Adult Multipotent Stromal Cell Technology for Bone Regeneration: A Review

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Since the discovery of bone marrow derived stromal cell osteogenesis in the 1960s, tissue engineering with adult multipotent stromal cells (MSCs) has evolved as a promising approach to restore structure and function of bone compromised by injury or disease. To date, accelerated bone formation with MSCs has been demonstrated with a variety of tissue engineering strategies. Though MSC bone tissue engineering has advanced over the last few decades, limitations to clinical translation remain. A current review of this promising field is presented with a specific focus on equine investigations.

ADULT MULTIPOTENT STROMAL CELLS (MSCS)

The promise of adult MSCs for tissue regeneration is a topic of intense interest. It is only relatively recently that reservoirs of cells with the capacity to differentiate into tissues derived from the same embryonic germ layer, defined as multipotent, were discovered. The Mesenchymal and Tissue Stem Cell Committee (MTSCC) of the International Society for Cellular Therapy established that to be defined as an MSC, a cell must: (1) adhere to plastic (plastic adherence) when maintained in standard culture conditions; (2) express cell surface antigens CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14, or CD11b, CD79α, or CD19, and HLA-DR; and (3) differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro*.[1]

There are numerous tissue sources of adult MSCs and an even greater number of proposed MSC-based therapies (Table 1).[2–5] Whereas the origins of "stem cell" science date back to the nineteenth century, a relatively recent resurgence in popularity has resulted in substantial advances in knowledge of the benefits and limitations of this novel treatment option.[6] Many early MSC discoveries were related to osteogenesis, and the ability to differentiate into osteoblasts remains one of their defining features. Hence, this area of MSC science has a long history that has promise to advance companion animal fracture treatment. Since an in-depth description of some relevant MSC terminology definitions has recently been presented,[6] this review will focus on the history, recent progress, and indispensable future endeavors surrounding MSC osteogenesis, beginning with a brief update on current naming conventions.

The nomenclature of MSCs reflects the dynamic nature of the field including, among other things, the discovery of numerous MSC sources, varying gene expression profiles among cell isolates, and a lack of universal MSC cell surface markers.[7–12] Caplan popularized the term mesenchymal stem cell to refer to the nonembryonic subpopulation of cells isolated from bone marrow and periosteum in 1991.[13] Subsequently, the MTSCC encouraged replacement of the term "stem" in MSC with "stromal" as well as addition of the descriptor "multipotent" for cells defined by the criteria above to give the moniker "multipotent mesenchymal stromal cells."[14,15] There is lack of consensus surrounding the potential redundancy of the terms mesenchymal and stromal since, by definition, stromal cells are derived from the embryonic mesoderm to which mesenchymal refers. For purposes of this review, the acronym MSC will be used for the name multipotent stromal cells.

ADULT MSC ISOLATION AND EXPANSION

MSCs have been isolated from numerous tissues including periosteum,[16] adipose,[17] synovial membrane,[18] skeletal muscle,[4] lung,[19] deciduous teeth,[20] umbilical cord,[21] blood,[22] skin,[5] pericytes,[23] and ear,[24] to name a few. Once harvested, relatively crude cell isolates are placed into specialized cultureware with nutrient medium. MSCs, a small fraction of the cell component of adult tissues, possess the characteristic of "plastic adherence" and, in contrast to mature tissue cells or hematopoietic cells, attach to cultureware, typically within 24 hours. The cells then divide to yield "clones." Percent confluence refers to the amount of the cultureware surface covered by cells. When cells nearly cover the cultureware surface, typically 70–80% confluence, they

Table 1 Representative Animal Models of MSC Bone Regeneration

Species	Application	Reference/Year	Cell Type	Scaffold	Follow-Up (Weeks)
Dog, Athymic mouse	Subcutaneous, intramuscular	Kadiyala et al/1997	BMSCs	HA/TCP, HA	4, 8 (dogs); 3, 6 (mice)
Dog	Femoral defect	Bruder et al/1998	BMSCs	HA/β-TCP	0, 4, 8, 12, 16
Sheep, Athymic mouse	Tibial defect, subcutaneous	Kon et al/2000	BMSCs	HA	0, 3. 6, 8
Mouse	Calvarial defect	Cowan et al/2004	ASCs, BMSCs	PLGA	2, 4, 8, 12
Sheep, Athymic mouse	Metatarsal defect, subcutaneous	Bensaid et al/2005	MSCs	Coral and CHA	0, 4, 8, 12, 16, 56 (sheep); 12 (mice)
Rabbit	Femoral defect	Dallari et al / 2006	BMSCs	FDBA, PRP	2, 4, 12
Dog	Mandibular defect	Ito et al/2006	BMSCs	Fibrin, PRP	2, 4, 8
Goat	Spinal fusion	Kruyt et al/2006	BMSCs	HA, BCP, and TCP	9
Dog	Mandibular defect	Yuan et al/2007	BMSCs	β-TCP	4, 12, 26, 32
Rabbit	Calvarial defect	Di Bella et al/2008	ASCs	PLA	6
Rat	Spinal fusion	Lopez et al/2008	ASCs	β-TCP/ COL1	0, 4, 8
Miniature pig	Mandibular defect	Zheng et al /2009	SHED	β-TCP	2, 4, 12, 24

ASCs, adipose derived multipotent stromal cells; BCP, biphasic calcium phosphate; BMSCs, bone marrow derived multipotent stromal cells; CHA, coralline-based HA; COL1, collagen type 1; FDBA, freeze-dried bone allografts; HA, hydroxyapatite; SHED, stem cells from human exfoliated deciduous teeth; PLA, polylactic acid; PLGA, poly lactic-co-glycolic acid; PRP, platelet-rich plasma; TCP, tricalcium phosphate.

are detached from the surface, counted, and then added to another culture vessel at a specific cell seeding density (cells/cm^2 vessel surface area). Each time cells are passed from one vessel to another is called a "passage." By "passaging" cells multiple times, and always reseeding at a relatively low density, the number of cells is increased exponentially because of cell division. Increasing the number of cells by culture of cell passages is referred to as the process of "expansion."

Many strategies to harvest MSCs from adult bone marrow[3,25–27] and adipose tissue[28–30] are reported. Isolation of MSCs from bone marrow aspirates typically involves segregation of a cell fraction containing the bone marrow derived stromal cells (BMSCs) with a low viscosity/osmolarity gradient followed by cell expansion for 7– 14 days (Fig 1A). Gradient separation selects cell subpopulations based on size, and canine BMSCs isolated by Percoll centrifugation gradient represent about 0.004% of the total nucleated cell population in bone marrow aspirates.[3] Osteogenic differentiation of BMSCs is reported in most species including dog,[31] cat,[32] human,[11] rabbit,[33] rat,[34] mouse,[35] nonhuman primate,[36] and horse,[37] among others. It is well established that BMSC expansion rates and osteogenic potential differ among bone sources and species.[38]

One of the earliest protocols for adipose-derived stromal cell (ASC) isolation from rat adipose tissue was described in 1966 by Rodbell in 3 consecutive articles published in the *Journal of Biological Chemistry*.[39–41] The procedure consisted of homogenization of adipose tissue, consecutive saline washes to remove erythrocytes and other hematopoietic cells, collagenase digestion, and separation of undigested adipose tissue from the pelleted stromal vascular fraction (SVF) containing the ASCs (Fig 1B). To date, ASC isolation from adipose tissue is very similar to the original method, with protocol changes generally related to adipose harvest site. *In vitro* expansion rates of ASCs are reported to differ between tissue sources.[42–44] Similar regional differences in ASC behavior have been documented in a number of species including people[43] and dogs.[44] For both species, subcutaneous isolates have higher expansion potential than those from mesenteric and omental tissues.[43,44]

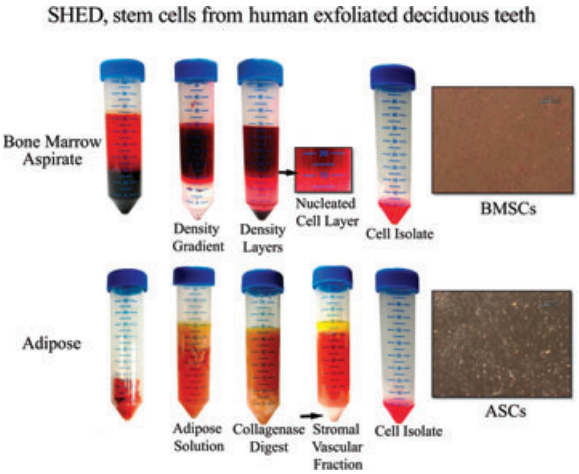

Figure 1 Multipotent stromal cell isolation.

ADULT MSC OSTEOGENESIS

Ossification of transitional bladder epithelium tissue autotransplanted into canine abdominal wall was reported by Huggins in 1931.[45] Friedenstein subsequently reported tightly coordinated bone formation with characteristic glycogen content and alkaline phosphatase (ALP) activity that resulted in rudimentary bone within autologous transitional epithelium grafted into guinea pig abdominal wall.[46] In subsequent years, Friedenstein explored osteogenic potential of other tissues, and, in 1970, reported *in vitro* osteogenesis of a subpopulation of bone marrow cells.[7] The nonhematopoietic osteoprecursor cells were unique in their inherent ability to adhere, proliferate, and develop as monolayer cultures. The fundamental concepts of velocity sedimentation separation of cells introduced by Friedenstein[7] and refined by Castro-Malaspina[47]

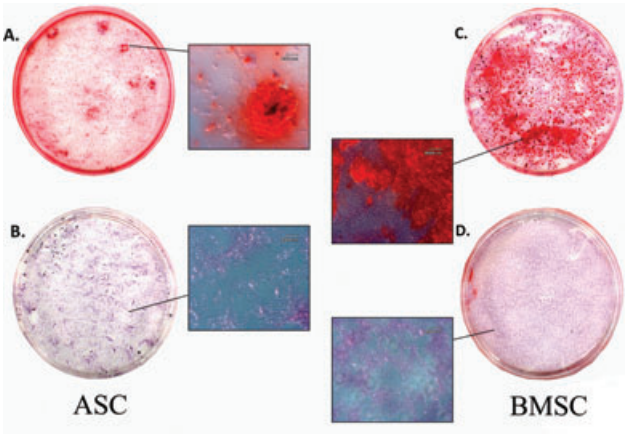

Figure 2 Adult equine BMSCs (A,B) and ASCs (C,D) after 21 days of culture in osteogenic (A,C) or control medium (B,D) after alizarin red staining. Calcium within cell colonies is stained red. (Inset magnification, 10×; scale bar, 600 μm).

remain the cornerstone of many current BMSC isolation protocols.

For *in vitro* osteogenic differentiation, culture medium containing ascorbic acid, dexamethasone, and beta-glycerol phosphate[48,49] is routinely used, and detection of mineralized matrix after differentiation is usually performed by colony staining with alizarin red or von Kossa stain (Fig 2). Major investigative efforts are directed toward elucidating the regulatory events responsible for the commitment of MSCs toward the osteogenic pathway using variations of these standard mechanisms.[50–53] MSC differentiation into bone precursor cells results in increased expression of genes specific to bone formation such as core binding factor alpha 1 (CBFA1), osterix, osteocalcin,[54] bone sialoprotein, ALP, and collagen type 1 alpha 1 (COL1A1). Typically, upregulation of bone matrix specific genes occurs before calcium deposition takes place during MSC osteogenesis.[54]

SCAFFOLD CARRIERS FOR MSC BONE REGENERATION

In vitro MSC osteogenesis supports the potential ability of the cells to augment natural bone formation *in vivo*. However, translation of the technology to patient care requires biocompatible carriers to implant and support the cells. There is a plethora of scaffolds from which to choose MSC carriers with no single best choice for all potential clinical scenarios. Biocompatibility and mechanical properties with biodegradability that parallels new bone formation as well as mediation of MSC osteogenesis are basic criteria for scaffolds designed to support bone engineering with MSCs.[55,56] Further, despite efforts to recreate physiologic environments in the laboratory, *in vitro* findings must be validated *in vivo* before clinical implementation. An important consideration surrounding MSC-mediated osteogenesis *in vivo* is the difference between orthotopic and ectopic osteogenesis.[57] Ectopic osteogenesis refers to ossification of tissue implanted outside of a normal site of osteogenesis (or outside of the origin of the implanted tissue). Orthotopic osteogenesis refers to bone formation in its correct anatomical location. Both orthotopic and ectopic ossification models are used in studies surrounding MSC osteogenesis, but the distinct biochemical and mechanical environment of orthotopic bone formation is likely most relevant for validating scaffold–MSC osteogenesis.

Given the importance of MSC–scaffold interactions, there is significant effort to develop scaffolds that support MSC osteogenesis. Bone is largely composed of hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, crystals distributed within an organic matrix. The porosity and mineralization varies between cortical and cancellous bone and regionally within bones. Autogenous bone grafts provide the 3 elements for bone generation including osteogenic progenitor cells, osteoinductive growth factors, and osteoconductive matrix.[58] However, limited quantity and the invasive harvest procedure can limit enthusiasm for graft harvest. Allogeneic tissue is often more readily available than autogenous bone, but cost, time-consuming banking procedures, and concerns about disease transmission often restrict use.[58] Additionally, because most allografts are essentially cell free from processing or immune targeting, their osteogenic potential is limited. These limitations led to the exploration of synthetic bone substitutes as alternatives to natural bone grafts.[59,60]

MSC–scaffold carriers for bone reconstruction are typically designed to replicate bone morphology, structure, and function to provide a suitable microenvironment for MSC adhesion, proliferation, and differentiation. Scaffold characteristics such as degradation rate,[61,62] mechanical properties,[63,64] and protein delivery[65–67] are major considerations for scaffold fabrication specific to bone tissue engineering. Porosity and pore size of biomaterial scaffolds affect cell migration, nutrient transport,[68] and osteogenesis.[69,70] Synthetic bone grafts typically have 2 of the 3 components for bone regeneration, osteoinduction, recruitment and direction of immature cells to develop into osteoblasts, and osteoconduction, promoting bone apposition by acting as a receptive scaffold.[58]

Optimization of scaffold biomaterials has been the subject of extensive studies using a number of materials like metals, ceramics, and glass as well as synthetic and natural polymers alone or in combination.[71–85] Calcium phosphate ceramics like HA and beta-tricalcium phosphate (β-TCP), $Ca_3(PO4)_2$, were once thought suitable for clinical use as MSC carriers because of their chemical and crystallographic similarities to native bone.[55,86] Biphasic calcium phosphate (BCP) refers to homogenous composites of HA and β-TCP.[56] Properties like solubility and resorption capacity of BCP formulations vary widely among different ratios of HA and β-TCP. In a caprine model, BCP and β-TCP scaffolds promote increased bone growth with the addition of BMSCs.[56] Unfortunately, calcium phosphate ceramics tend to have poor mechanical properties, predisposing them to fragile failure.[87] Scaffolds

of calcium, magnesium, and silicon containing ceramics like akermanite ($Ca_2MgSi_2O_7$) have better mechanical properties and degradation rates than other bioceramics, and are reported to enhance osteogenic commitment of MSCs.[55,86] Human ASC attachment and proliferation were observed to be similar on akermanite and ß-TCP *in vitro*, and osteogenic ASC differentiation was enhanced on the akermanite over the β-TCP after 10 days of culture.[55] This information highlights the potential for MSCs to enhance osteogenesis over scaffold alone.

Scaffolds can also serve as vehicles to deliver various factors to enhance implant integration[58,68] and osteogenic commitment of native and exogenous precursor cells.[88] Bone metabolism and homeostasis are regulated by a plethora of hormones and growth factors such as parathyroid hormone (PTH), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), vascular-endothelial growth factors (VEGFs), transforming growth factors (TGF), and bone morphogenetic proteins (BMPs).[89] Knowledge of the physiologic roles of specific proteins in bone formation makes them target candidates to maximize MSC differentiation and osteogenesis before and after implantation. Kadiyala et al tested the osteogenesis of autologous canine BMSCs on 3 different scaffolds coated with various bioactive factors compared to scaffolds without cells after subcutaneous or intramuscular implantation.[3] Greater osteogenesis was observed in HA/TCP implants coated with human fibronectin than HA granules coated with autologous gelatinous fibrin or HA disks treated with autologous serum, and all had greater osteogenesis than implants without BMSCS 8 weeks after implantation. Similarly, Dallari et al tested autologous rabbit BMSCs with platelet-rich plasma (PRP) and freeze-dried bone allografts (FDBA) alone or in different combinations to accelerate healing of femoral cancellous bone defects.[90] Bone healing was significantly greater 12 weeks postimplantation in cohorts treated with BMSC + PRP + FDBA, suggesting that combinations of cells, growth factors, and matrices are a viable approach to facilitate bone healing. Given differences between BMSCs and ASCs, scaffold composition should be tailored to each cell type for different species. To date, a gold standard scaffold carrier for MSC osteogenesis has yet to be identified. Age, nutritional state, activity level, and comorbidities are additional factors to be considered with regard to bone forming potential.[68] Optimized scaffold–MSC constructs may become an integral part of treatment strategies to overcome reduced bone forming potential because of patient injury or disease.

PERFUSION BIOREACTORS FOR MSC–SCAFFOLD OSTEOGENESIS

The term "bioreactor" refers to a wide variety of culture systems. Bioreactors provide a mechanism to maintain cell–scaffold constructs in a biocompatible environment during application of defined chemical and physical stimuli. The goal is usually to induce specific cell behavior under controlled and repeatable conditions, often by recreating a complex natural environment. Some common mechanical stimuli include scaffold tension and compression as well as shear forces from nutrient medium motion. Perfusion bioreactors are culture systems in which nutrient medium is repeatedly forced or "perfused" through cell–scaffold constructs. Their categorization as "dynamic" culture systems distinguishes them from "static" culture systems in which there is no fluid motion. An example of "static" culture is a standard culture flask or plate.

Tissue formation in three-dimensional scaffolds is significantly affected by nutrient transport, physical stress, cell density, and gas exchange.[91,92] For the best possible tissue regeneration, postimplantation cell viability and homogenous cell distribution throughout the scaffold are crucial.[93] Dynamic systems like perfusion bioreactors facilitate optimal seeding under controlled conditions.[93] The nutrient medium passing directly throughout the pores of the scaffold simultaneously provides sheer stresses, gas exchange, nutrient delivery, and waste removal to support cell proliferation and differentiation within the scaffold.[93,94] Janssen et al observed that oxygen consumption during perfusion was directly related to the number of viable goat BMSCs on the scaffold.[93] The potential augmentation of bone formation in MSC–scaffold constructs by perfusion bioreactors is often assessed by cell viability as well as bone-specific protein and mRNA levels. The amount of bone generated by human osteoprogenitor cells on calcium carbonate ($CaCO_3$) scaffolds did not differ between dynamic and static cultures after 14 days, but osteocalcin expression was statistically greater in dynamic cultures.[95] Similarly, perfusion of mouse osteoblastic-like cells on ß-TCP scaffolds promoted better cell attachment and increased ALP activity over static cultures.[96] Hence, perfusion bioreactor culture may increase cell proliferation and promote more consistent osteogenesis over manual seeding and static culture by equalizing cell distribution and environmental conditions throughout the scaffold.

ADULT MSC OSTEOGENESIS FOR THE VETERINARIAN

The unique MSC characteristics of cell expansion, ability to differentiate into multiple cell types, and immune privilege[97–99] make bone regeneration with MSCs an attractive technique to enhance traditional procedures[3,100] without the limitations of autogenous grafts or risks of allogeneic tissues.[58] As described earlier, the ability of various scaffold compositions and structures with and without growth factors must be tested and customized for specific cell type, species, and intended use (Table 1). Scaffold–MSC combinations are often implanted in critical size bone defects to test their safety and efficacy. A critical-size bone defect is defined as the smallest size intraosseous wound

in a particular bone and species that will not heal spontaneously during the lifetime of the animal. Comprehensive *in vivo* models that provide accurate information about the performance of MSC–scaffold constructs are important to the continued progress of MSC technology.

Rats, mice, and rabbits are established animal models for targeted osteogenesis therapies, and there are abundant studies surrounding MSC osteogenesis in rabbit and rodent models.[101] Results from the models vary widely with inconsistent effects of MSC implantation on osteogenesis among studies, in part because of differences among models and cell preparation methods.[57,102,103] With respect to models, distinctions should be made between ectopic bone formation in subcutaneous implants versus flat and long bone healing. Additionally, cell harvest, expansion, and preimplantation conditions range widely. In light of these considerations, promising reports include that ASCs implanted into mouse and rabbit skull defects promote healing of critical-size defects without genetic manipulation or exogenous growth factors.[28,29] Additionally, syngeneic and allogeneic adult rat ASCs on 80% β-TCP/20% bovine COL1 scaffolds result in accelerated spinal fusion compared to scaffold alone.[104] Given distinct differences between rabbit/rodent and large animal models, species-specific investigations are necessary.

Large animal MSCs have osteogenic potential *in vivo*.[31,105] In dogs, autologous MSCs were used to repair critical-size flat and long bone defects.[31,106,107] Implantation of porous ceramic constructs (65% HA 35% ß-TCP) with autologous BMSCs promoted femoral defect (21 mm) healing in dogs over scaffold alone.[31] Additionally, osteogenically induced autologous BMSCs on ß-TCP scaffolds promote healing of canine segmental mandibular defects better than ß-TCP alone and comparable to autologous bone.[106] Alveolar augmentation with autologous BMSC–PRP–fibrin gel promoted osteointegration of dental implants in dogs.[108] Recently, repair of critical-size mandibular defects was demonstrated using autologous stem cells isolated from deciduous teeth in miniature pigs.[109] These studies are just some of the recent work on adult MSC–scaffold constructs to promote osteogenesis in monogastric animals.

Small ruminant models have also been important to advances in adult MSC applications for bone regeneration. Isolation of ovine BMSCs was described as early as 1994,[110] and use of adult MSCs for repair of bone defects has been explored in a number of small ruminant models.[111–113] In ovine critical-size tibial segmental defects (3.5 cm), bone formation was more extensive with HA scaffolds containing autologous BMSCs compared to scaffold alone 2 months after surgery.[114] In scaffolds with BMSCs, bone formation occurred both in the pores and outer surface of the cylinders compared to those without BMSCs in which bone formed only on the surface.[114] The natural exoskeleton of coral has an interconnected porous architecture similar to spongy bone and the best mechanical properties of porous, calcium-based ceramics.[87] Natural coral coated with HA has the same architecture as uncoated coral but a lower resorption rate. Segmental metatarsal defects 6, 12, 15, and 25 mm long healed after 16 weeks in 43% of ovine limbs when treated with autologous BMSCs on coral-HA scaffolds compared to none of those treated with coral-HA alone or with autologous single cell suspensions from fresh whole bone marrow (FBM).[87] Similarly, in an ovine metatarsal defect model, bony surface area was better in coral-HA scaffolds with autologous BMSCs compared to coral-HA scaffolds alone or in untreated defects, but inferior to corticocancellous bone autografts.[115] A scaffold composed of 67% Si-TCP and 33% HA/ß-TCP loaded with autologous BMSCs had progressive bony ingrowth from the periosteal bone remnants to the inner scaffold with simultaneous scaffold degradation in an ovine critical-size tibial defect (4.8 cm).[105] In addition to ovine models, caprine models, have been used to test MSC–scaffold constructs for bone regeneration.[116] The larger size of small ruminants compared to rodents and smaller mammals allow evaluation of MSC applications in challenging anatomic environments characteristic of larger patients. However, anatomic and size differences are clear impetus for species-specific studies.

EQUINE ADULT MSC OSTEOGENESIS

Musculoskeletal injuries are among the most challenging problems in horses[117] and fracture repair is fraught with complications.[118] Repeated cycling before development of stable fracture callus contributes to implant failure after long bone fracture stabilization, and the incidence of life-threatening complications increases with time. Methods to accelerate fracture healing could significantly reduce complications and improve successful treatment of equine fractures. As alluded to earlier, biomechanical loading of long bones in many of the common animal models have little similarity to the horse. This is especially relevant given the importance of mechanotransduction, the process by which cells convert mechanical stimuli into a chemical response, on stromal cell activation, proliferation, differentiation, and osteogenesis.[119] Hence, equine investigations are most relevant to potential MSC therapies targeted for the horse.

Adult MSCs have been isolated and characterized from many equine tissues including bone marrow, adipose, and blood, among others.[49,120–122] Arguably, the most popular and relatively established sources of equine MSCs are bone marrow and adipose tissue.[123] Notably, equine BMSCs were isolated and characterized almost 10 years earlier than equine ASCs, affording greater experience with the former.[37,49,124] Osteogenesis of equine BMSCs has been reported to be superior to that of ASCs,[49,125,126] though ASCs have been shown to have robust osteogenic potential.[125,127,128] It is possible that cell-specific culture and induction conditions may augment the osteogenic potential of equine ASCs. An immediate goal of tissue regeneration in the horse is to use MSC technology to accelerate long bone fracture healing in combination with traditional instrumented internal and external stabilization procedures

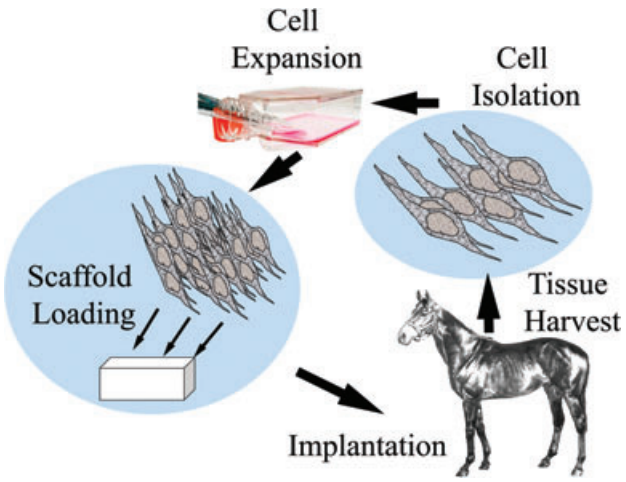

Figure 3 Equine multipotent stromal cell bone tissue engineering paradigm.

(Fig 3). Eventually, it may be possible to implant viable, customized tissue implants to replace damaged tissue and thereby facilitate the healing process in combination with new or established surgical procedures.

Current work includes the use of cell surface antigen markers to confirm the identity of the cells.[127,129,130] Two key transcription factors, octamer-4 (OCT-4) and Nanog homeobox (NANOG) are required for maintenance of embryonic cell pluripotency.[131,132] In horses, both OCT-4[21,133–135] and NANOG[135] antibodies have been used to label embryonic[21,133,134] and adult stromal cell lines.[135] Limited availability of equine-specific antibodies complicates this process. However, increasing numbers of available equine-specific antibodies as well as those directed against other species' antigens validated for equine use provide a fairly comprehensive panel with which to identify and characterize adult equine MSCs.[129,136,137]

Cell banking of cryopreserved MSC aliquots is an appealing mechanism to increase cell accessibility and obviate the need for autologous cell harvest from sick or injured patients. Recent research suggests that there is no difference in osteogenic potential between fresh and cryopreserved multipotent cells isolated from equine adipose[128] or peripheral blood.[137] The ability to use cryopreserved multipotent cells to augment natural osteogenesis will contribute significantly to clinical availability of the technology.

There are numerous studies on the use of equine MSCs to treat tendon and cartilage injuries[37,123,126,138–141]; however, information on use for bone regeneration is limited.[142–145] It is clear that equine MSCs undergo promising osteogenesis *in vitro*. However, it is far more challenging to monitor MSC osteogenesis *in vivo*, and even more so to separate cell effects from those of the scaffold carriers. For MSC bone regeneration to be a feasible therapeutic approach in the horse, direct cell applications as well as cell seeding and interactions with carrier scaffolds must be established before and after implantation (Fig 3). To date, there is limited empirical evidence surrounding equine MSC contributions to bone healing. A recent publication indicates superior bone healing of experimentally induced fourth metatarsal and metacarpal defects after percutaneous administration of autologous dermal fibroblasts genetically engineered to express BMP 2.[143] Another report suggests effective *in vivo* osteogenesis by culture expanded equine ASC xenografts in a rat calvarial defect model.[144] Continued, focused research efforts will significantly augment equine-specific MSC therapies to facilitate bone healing.

CONCLUSIONS

The enduring interest in MSC osteogenesis is strong evidence of the appealing potential to harness the natural phenomenon and enhance standard therapies. Because of this intense research focus, knowledge about the role of diverse cell types involved in bone homeostasis and repair has grown rapidly since the early days of ectopic osteogenesis by implanted tissues. The tools with which to isolate and identify MSCs from nearly any tissue in domesticated animals are increasingly available. Abundant evidence supports the osteogenic potential of MSCs isolated from numerous tissue reservoirs in a number of animal species. Clinical application of fresh and cryopreserved autologous and allogeneic MSCs is becoming standard clinical fare in many animals. Unfortunately, comparable progress surrounding MSC bone regeneration has yet to be achieved in horses. The unique complications associated with fractures because of equine anatomy and athleticism is compelling incentive for continued efforts to shift this paradigm.

The information presented in this review provides only a glimpse of the complex process of MSC osteogenesis. This complexity may, in part, explain the wide variation in reported MSC treatment outcomes. However, limited standardization of adult MSC harvest, isolation, expansion, and administration procedures also contributes to variability among investigations and trials. As presented, *in vitro* and ectopic osteogenesis does not represent orthotopic osteogenesis, the most clinically relevant of the three. To establish the benefits of adult MSC-mediated osteogenesis over grafting procedures, growth factor delivery and other standard therapies, direct comparisons are required. Rigorous preclinical investigations and clinical trials with appropriate controls will solidify and condense available research results into viable treatments that are safe, effective, and reproducible. Based on current momentum in the field, use of adult MSCs to augment fracture repair in companion animals may soon become the standard of care.

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