, requiring cell expansion *ex vivo*. Although MSCs are capable of self-renewal and proliferation, these abilities decrease with time when the cells are cultured *in vitro* along with other functional changes^[56]. With cell cycle arrest and the loss of proliferation, some MSCs may undergo senescence^[56,57]. Oxidative stress and the dysregulation of regulatory factors associated with differentiation are related to the decreased differentiation potential of senescent MSCs. Bork *et al*^[58] revealed that DNA methylation identified at specific CpG sites is a typical epigenetic signature. When expanded *in vitro*, MSCs experience genomic DNA damage, and attempts to repair DNA damage seem to promote senescence, which could help defend against cell death. Other studies have shown that the functional decline associated with age can be reversed by manipulating epigenetic factors that are dysregulated. This could shed new light on the epigenetics of cell aging to improve therapeutic potential^[57].

Cells cultured *in vitro* are exposed to various conditions. The properties of the biomaterial interface, such as its topography, stiffness, and chemistry, can lead to transcriptional variations^[59-61]. Darnell *et al*^[62] found that the stiffness of hydrogels could regulate cytokine secretion by mouse mesenchymal stem cells. These authors further revealed many stiffness-sensitive genes by RNA-seq, which showed that stiffness can regulate the expression of MSC immunomodulatory markers. In addition, MSC signatures change as cell confluence increases *in vitro*, and 100% confluent MSCs may exhibit compromised pro-angiogenesis properties^[63]. Other studies are building upon the complexity of the niches produced *in vitro* to create a tissue-like system^[59]; however, there is a lack of research at a single-cell resolution regarding how cells sense and respond to the *ex vivo* culture microenvironment.

MSC THERAPY IN CLINICAL TRIALS

Lazarus *et al*^[64] reported the first use of MSCs as a cellular pharmaceutical agent in humans in 1995. Since that time, the number of clinical trials of MSCs has increased worldwide. The niche-like regenerative properties and anti-inflammatory ability of MSCs make them candidates for the treatment of acute tissue injury, chronic degenerative diseases, and inflammatory diseases^[1]. Conditional approval for MSC treatment in children with GvHD (graft-versus-host disease) was secured in 2012 in Canada, which was a historic event in the application of MSC therapy in the clinic. In addition, the use of MSCs to treat Crohn's-related enterocutaneous fistular disease was approved by the European Commission in 2018. Other high-quality clinical trials have focused on heart disease. Bartunek *et al*^[65] performed an MSC trial (NCT00810238) of heart failure secondary to ischemic cardiomyopathy in 2013, which showed improved cardiac outcomes. However, three years later, another phase 3 trial (NCT01768702) of the same MSC therapy for the treatment of chronic advanced ischemic heart failure was performed. No significant difference was found between the MSC-treated and placebo groups from baseline to 39 wk in the primary endpoint. The authors also evaluated ventricular remodeling at 52 wk. The data revealed an inverted U-shaped dose curve with worse outcomes at a higher dose delivery of MSCs^[66].

Although MSCs are effective for treating some cardiac patients, the underlying molecular mechanism is poorly understood. Lescroart *et al*^[67] revealed that the population of Mesp1 cardiovascular progenitors are the progenitors of distinct cell lineages and regions of the heart, identifying molecular characteristics in the early stage of mouse gastrulation. In addition to cardiomyocytes, interstitial cells, including fibroblasts and vascular and immune cells, are also important for heart repair. Farbehi *et al*^[68] identified more than 30 populations, representing nine cell lineages. The novel fibroblast lineage trajectory observed under both sham and myocardial infarction and in myofibroblasts leads to a uniquely activated cell state. To evaluate whether novel cardiovascular progenitors can differentiate into cardiomyocytes, scRNA-seq was

used, and laminin-221 was identified as the most likely cardiac laminin that was highly expressed during development. Moreover, cells coated with laminin-221 could be reproducibly differentiated at different time points^[69]. Future sc-RNA-seq studies will help to identify disease-related phenotypes and the transition trajectories of cells during disease development.

CONCLUSION

scRNA-seq has been applied and improved for more than ten years since 2009; it was highlighted as the “Method of the Year” in 2013^[70] and has recently become a routine approach for investigating cell heterogeneity. The challenges and limitations of the technology should also be considered. Eleven grand challenges in single-cell data science were reviewed by Lahnemann *et al*^[71]. For example, scRNA-seq data may show zero results, where a given gene has no unique molecular identifiers or reads mapping to it in a given cell. Such sparsity of scRNA-seq results can affect downstream analyses, which necessitates further methodological development. Regarding the application of MSCs, the lack of information other than transcript levels could lead to inaccurate analysis. Then, although novel cell surface markers and cell subpopulations may be identified by scRNA-seq, few interactions with surrounding cells and organisms can be demonstrated.

In the future, more efforts are needed to explore methods that provide multimodal data^[35]. Such methods may include the simultaneous profiling of multiple types of molecular data within a single cell, for instance, scRNA-seq coupled with DNA sequence or protein information. Other types of technologies may allow the detection and analysis of different kinds of data together. If the combination of multiple types of single-cell data across different experiments or experimental conditions can be achieved, we will obtain new information about MSCs from a transcriptome-centric perspective, which will undoubtedly improve the efficiency of MSC therapy.

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