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MINIREVIEWS

Isolation and characterization of mesenchymal stem cells in orthopaedics and the emergence of compact bone mesenchymal stem cells as a promising surgical adjunct

Albert Anastasio, Marina Gergues, Michael S Lebhar, Pranela Rameshwar, Joseph Fernandez-Moure

ORCID number: Albert Anastasio 0000-0001-5817-3826; Marina Gergues 0000-0003-4464-3756; Michael S Lebhar 0000-0001-6560-3329; Pranela Rameshwar 0000-0003-0434-9034; Joseph Fernandez-Moure 0000-0003-1397-6320.

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Albert Anastasio, Department of Orthopedic Surgery, Duke University Health System, Durham, NC 27710, United States

Marina Gergues, Department of Medicine, Hematology/Oncology, Rutgers University, New Jersey Medical School, Newark, NJ 07103, United States

Michael S Lebhar, School of Medicine, Duke University School of Medicine, Durham, NC 27710, United States

Pranela Rameshwar, Department of Medicine-Hematology/Oncology, Rutgers School of Biomedical Health Science, Newark, NJ 07103, United States

Joseph Fernandez-Moure, Department of Surgery, Division of Trauma, Acute, and Critical Care Surgery, Duke University School of Medicine, Durham, NC 27710, United States

Corresponding author: Joseph Fernandez-Moure, MD, MSc, Surgeon, Department of Surgery, Division of Trauma, Acute, and Critical Care Surgery, Duke University School of Medicine, 2301 Erwin Rd, Durham, NC 27710, United States. joseph.fernandezmoure@duke.edu

Abstract

The potential clinical and economic impact of mesenchymal stem cell (MSC) therapy is immense. MSCs act through multiple pathways: (1) as "trophic" cells, secreting various factors that are immunomodulatory, anti-inflammatory, anti-apoptotic, proangiogenic, proliferative, and chemoattractive; (2) in conjunction with cells native to the tissue they reside in to enhance differentiation of surrounding cells to facilitate tissue regrowth. Researchers have developed methods for the extraction and expansion of MSCs from animal and human tissues. While many sources of MSCs exist, including adipose tissue and iliac crest bone graft, compact bone (CB) MSCs have shown great potential for use in orthopaedic surgery. CB MSCs exert powerful immunomodulatory effects in addition to demonstrating excellent regenerative capacity for use in filling boney defects. CB MSCs have been shown to have enhanced response to hypoxic conditions when compared with other forms of MSCs. More work is needed to continue to characterize the potential applications for CB MSCs in orthopaedic trauma.

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Core Tip: The use of stem cell therapies continues to emerge as available therapy for tissue engineering of orthopedic trauma. Compact bone mesenchymal stem cells (MSCs) have been studied for many years and have been found to have a greater orthopedic regenerative capacity compared to other autologous sources. Herein, we describe and review a novel source of MSCs from compact bone and their uses in orthopedic regeneration. This review is best suited for the traumatologist in search of a comprehensive review of this novel sources of MSCs and their potential uses *in vitro*, *in vivo*, and clinically.

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INTRODUCTION

Background on mesenchymal stem cells

The osteogenic ability of bone marrow aspirate was first demonstrated by Jones *et al*[1,2] in 1869. This ability was later attributed to the presence of mesenchymal stem cells (MSCs), which are a heterogeneous population of cells defined by surface markers[1,3,4]. MSCs isolated from animal models have consistently demonstrated capacity for multi lineage differentiation, pluripotency, and myriad MSC surface markers[5]. MSCs have also been extracted from human sources, and can be harvested from bone marrow, blood, or mesenchymal tissues (including bone, cartilage, meniscus, ligaments, and tendons). After harvest, they undergo isolation, expansion, and differentiation to create a lineage aimed at modulating the immune system and the regenerative response[6-9]. A common harvest site is from the bone marrow (iliac crest), and this specimen contains mixtures of mesenchymal progenitors with variable differentiation potential[10]. Hernigou *et al*[11] reported concentrations of approximately 400 mesenchymal stem cells/mL of iliac crest aspirate. Another site for harvest of MSC is adipose tissue, which is obtained through standard liposuction methods[12-15]. While these sites are capable of generating a robust number of MSCs, they are both surgically morbid procedures with their own sets of complications.

MSC isolation began in the 1970s, where initial investigations demonstrated that bone, cartilage, muscle and other mesenchymal tissue could be differentiated from embryonic stage chick limb bud mesenchymal progenitor cells[16,17]. MSCs were found to have the capacity to differentiate into cartilage, muscle, marrow stroma, tendons, ligaments, fat, and other connective tissues[15]. Recent technology developments for isolating and culture expanding adult marrow-derived MSCs has led to an explosion of research opportunities with current and future clinical applications[18-20]. Most recently, MSCs isolated from trabecular and cortical bone portions, referred to as cortical bone fraction MSCs (CB-MSCs), have emerged[4]. CB-MSCs have shown to have higher biosynthetic activity *in vitro* under hypoxic conditions when compared to MSCs of other sources[21]. These findings have profound implications since CB-MSCs have the unique ability to withstand the harsh conditions that may exist in recipient tissue. The mechanisms behind enhanced survivability in the hypoxic condition are numerous, and may include the enhanced production of a variety of protective cytokines[21,22]. Regardless, post-traumatic inflammation, reactive oxygen species, and compromised blood flow inducing hypoxic tissue state complicate the *in vivo* environment after fracture, and thus, CB-MSCs may be better suited for orthopedic tissue engineering than their bone marrow-derived counterparts[21].

From enhancing current techniques used to treat fractures or bolster fusions, to

tissue engineering and the opportunity to impact genetic diseases such as Osteogenesis Imperfecta or muscular dystrophy in cell replacement therapy, the number of patients that could be positively impacted by use of MSCs is wide-ranging. Prior to exploring current uses of mesenchymal stem cells in orthopaedic surgery and discussing emerging evidence in support for further research of CB-MSCs within orthopaedics, we will survey current source isolation and characterization techniques of MSCs.

Sourcing of MSCs

Today we have many sources of MSCs, including the two most commonly discussed – iliac crest bone marrow aspirate and adipose tissue. These have shown some benefit in achieving osseous regeneration in some clinical applications. However, there is a wide variation in refining methods and administration techniques within the current literature, and there has yet to be a standardized volume or concentration of MSCs within published data, which has led to varied results[23-25].

Mesenchymal progenitor cells have a prevalence of approximately one per 30000 nucleated cells from iliac crest bone marrow aspirate in some studies[9]. This calculates to around 600 progenitor cells per milliliter. This could be further increased to 2500 per milliliter by concentration techniques, such as centrifugation or freezing, or by small volume aspiration[4,9]. Large amounts of progenitor cells are required for most orthopedic applications, though, which makes bone marrow aspiration impractical. Thus, alternative sources of MSC where yield and osteogenic potential are greater is sought.

Adipose tissue, dental pulp, and umbilical cord MSCs are additional sources that have proven reliable sources of MSCs[16]. All these sources have their own advantages and disadvantages, but one common drawback shared by these sources is donor site surgical intervention required to acquire the cells. Further, while many sources have been identified and used experimentally in orthopedic regeneration what lacks is a consensus on what source is best suited for bony repair. Some studies have shown bone marrow MSCs to be equal to umbilical MSCs, but superior to adipose MSCs[26]. However, there is newer research showing extraction of MSCs from compact or cortical bone[27]. The benefit of this therapy is that it can harvested intra-operatively and can potential yield a population of cells predisposed to promoting an osteogenic niche. Compact bone has been identified as a viable and reliable source for MSCs. Using discarded bone from laminectomy specimens, Fernandez-Moure *et al*[28] demonstrated that CB-MSCs were found in the spine.

Given their origin, this unique population of cells holds significant potential for orthopedic regeneration. With intra-operative refinement techniques, this harvested compact bone could be a source of MSCs that could be administered during the same operative procedure[28]. Theoretically, a surgeon could derive cells from extracted bone and reimplant in the same procedure from the same source, thus overcoming many of the regulatory hurdles associated with a donor procedure. This will decrease costs, anesthesia time, and patient morbidity while giving improved outcomes in skeletal reconstruction. While determination of an ideal source remains an ongoing debate, isolation techniques once a source has been identified is an additional hurdle to the effective implementation of MSCs from various sources.

Isolation techniques of CB-MSCs

Since Friedrichstein identified the isolation of cells of the mesenchymal lineage, many methods for the isolation of mesenchymal progenitor cells have been described (Table 1). The isolation of bone derived MSCs was first described by Robey *et al*[29]. In this study human bone cell cultures were established by maintaining collagenase-treated, bone fragments in low Ca^{2+} medium and the technique described provided a useful system for the study of osteoblast metabolism *in vitro*. This method of isolation has been adapted for use in clinical samples. Tuli *et al*[30] described a method where reaming debris was taken from the intramedullary canal of femurs undergoing total hip arthroplasty. Reaming debris was then taken and underwent collagenase XI digestion for 3-4 d until cellular material had disappeared. Boney fragments were then transplanted into new flasks and allowed to culture so that cells would migrate out from the bone and onto the plastic surface. Those cells underwent multilineage characterization and immunohistochemical analysis. Similarly, others have used mono-enzyme digest using collagenase I or II as the sole processing agent for MSC isolation[4,31,32]. In order to enhance the selectivity of the cell population isolated Gangji *et al*[33] coupled a Collagase and Dipase digest with fluorescent assisted cell sorting for MSC specific makers. This method of characterization lead to a homogenous population of MSCs as defined by the standards of the International Society for

Table 1 Source and isolation methods of mesenchymal stromal cells

Source	Isolation method	Ref.
Bone marrow	Aspirates cultured and media changed every 3-4 d to select for MSCs	[34]
	Aspirates layered over Ficoll-Paque density-gradient and plates in tissue culture dish. Adherent cells maintained with periodic passaging	[82]
	Bone marrow mononuclear cells seeded from single colony-forming unit fibroblasts and selected for by CD105(+)/CD45(-)	[83]
	Sort bone mononuclear cells based on aldehyde deydrogenase expression (ALDHhighCD45-)	[84]
	Sort based on CD45−/lowCD271+ phenotype following a microbead-based pre-enrichement	[85]
	Layer bone marrow over hyluronic acid followed by centrifugation, collect most superficial layer containing the mononuclear cells	[86]
Compact bone	Trabecular bone fragments rinsed and placed in complete α-MEM/Ham's F12, confluent monolayers were obtained within 10-20 d	[87-89]
	Bone cell cultures established by treating bone fragments with collagenase in low Ca^{2+} medium	[9,90, 30]
	Compact bone fragments obtained, cultured, and isolated. CB-MSCs then undergo trypsinization to reveal enhanced osteogenic capacity	[43]
Adipose	Place 10-20 mL of washed adipose tissue in 100 mm Petri dish; dissect out yellow tissue; mince tissue finely and place in enzymatic digestion solution; centrifuge and collect pellet for wash; resuspend in complete culture medium	[14,13]
	Wash lipoaspirate with PBS; enzymatically digest using collagenase 1A solution; spin down cells, wash and plate in complete medium	[12]
Bone marrow	Aspirates cultured and media changed every 3-4 d to select for MSCs	[34]
	Aspirates layered over Ficoll-Paque density-gradient and plates in tissue culture dish. Adherent cells maintained with periodic passaging	[82]
	Bone marrow mononuclear cells seeded from single colony-forming unit fibroblasts and selected for by CD105(+)/CD45(-)	[83]
	Sort bone mononuclear cells based on aldehyde deydrogenase expression (ALDHhighCD45-)	[84]
	Sort based on CD45−/lowCD271+ phenotype following a microbead-based pre-enrichement	[85]

MSC: Mesenchymal stromal cell; CB: Compact bone; PBS: Phosphate buffer saline.

Cellular Therapy[34]. Relied solely on cellular migration outward from the boney reamings and the inherent property of stem cell plastic adherence to isolate their cell population. They used the bone reamings of patients with closed diaphyseal femur fractures who were undergoing internal intramedullary nail fixation for cellular extraction without any additional agents. Cells adherent after ten days were transferred to a new flask and grown to confluence prior to phenotypic characterization. While no comparison was made to other methods of isolation the authors did demonstrate the capability of the bone reaming derived cells to transform into both neuron-like cells and functional osteoblasts. This suggested that cells derived from the bone itself were capable of transdifferentiation, a characteristic of MSCs.

Various processing agents and isolation methods have been described for the isolation of CB MSCs as well. Zhu *et al*[25] 2010 described a protocol for the isolation and culture of large numbers of murine MSCs (mMSCs) from compact bones in contrast to mMSCs culture from bone marrow, the bone marrow cavities are flushed at least three times in order to thoroughly deplete hematopoietic cells[35-37]. The mouse compact bones are then dissected into fragments of 1-3 mm^3 and digested with collagenase II. The released cells are discarded and the digested bone fragments are cultivated in an MSC culture medium. In contrast to the frequent medium changes in primary culture required in the mouse bone marrow culture technique, the culture medium is not changed until the third day after the initiation of culture[35]. During cultivation, fibroblast-like cells are observed around the collagenase-digested bone fragments within 48 h of cultivation. The mMSC cultures reach 70%-80% confluence within 5 d in the first passage and significant numbers (> 10^7) of mMSC can be harvested in a short time from one mouse.

This protocol has been modified from the original description by Guo *et al*[24]. Where muMPC, murine mesenchymal progenitor cells, culture was developed by addition of bone fragments with mouse bone marrow cells in the presence of basic fibroblast growth factor and bone fragment-conditioned medium. They postulate that murine

counterparts were able to be purified with adherent culture of either enzyme-treated bone fragments or the released cells[38-42]. In the protocol the femurs and tibiae were collected from 2 to 3-wk old C57BL/6 female mice. The epiphyses were removed, bone marrow was flushed out, and the bone cavities were washed thoroughly. In the presence of collangease II the cells were allowed to migrate out and seed culture plates. Cai *et al*[43] expanded upon these methodologies by obtaining compact bone fragments, culturing them, and isolating CB-MSCs. After trypsinization of these cell lines, the cultured fragments exhibited significantly higher proliferation and were accompanied with less CD45 expression but more CD90 and CD44 expressions. Moreover, the capacity for osteogenic and adipogenic differentiation of the MSCs obtained from the cultured compact bone was enhanced when compared to the cells harvested from bone marrow[43].

Varying methods of cell isolation have been described yet none has been shown to be superior to another. After isolation of MSCs from a donor source, characterization of the obtained cells is necessary to maximize downstream utilization as well as to be able to standardize methodology for future investigation (Table 1).

Characterization techniques

Techniques have been developed to source, isolate, and experimentally assess CB-MSCs.

Many laboratories have developed methods for the extraction and expansion of MSCs from bone. Once isolated, the characterization of the cell population is critical to its downstream uses and potential. The variation of methods used and the variety of tissue sources used has lead to significant differences in the cell population isolated and the need for minimal criteria for the definition of a mesenchymal stromal cell was required to standardize investigation. To address this need, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy established the minimal criteria by which a cell could be characterized and thus named a mesenchymal stromal cell[44]. Three criteria were proposed: (1) cell adherence to plastic; (2) specific surface antigen expression; and (3) multipotent differentiation.

These criteria allowed for a standard to be set for those working in isolation of CB-MSCs. First, the isolated cells must be adherent to plastic; Second, > 95% of the isolated cells must express CD 105, CD73, and CD90 measured with flow cytometry. In addition, the absence of CD45, CD34, CD14 or 11b, CD79 or CD19 and human leukocyte antigen class II must be documented in < 2% of the cell population; Lastly, the cells must be able to differentiate, under appropriate conditions, into adipocytes, chondrocytes, and osteocytes. Prior to the establishment of the aforementioned standard by the International Society for Cellular Therapy many characterized the cell isolated from the bone by their ability to tri-lineage differentiate. While this proved useful for their translational potential it underestimated the true potential and full character of the cell itself. CB-MSC identified have shown all these characteristics. Further, studies into source specific markers for MSC in addition to those identified are currently underway.

Clinical applications of MSCs in orthopaedic surgery

MSCs have long been utilized in orthopaedic surgery, and their clinical applications are robust. The economic impact that these therapies have the potential to affect is immense. Public health investigations have shown that osteoarthritis is associated with an economic burden of approximately $150 billion (2007 dollars), and is projected to affect 25% of the adult United States Population by 2030[11,45]. From the standpoint of orthopedic trauma, it has been estimated that 100000 fractures will go onto non-union each year in the United States alone[17]. Zull *et al*[46] looked at direct and indirect costs of reamed intramedullary nailing for tibial fractures compared to casting, casting plus ultrasound, and non-reamed intramedullary nailing. They found that reamed intramedullary nailing led to significant reduction in non-union rates, and calculated an approximate cost of $11800 (Canadian) per non-union case[46]. Beaver *et al*[47] looked establish tibial non-unions and calculated their surgical and medical care to cost United States $ 11333 per case. From increasing union rates, enhancing fusions, minimizing bone stock defects after total joints to treating articular cartilage defects, tendinopathies, avascular necrosis or bone cysts, the full impact of applications of MSCs is difficult to quantify both clinically and economically. The possibilities are as diverse as they are fascinating, and the ability to consistently improve even one of these complications or disease processes would have a great impact on the patients that are involved and the healthcare system.

While the field of orthopaedic surgery has pioneered the use MSCs many other fields within medicine have the potential to benefit from the knowledge gained from

working with MSCs (Table 2). Within the field of head and neck surgery, animal research has shown promise with the use of MSCs along with mandibular distraction osteogenesis producing improved total bone production and compact bone ratios in the "regenerate" or bone formed at the distraction site[48]. Liu *et al*[49] published a case report of their success using bone marrow aspirated MSCs to treat a patient with poorly controlled diabetes mellitus and a severe post-traumatic infection causing non-healing skin defect in the hand. The wound demonstrated complete healing 10-d after treatment saving the patient from wrist amputation[49]. Using a rat-model of ischemic cardiomyopathy, Tano *et al*[50] showed improved cardiac function after application of a pericardial MSCs embedded within a biodegradable carrier membrane.

The use of MSCs for regeneration of the musculoskeletal system is an area of much basic science research and evolving clinical applications[51-54]. Over the past twenty years, MSCs have been used clinically in a variety of scenarios to enhance the outcome for orthopedic patients from the standpoint of both wound healing and pain[55]. Dating back to the 1980s, bone grafting and bone marrow injection into fracture sites has been utilized to enhance union[4]. The use of MSCs in the setting of non-unions and critically sized defects, where bone's natural regenerative capacity to heal without scarring is impaired due to poor biology and/or biomechanical environment, was the focus of early investigations[6,16]. Connolly *et al*[32] published a case series of 20 tibial non-unions treated with unprocessed bone marrow with 90% demonstrating union at 6-8 mo follow-up. Hernigou *et al*[56] discussed the use aspirated iliac crest marrow with centrifugation to separate out osteoprogenitor cells (based on fibroblast colony forming units, CFU-F) for re-injection in sixty tibial non-unions. Fifty-three of the 60 patients went on to union at an average of twelve week[56]. Clinical applications of MSCs have since expanded to include healing of high-tibial osteotomies, large bone defects after total hip arthroplasty and trauma, distraction osteogenesis, treatment of avascular necrosis, articular cartilage defects, tendinopathy (patellar, lateral epicondylitis), spinal fusion, and treatment of bone cysts[2,4,31,55,57-60]. These findings paved the way for future use and investigations to understand the mechanisms underlying the observed wound healing effects. While MSCs have certainly proved to have widespread efficacy in orthopaedics, CB-MSCs, remain underutilized and under-investigated within the field.

The potential of CB-MSCs

MSCs have widespread uses within orthopaedics, but traditionally, the cortical bone fragment remains underutilized. Emerging developments have allowed for the isolation and characterization of CB-MSCs. Thus, investigation of the efficacy of CB-MSCs in murine models has been undertaken with very promising results. CB-MSCs appear to have beneficial immunomodulatory properties, which indicates that they may have great potential for utilization in cases requiring boney augmentation.

Repair of large bone defects still poses a major challenge for the orthopedic surgeon. For instance, it is widely known that these defects cannot heal on their own or repair themselves to a fully functional tissue. To overcome these issues, orthopedic surgeons generally implant a section of bone tissue. Unfortunately, this can lead to immune rejection or infections. The severity of these potential complications necessitates careful consideration of the immunological milieu surrounding the boney defect. With their diverse biological properties and efficacy, MSCs have long been considered as the ideal cells for cellular therapy. MSCs have the potential of secreting factors such as cytokines and exosomes, to produce varied effects within a specific micro-environment[61-64]. Once educated, a process also called "licensing", MSCs turn into anti-inflammatory cells within their niche, which can exert immunosuppressive functions to affect other cells within the immune system[6,7,65,66].

MSCs have obvious beneficial effects on the inflammatory milieu, and traditionally, these cells have been isolated from bone marrow and adipose derived MSCs. Emerging techniques have allowed for the additional isolation of MSCs from the cortical or compact bone. MSCs harbored within these tissues have been shown to be isolated in large quantities making re-transplantation a clinical reality. Once isolated their osteogenic regenerative potential has been measured and, *in vitro*, has been shown to be superior to adipose or bone marrow derived MSCs. Blashki *et al*[67] demonstrated that MSCs isolated form the cortical bone had greater potential for colony forming unit formation *in vitro* and greater osteoid generation *in vivo*. Similarly, Murphy *et al*[68] demonstrated an enhanced potential for CFU formation when cortical bone MSCs were cultured on bioactive osteogenic scaffolds. While the potential for osteogenesis was demonstrated in these studies a direct comparison had not established which tissue surface was ideal for osteogenic regeneration.

Moreover, like MSCs obtained from other sources, CB-MSCs appear to have

Table 2 Potential clinical scenarios for use of mesenchymal stromal cell therapy

Ref.	Animal model/methods	Findings	Conclusion	*In vitro/in vivo*
Ogulur *et al*[91]	mCB-MSCs isolated from 6–8 wk old BALB/c mice	mCB-MSCs significantly reduced cellular immune infiltration and presence of goblet cells as well as the thickness of epithelium, smooth muscle layers, and basement membrane in ovalbumin induced chronic asthmatic mice	Inflammation in distal and proximal airways of ovalbumin induced asthmatic mice can be suppressed by use of IV mCB-MSCs	*In vivo*
Qiao *et al*[76]	CB-MSCs isolated from C57BL/6 mice administered to 8–10 wk old BALB/c mice	BALB/c mice exposed to 8 Gy TBI and treated with CB-MSCs showed improved survival, body weight, and CFU-GM counts of bone marrow cells coupled with suppressed Th1 immunity with increased Treg percentages and decreased IFN-γ, CXCR3 and CCR5	CB-MSC transplantation post total body irradiation attenuates radiation-induced hematopoietic toxicity and provides immunoprotection	*In vivo*
Duran *et al*[92]	Cortical bone–derived stem cells from 12-wk-old EGFP+ transgenic mice	Improved 6 wk survival post MI procedure (50.4% to 76.5%) from saline to CB-MSC therapy. Increased expression of proangiogenic paracrine factors (bFGF and VEGF) and differentiation into infarct zone	Treatment with CB-MSCs post MI leads to enhanced survival, cardiac function, and remodeling	*In vivo*
Cheng *et al*[93]	MSCs isolated from compact bone of Tg26 HIV-1 transgenic mice	Transplanted Tg26 HIV-1 MSCs were less effective in protecting renal tubular cells compared to healthy mice MSCs in a cisplatin-induced AKI model due to inferior proliferation and decrease in secretion of protective cytokines	Compact bone MSCs infected with HIV-1 had impaired proliferation, differentiation, and function resulting in less therapeutic potential	*In vivo*
Yamachika *et al*[94]	MSCs from compact bone of 5-week-old C57-GFP male mice	Cells cultured in bFGF-conditioned medium demonstrated trilineage differentiation potential even at passage 24 in contrast to leukemia inhibitory factor-conditioned medium	Compact bone MSCs cultured in bFGF-conditioned medium demonstrated bone formation ability *in vivo*	*In vivo*
Bakker *et al*[95]	Tibial reaming debris from adult female sheep	Treatment with reaming debris, similar to iliac crest, revealed larger callus volume with decreased cartilage in the fracture gap, increased bone volume, and improved toughness at 3 wk with greater torsional stiffness at 6 wk	Reaming debris has characteristics similar to iliac crest bone that allow it to be an excellent replacement for enhancing healing of bone defects fixed with an intramedullary nail	*In vivo*
Guo *et al*[24]	Murine mesenchymal progenitor cells (muMPCs) isolated from 2-3 wk old C57BL/6 female mice tibia/femur compact bone	Collagenase-digested bone fragments produced muMPCs that inhibited Con A-stimulated splenocyte proliferation and suppressed lymphocyte activation by allogeneic cellular stimuli *in vitro*. In addition, muMPCs improved survival of allogeneic skin grafts *in vivo*	Using this protocol allows acquiring of muMPCs with similar properties to marrow counterparts, which allows them to be used in future investigations with mouse models	*In vivo*
Lim *et al*[96]	hABMSCs	hABMSCs exposed to low-intensity pulsed ultrasound revealed increased ALP, expression levels of CD29, CD44, COL1, and OCN, and calcium deposition	Treatment with LIPUS could improve the cell viability and osteogenic differentiation of hABMSCs	*In vitro*
Lim *et al*[97]	hABMSCs	hABSMSCs treated with extremely low frequency pulsed electromagnetic fields (ELF-PEMFs) revealed 15% increased proliferation at day 5, increased ALP, vinculin, vimentin, and CaM expressions, and enhanced mineralization during osteogenesis	Exposing hABMSCs with ELF-PEMFs could improve and accelerate the process of early cell proliferation mediated osteogenesis	*In vitro*
Lim *et al*[98]	hABMSCs harvested from human mandibular alveolar bone	hABSMSCs exposed to LFDSS for 10–60 min/d demonstrated improved viability, proliferation, and mineralization in culture with osteoblasts. ALP activity and gene expression of IBSP, COL-I, OCN, and OPN increased	Proper intensity and exposure time of LFDSS to hABMSCs can improve their differentiation and maturation	*In vitro*
Soleimani *et al*[35]	MSCs isolated from 6-8 wk old BALB/c mouse tibial and femoral bone marrow	The protocol states MSCs should be cultured in Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) in a 37 °C–5% CO_2 incubator with passage at 2 wk of culture	This protocol allows development of a purified population of MSCs 3 wk after the initiation of culture	*In vitro*
Dominici *et al*[44]	Human multipotent MSC	In standard culture, MSC must be plastic-adherent, express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR and demonstrate tridifferentiation *in vitro*	Standard criteria for MSC characterization, will allow for exchange of more uniform data between researchers	*In vitro*
Wenisch *et al*[99]	Mesenchymal stem cells harvested from HRD of 12 adult patients with closed diaphyseal femoral fractures	With neuronal induction, MSCs assumed neuronal morphologies and expressed neuron-specific enolase, beta-III-tubulin, neurofilament-H and HNK-1. Similar to immature neurons, MSCs had features of neuritogenesis and synaptogenesis and lacked electrical signaling	Neuronal induction allowed initiation of the early neuronal differentiation, but exposure to non-neurological stressors led to necrotic alterations	*In vitro*

Wenisch *et al*[100]	Mesenchymal stem cells harvested from HRD of 12 adult patients with closed diaphyseal femoral fractures	After multiple passages, HRD-derived cells and MSCs maintained a nondifferentiated phenotype and showed osteogenic and neuronal pathway differentiation ability after induction	Human reaming debris provides a multipotent stem cells which have the ability to grow and proliferate *in vitro*	*In vitro*
Tuli *et al*[90]	Collagenase-treated human trabecular bone chips	Collagenase-treated trabecular bone fragments contain cells that stain positive for CD73, STRO-1, and CD105, and negative for CD34, CD45, and CD144 with tridifferentiation potential	Trabecular bone-derived cells maintain a nondifferentiated phenotype and display tridifferentiation potential with long-term *in vitro* culture	*In vitro*

AKI: Acute kidney injury; bFGF: Basic fibroblast growth factor; CBSC: Cortical bone stem cell; CB-MSCs: Compact bone mesenchymal stem cells; CFU-GMC: Colony-forming unit granulocyte/macrophage; LIPUS: Low intensity pulsed ultrasound; MI: Myocardial infarction; TBI: Total body irradiation; VEGF: Vascular endothelial growth factor; HRD: Human reaming debris; hABMSC: Human alveolar bone-derived mesenchymal stem cell; LFDSS: Low fluid dynamic shear stress; ALP: A lkaline phosphatase; HLA: Human leukocyte antigen; HIV: Human immunodeficiency virus.

excellent immunomodulatory properties. Previously, Guo *et al*[24] performed skin grafting in a mouse model (C57BL/63BALB/c) with or without murine CB-MSCs pre-transfusion in order to determine if these cells had immunosuppressive effects *in vivo*. Their findings indicated that the delivery of these cells caused a significant increase in survival of allogeneic skin grafts further ascertaining the anti-inflammatory role that these cells exert on the *in vivo* immune response. Recently, some studies have shown that CB-MSCs are both multipotent and capable of extensive *in vitro* expansion similar to BM-MSCs, enhancing their therapeutic appeal in the field of orthopedics[69,70]. Besides phonotypical properties, CB-MSCs have been shown to share with BM-MSCs functional properties such as tri-differentiation potential in adequate conditions and immune suppression both *in vitro* and *in vivo*[71-73]. For instance, in a mouse model of acute graft-versus-host disease, Zhu *et al*[25] reported a decrease in tissue damage after transfusion of murine CB-MSCs, potentially altering the phenotype and function of splenic lymphocyte[74]. Similarly, these authors inferred that CB-MSCs also affected functional properties of T lymphocytes and dendritic cells by modulating their migratory behavior leading to a delayed lethal acute graft-versus host disease reaction[75]. Furthermore, Qiao *et al*[76] demonstrated a significant protective benefit of CB-MSCs in a radiation-induced hematopoietic toxicity mouse model. CB-MSCs acted to alleviate lymphocyte-mediated CFU-GM, colony-forming unit granulocyte/macrophage, inhibition and expand regulatory T cell lineages. They also mitigated T cell chemokine receptor expression and shifted the Th1/Th2 balance toward anti-inflammatory Th2 polarization[76].

In another study, authors investigated the impact of CB-MSCs in airway remodeling and inflammation in experimental ovalbumin-induced mouse model of chronic asthma. The authors infused GFP-labeled murine CB-MSCs which were located in the lungs of OVA group 2 wk after intravenous induction accompanied with a significant Treg response in ovalbumin-treated mice. It is worth noting that increase in Treg cell numbers along with other factors such as cytokines, to be linked to MSC-mediated immunomodulation[77]. Thus murine CB-MSCs could be effective at reducing an allergic inflammation. Furthermore, Shan *et al*[78] demonstrated mitigation of prion disease in brain extracts from infected mice after administration of CB-MSCs by enhancing microglial activation. Remarkably, the Intra-hippocampus transplantation of CB-MSCs had a small but statistically significant effect on prolonging the survival of mice inoculated with the Chandler prion strain[78].

CB-MSCs appear to not only have efficacy from an immunomodulatory standpoint, but may serve as an ideal scaffold material adjunct to repair boney defects. CB-MSCs when compared with BM-MSC, are bigger in size, show a lower proliferation rate at early passages, and have a greater commitment toward the osteogenic lineage. This cell source has been shown *in vitro* to generate greater alkaline phosphatase and calcium deposition in both normoxic and hypoxic conditions[23]. MSCs attached to three-dimensional scaffold designed to mimic the biological and mechanical role of extracellular matrix can be a faster approach to promote bone regeneration[79]. To date, several scaffolds have been used in MSC-based bone augmentation procedures. For these scaffolds, most of the literature reports on hydroxy apatite, b-tricalcium phosphate or a mixture of the two as mineral component interacting with MSC[80,81]. These scaffolds for bone engineering should possess key characteristic specifications including: osteo-conductivity, biocompatibility (adequate biological response), biodegradability, easily manufactured and sterilized, easily handled in the surgery room, and cost effective[82-85]. Moreover, the scaffold should have an architecture that resembles the structure of bone.

Thus, with their vast appealing functional roles, including immunosuppression, CB-

MSCs are ideal cells for cellular therapy in bone tissue engineering. Several researchers have proposed using CB-MSCs and three-dimensional scaffolds and implanting this combination into donor patients. To date, however, very few studies have looked into the use of CB-MSCs and scaffolds for compact bone regeneration. Perhaps with future research, CB-MSCs will be also considered as promising candidates for use in development of bioengineered bone to potentially impact clinical therapy and possibly beneficial to in bone engineering and regeneration[86-100].

CONCLUSION

MSCs hold great promise for regenerative therapies in osteogenic surgery. While there is still debate on the ideal source of MSCs to use in tissue regeneration, the field is still moving in the right direction for clinical applications. Previous work from our lab shows that compared with BM-MSCs and AD-MSCs, CB-MSCs have superior ability to survive in hypoxic conditions while remaining biosynthetically active[27]. CB-MSCs have been demonstrated to have excellent immunomodulatory efficacy in various animal models. More work needs to be continued both *in vitro* and *in vivo* to properly characterize these cells and make them functional for tissue engineering and regeneration.

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