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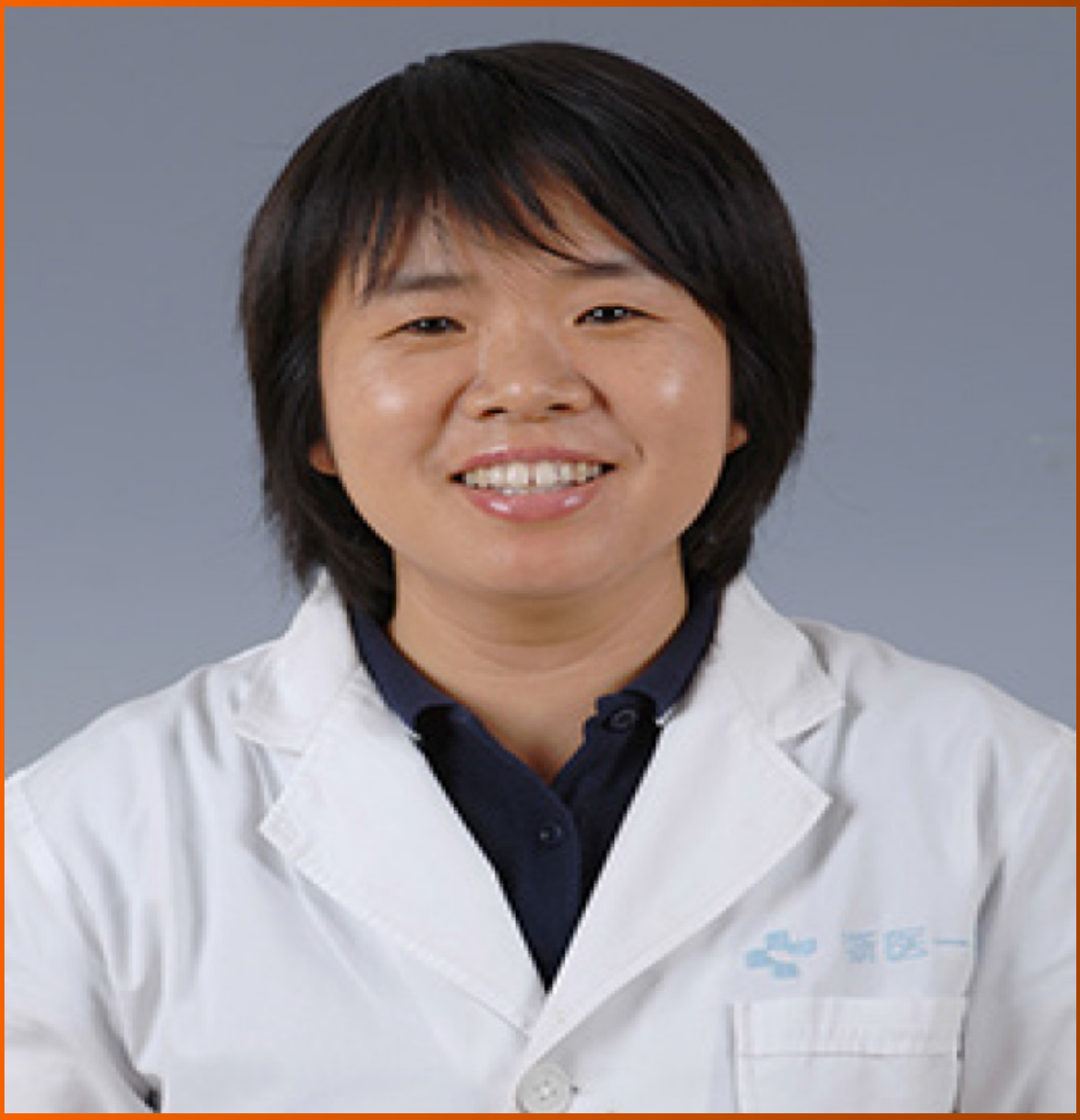

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REVIEW

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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