Concise Review: Musculoskeletal Stem Cells to Treat Age-Related Osteoporosis

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ABSTRACT

Age-related (type-II) osteoporosis is a common and debilitating condition driven in part by the loss of bone marrow (BM) mesenchymal stromal cells (MSC) and their osteoblast progeny, leading to reduced bone formation. Current pharmacological regimens targeting age-related osteoporosis do not directly treat the disease by increasing bone formation, but instead use bisphosphonates to reduce bone resorption—a treatment designed for postmenopausal (type-I) osteoporosis. Recently, the bone regenerative capacity of MSCs has been found within a very rare population of skeletal stem cells (SSCs) residing within the larger heterogeneous BM-MSC pool. The osteoregenerative potential of SSCs would be an ideal candidate for cell-based therapies to treat degenerative bone diseases such as osteoporosis. However, to date, clinical and translational studies attempting to improve bone formation through cell transplantation have used the larger, nonspecific, MSC pool. In this review, we will outline the physiological basis of age-related osteoporosis, as well as discuss relevant preclinical studies that use exogenous MSC transplantation with the aim of treating osteoporosis in murine models. We will also discuss results from specific clinical trials aimed at treating other systemic bone diseases, and how the discovery of SSC could help realize the full regenerative potential of MSC therapy to increase bone formation. Finally, we will outline how ancillary clinical trials could be initiated to assess MSC/SSC-mediated bone formation gains in existing and potentially unrelated clinical trials, setting the stage for a dedicated clinical investigation to treat age-related osteoporosis.

SIGNIFICANCE STATEMENT

This review makes a strong case for the use of cell therapy for the treatment of human age-related (type II) osteoporosis. The authors highlight that age-related bone loss is associated with the decline of musculoskeletal progenitors, and transplantation of such progenitors may alleviate the disease. Preclinical animals studies are integrated into the design and, as available, clinical human data into a proof of principal that cell therapy should be explored to treat age-related bone loss. Finally, the authors make the case for the use of ancillary clinical trials to partner with existing human cell therapy trials to maximize the scientific impact these expensive experimental interventions.

INTRODUCTION

Osteoporosis and low bone mineral density (BMD) affect more than 55 million people in the U.S. [1] and Canada [2]. These conditions are responsible for over two million fragility fractures and approximately $20 billion in related costs every year [3]. Osteoporotic hip fractures are of particular concern, as over 300,000 occur yearly in the U.S., leading to 31,000 associated deaths, and disabling half of those who survive [4]. Associated health care costs and morbidity are expected to rise exponentially due to an aging demographic [5].

Osteoporosis is caused by an imbalance between the tightly regulated process of bone formation by osteoblasts and resorption by osteoclasts. Primary osteoporosis is defined as bone loss attributed to aging or a decline in sex hormones associated with aging, whereas secondary osteoporosis is bone loss caused by an unrelated pathological condition [6–8]. Primary osteoporosis is classified as two categories, postmenopausal osteoporosis (type I) and age-related osteoporosis (type II, or senile). The mechanism underlying postmenopausal osteoporosis is associated with excessive bone resorption, driven by estrogen deficiency, and often managed with anti-resorptive bisphosphonate therapy. On the contrary, age-related osteoporosis involves the gradual loss of bone caused by insufficient bone formation.

The lack of an overall mechanistic understanding of what drives age-related osteoporosis has...
hindered the development of anabolic therapy appropriately targeting the etiology of the disease. It is hypothesized that decrease in the number and function of bone and bone marrow (BM)-derived mesenchymal stromal cells (MSCs)—a heterogeneous population comprising skeletal stem cells (SSCs), osteoblastic cells, and fibroblasts—lies at the root of age-related bone loss [9–20]. Specifically, age-related changes in the proliferative and differentiation capacity of BM-MSCs are suspected [21], and recent evidence suggests that the loss of SSCs, which are a rare subset of MSCs, could be the most relevant event in the progression of senile bone loss [22, 23]. Thus, treatment strategies aimed at replenishing the MSCs compartment—and by extension SSCs—or augmenting endogenous populations of these cells, could result in bone growth and combat age-related osteoporosis.

**Bone Loss and Osteoporosis**

Bone is a dynamic tissue, with remodeling-mediated maintenance regenerating the entire skeleton every 10 years [24]. Homeostasis is achieved when bone formation by osteoblasts, the terminally differentiated phenotype from skeletal progenitors within the MSC pool, equals resorption by osteoclasts derived from the hematopoietic compartment. Perturbation of this balance caused by reduced formation, or excessive resorption, can manifest itself in osteopenia or, more seriously, osteoporosis.

The etiology behind postmenopausal osteoporosis is well characterized. Loss of estrogen after menopause due to ovarian atrophy results in an anti-osteoblast, pro-osteoclast environment. Postmenopausal osteoporosis is characterized by an increase in the frequency and length of resorption periods and a shortening of formation periods. Net bone loss occurs, primarily in trabecular sites including the vertebral column and femoral neck. Bisphosphonates are usually the first line treatment for postmenopausal osteoporosis; however, estrogen replacement is also an option (Fig. 1A).

Following menopause, a slower phase of bone loss persists, and continues for the remainder of the woman’s life, and equally affects cortical and trabecular bone (Fig. 1B) [25]. In comparison, the decline of BMD in men occurs from midlife onward, with progressive trabecular and cortical bone loss nearly equivalent to the second phase of bone loss in women. However, the lack of estrogen-mediated postmenopausal bone loss results in less overall BMD decline (Fig. 1B) [25]. As such, if BMD decline due to the gradual bone loss beyond midlife falls far below the healthy adult average, age-related osteoporosis is diagnosed. Age-related osteoporosis is linked with low bone formation and turnover, with resorption exceeding formation [26]. Age-related osteoporosis affects both cortical and trabecular bone, causing a higher risk of hip, vertebral, and radial fractures (Fig. 1A) [27]. Nevertheless, the molecular mechanisms underlying type II osteoporosis are not well understood, and patients usually receive bisphosphonates, a
Pathology of Age-Related Osteoporosis

For years it has been thought that sex steroid decline was the transformative event causing age-related osteoporosis. A recent study showed that reduced sex steroid levels had a strong correlation with reduced trabecular microarchitecture in the proximal radius of subjects over 60 years of age [30, 31]. Interestingly, longitudinal studies of BMD at both the distal radius and proximal tibial sites documented substantial loss of trabecular bone starting shortly after the completion of puberty in both men and women, an age when sex steroid levels are normal [32], suggesting a mechanism underlying age-related bone loss that was sex steroid independent [33]. As discussed further below, the loss of MSCs, particularly the SSCs present within that population is manifest in a reduction of osteoblasts and a concomitant decrease in bone formation that, if severe enough, can result in osteoporosis.

Mesenchymal Stromal Cells

In addition to the hematopoietic cells residing within BM, there is a population of resident MSCs—perivascular cells that contribute to tissue repair and contain osteoprogenitors critical to bone maintenance. Clonogenic MSCs comprise approximately 1:100,000 and 1:1,000,000 BM cells in humans [34] and mice [35], respectively.

In the 1970s, Friedenstein and colleagues were the first to identify the osteogenic element of BM [36]; these clonogenic fibroblast colonies were the first documentation of the cells now known as MSCs. Since then, the multipotency of the BM-MSCs population as a whole has been well characterized, showing that particular subsets of cells within the total population possess the ability to differentiate into skeletal phenotypes, including bone, muscle, fat, tendon, cartilage, and marrow stroma [37, 38]. While MSCs were originally isolated from BM [39, 40], they were later isolated from adipose tissue [41], muscle [42], liver [43], umbilical cord [44], and dental pulp [45], suggesting their tissue residence was ubiquitous. Once considered a homogeneous stem cell population, discreet differences exist between these tissue sources that are only realized with detailed in vivo analyses [42, 45]. Recent breakthroughs have revealed that the larger MSC pool contains a heterogeneous mix of progenitor and terminally differentiated cell types. Importantly, a highly potent and dynamic progenitor, the SSC, has been isolated and is the focus of extensive investigation.

Skeletal Stem Cells

The hierarchical organization of musculoskeletal stem cell compartment contained within the heterogeneous MSC pool has been documented [46]. However, the exact identity of the various self-renewing, multipotent, and lineage committed stem cells within this hierarchy remains undefined. Paradigm shifting work has uncovered rare subsets within the MSC population that harbor specific and robust stem cell activity. A subset of BM cells that tracks with CD146 (human) and nestin (mouse) are capable, under single colony transplantation into a biocompatible scaffold, of forming bony organoids (ossicles) that contain: bone tissue, cartilage, adipocytes, fibroblasts and hematopoiesis-supporting stroma. Upon serial transplantation into secondary recipients, these cells have the potential to form a similar bony organ in their new host [47–49]. Further, within the defined stem cell hierarchy, the discovery of SSCs marks an initial dissection of the specific organized members of the musculoskeletal stem cell compartment. SSCs are thought to express the BMP antagonist Gremlin 1 [23] and are capable of forming bone, cartilage and marrow stroma, but incapable of adipogenic differentiation [22, 23]. SSCs are concentrated within the metaphysis of long bones, and are needed for bone development, remodeling, and fracture repair [23]. Interestingly, the loss of these cells is associated with impaired bone length, and an overall reduction in bone volume [23]. A putative SSC, a self-renewing cell displaying tri-lineage differentiation capacity (cartilage, bone, fibrous stroma) had been previously documented by our group using clonal expansion and differentiation, albeit yet to be named SSCs at the time [46]. Following the current paradigm, that work suggests that there are several putative, although yet to be characterized, multipotent cells within the larger “MSC” population.

Evidence for BM-MSC Decline in Osteoporosis

Numerous studies have documented an age-dependent decrease in frequency and osteogenic potential of BM-MSCs in both mice [19, 20, 50] and humans [9–14, 18]. These findings have led to the hypothesis that an overall decrease in the number and function of BM-MSCs and their osteoblast progeny lies at the root of age-related bone loss. Specifically, age-related changes in the proliferative and differentiation capacity of BM-MSCs [21], including a shift toward increased adipocyte differentiation [51], as well as MSC [15, 51] and osteocyte senescence [24], have been implicated in the overall decline of skeletal progenitors and terminally differentiated osteogenic cells, reducing bone formation.

Mouse models of aging also implicate MSC loss as the transformative event that drives age-related bone loss. The well-characterized murine model of accelerated senescence and osteopenia (SAMP6) was discovered to display reduced osteoblast numbers [15, 52]. These mice also exhibit reduced MSC numbers, along with the tendency of MSCs to differentiate toward the adipocyte lineage [53]. The stem cell antigen-1 (Sca-1) deficient mouse model of age-related osteoporosis displays an MSC self-renewal deficiency, and an age-associated loss of MSCs, osteoprogenitors, and adipoprogenitors. Progenitor loss coincides with the onset of the osteoporotic phenotype, and is characterized by a low-turnover phenotype driven by an initial loss of bone formation, which causes an associated loss of osteoclast numbers and decreased bone resorption [19, 54]. Similarly, the biglycan (bgn) deficient mouse also displays age-related osteoporosis driven by decreased proliferation and increased apoptosis of BM stromal cells. Mechanistically, bgn deficient MSCs are unable to respond to transforming growth factor beta (TGFβ) induced colony proliferation, which has been attributed to the MSC defects [16]. Finally, with the discovery of SSCs, it was documented that if these cells are ablated, a severe bone formation deficit occurs [23]. As such, therapies to replenish the BM-MSC pool, particularly SSCs, could be useful for osteoporosis therapy.

Current Definition of an MSC Used in Clinical Studies

In 2006, the International Society for Cytotherapy (ISCT) defined the minimal criteria to define a human MSC. First, an MSC must be plastic-adherent when maintained in standard culture
conditions. Second, MSCs must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Third, MSCs must differentiate to osteoblasts, adipocytes and chondrocytes in vitro [55]. Early clinical trials were based on whole bone marrow (WBMM), or rudimentary isolation procedures with only plastic adherence, osteogenic capacity, and lack of hematopoietic/lymphoid cell markers being assessed [56–58]. Although MSCs can mediate tissue repair directly via differentiation [46, 49, 56, 58–61], most transplantation studies have established that much of the tissue repair attributed to MSCs, in both translational [46, 62–64] and clinical contexts [60], is due to paracrine action. The ISCT has recognized that previously agreed upon minimal criteria did not focus on the immune-modulatory properties of MSCs. In response, they have initiated a discussion, aimed at standardizing the immunological characterization of MSCs for clinical use [65]. Standard MSC priming protocols and the use of cellular markers along with defined responder cell assays, as a measure of immunomodulatory potency to define release criteria, will soon be in place to permit more refined and rigorous clinical analysis. The identification of SSCs within the MSC hierarchy represents an opportunity of dissecting the potential variances of regenerative and immunomodulatory properties within the bulk BM-MSC pool. Development of robust protocols to isolate and grow individual MSC populations such as SSCs, while maintaining their in vivo properties, will be an important step in unlocking their regenerative potential.

**Proof of Concept: Evidence that MSCs Can Be Used to Treat Age-Related Osteoporosis**

Preclinical MSC Transplant-Based Interventions to Treat Osteoporosis

A number of preclinical studies have been undertaken to determine whether MSC-based cell transplantation can induce bone formation (Table 1). These studies, which used mouse or human MSCs, or WBMM transplantation, have been carried out in various murine models of MSC dependent osteoporosis, including aged-related bone loss, as well as secondary osteoporosis due to glucocorticoid use or systemic lupus erythematosus and are discussed below. However, MSC have also been shown to increase bone formation when transplanted into ovariectomized (OVX) mice—a model of postmenopausal osteoporosis that is considered MSC independent.

We have recently reported that transplantation of unmodified, low-passage MSCs prevent age-related osteoporosis in Sca-1<sup>+</sup> mice [72]. Low-level donor engraftment of between 0.1 to 4.5 donor MSCs per million total BM cells was documented, as well as approximately 2,300 donor MSCs per million total recipient lung cells, 6 months after a single systemic bolus injection. At the 6-month time point, we showed that transplanted animals displayed markedly increased bone formation, as well as higher osteoclast numbers. This led to improved bone quality and turnover, and importantly, sustained microarchitectural competence, particularly in the connectivity and orientation of trabeculae.

Complementary to our work, studies documenting proof of principal that MSC transplantation can prevent senile osteoporosis in mouse models of accelerated aging present consistent findings. Using inframensurale injection of WBMM into 4-month-old SAM<sub>P6</sub> mice, Ichikawa et al. documented site-specific phenotypic relief of the osteoporosis phenotype for at least 8 months after transplant. Histological analysis revealed that treated mice were able to retain similar trabecular bone mass to controls; and BMD, assessed via microdensitometry, further revealed the prevention of the SAM<sub>P6</sub> age-related osteoporotic phenotype. Donor cells replaced a significant number of native mutant MSCs, and the BM microenvironment was normalized. The authors attributed this improvement to increased levels of IL-11 and IL-6, correcting the imbalance between bone resorption and formation, preventing the onset of osteoporosis [66]. In another study targeting age-related osteoporosis in a mouse model of accelerated aging, Singh et al. transplanted WBMM into the Wrn<sup>−/−</sup>;Terc<sup>−/−</sup> compound mutant mice. Mice that were transplanted at 3-months of age displayed a survival advantage compared to nontransplanted mice, and engraftment of MSCs was documented 10.5 months after transplant. At the 10.5-month time point, approximately 20% of cortical osteocytes and 6% of trabecular osteocytes were donor derived. Additionally, 15% of cortical, and 5% of trabecular, endosteal lining osteoblasts were derived from donor MSCs. Importantly, transplanted animals showed delayed onset of phenotypic microarchitectural loss, as well as improvements in endocortical bone mass [71].

Proof of concept has also been documented in mouse models of other forms of osteoporosis. Hsiao et al. isolated an enriched population of MSCs for transplant to OVX mice. The authors demonstrated that plating BM for a short time (3 hours) was sufficient for MSC attachment, but insufficient for contamination by hematopoietic cell attachment. The resulting cell population was capable of engrafting for at least 2 months to trabecular and cortical bone, and increased bone density in the distal femoral compartment of OVX mice [67]. Analogous to age-related osteoporosis, secondary osteoporosis in the fas<sup>−/−</sup> mouse model of systemic lupus erythematosus is mediated by suppression of osteoblasts, with an additional induction of osteoclasts not usually present in age-related osteoporosis—excessive IL-17 expression causing a hyperimmune condition that damages BM MSCs—mediates this phenotype. To combat lupus mediated secondary osteoporosis, Ma et al. transplanted human MSCs (isolated from BM and dental pulp) into fas<sup>−/−</sup> mice. The authors reported that the human MSC transplant mediated recovery of the impaired endogenous BM-MSC function of the recipient, suppressing IL-17 to normalize osteogenesis and osteoclastogenesis [68]. Another recent study also demonstrated phenotypic correction of secondary osteoporosis in fas<sup>−/−</sup> mice via systemic WT BM transplant. The authors concluded that Fas was donated to the recipient BM MSCs of fas<sup>−/−</sup> MSCs through exosome transfer, initiating signaling events that improved long-term fas<sup>−/−</sup> BM MSC function [75]. Glucocorticoid induced secondary osteoporosis shares a pathology common to age-related osteoporosis, since the disease is driven by defective or diminished osteoblasts. Sui et al. revealed that mice transplanted with MSCs combined with glucocorticoid treatment prevented the loss of bone volume and strength observed in mice that only received glucocorticoids. The benefit was mediated by transplanted MSCs inhabiting the BM and enhancing osteoblastogenesis [76].

Modification of MSCs to improve osteogenic differentiation or homing to BM have also been proven beneficial to relieve osteoporosis or the general contribution to local bone formation in vivo. Lien et al. genetically modified the MSC-like C3H10T1/2 embryonic cell line to enhance their ability to contribute to bone formation. C3H10T1/2 cells underwent modifications to express the SDF-1 ligand CXCR4, to increase MSC vascular adhesion and
Table 1. Preclinical animal studies testing MSC transplantation to augment bone formation

| Author           | Ref. | Animal model                               | Source/delivery/dose | Engraftment | Outcome                                                                 |
|------------------|------|--------------------------------------------|----------------------|--------------|--------------------------------------------------------------------------|
| Ichioka (2002)   | [66] | SAMP6 (age-related osteoporosis)           | WBM (M) / intrafemoral/ 3 × 10^6 cells | Not quantified | Increase in trabecular bone. Normalization of BMD and BM environment     |
| Hsiao (2010)     | [67] | OVX mice (Postmenopausal osteoporosis)     | Transgenic MSCs (M) (GFP)/IV/1.5 × 10^6 GFP-MSCs on day 8, 6, 12, 18, 24, and 30 | Not quantified, GFP signal present in trabecular and cortical bone (2 months) | Improvement in endochondral BMD and slight improvement in BV/TV          |
| Ma (2015)        | [68] | MRL/lpr mice (Secondary osteoporosis)      | Human BMSCs/IV/1 × 10^4 cells per g | Not quantified | Improvement of BMD and trabecular bone formation                         |
| Cho (2009)       | [69] | OVX mice (Postmenopausal osteoporosis)     | Transgenic MSCs (M) (CXCR4 and Rank-Fc)/IV/2 doses (6–7 × 10^5 cells; day 0/7) | 2% (48 hours) | Prevention of BMD decline                                                |
| Lien (2009)      | [70] | Glucocorticoid-induced secondary osteoporosis (C3H/HeN) | Transgenic MSC-like cell line (M) (CXCR4 and Cbfa-1)/IV/1 × 10^6 | 1.5% (7 Days) | Restoration of bone formation, stiffness and strength                     |
| Singh (2013)     | [71] | Wm1-/-/Terc-/- accelerated aging (Age-related osteoporosis) | WBM (M)/IV/S × 10^6 | MSCs present in bone marrow. 6%-20% of femoral osteocytes and 5%-15% of femoral osteoblasts were donor derived (10.5 months) | Delay in microarchitectural deficiencies associated with Wm1/ Terc mice |
| Kiernan (2016)   | [72] | Sco-1+ mouse (Age-related osteoporosis)    | WT/Transgenic (GFP) MSCs (M)/IV/2 × 10^6 | Bone marrow (0.1 to 4.5 cells/million), lungs (2,300 cells/million) documented by flow cytometry and qPCR (6 months) | Improvement in bone formation and overall turnover. Improved microarchitecture |
| Sackstein (2008) | [73] | NOD/SCID mice                              | MSCs (H) (Modified CD44)/IV/5 × 10^6 | Not quantified, present on endosteal surface (12 weeks) | Small amount of human osteoid documented                                |
| Guan (2012)      | [74] | OVX (Postmenopausal) and aged C57BL/6 mice (Age-related osteoporosis) | Transgenic MSCs (H) (GFP w/ LLP2A ligand/IV/dose not specified) | Not quantified, large numbers of transplanted cells present on trabecular surface | Complete resolution of osteoporosis. Increase in bone formation          |
| Liu (2015)       | [75] | MRL/lpr mice (Secondary osteoporosis)      | WT (M) MSCs/IV/0.1X10^6 cells per 10 g body weight | Not quantified | Amelioration of osteopenia, restoration of native BMSC function in MRL/lpr mice |
| Sui (2016)       | [76] | Glucocorticoid-induced secondary osteoporosis (C57Bl/6) | MSCs (M) from untreated C57Bl/6 mice/IV/1 × 10^6 | Not quantified, but present for at least 4 weeks | MSC transplant prevented the loss of bone volume and strength.          |

Table 1 documents specific reference, animal model, donor engraftment, MSC source/delivery/dose, and outcome for all preclinical studies using MSC transplant to increase bone formation in various rodent models of osteopenia. Abbreviations: BMSCs, bone marrow stromal cells; BMD, bone mineral density; BVTV, Bone volume fraction; GFP, green fluorescent protein; MSC, mesenchymal stromal cells; OVX, ovariectomized; WBM, whole bone marrow; WT, wild type.

homing and RUNX2 to promote osteogenic differentiation. The authors injected these engineered cells into a glucocorticoid induced mouse model of secondary osteoporosis, a phenotype partially driven by apoptosis of bone forming osteoblasts. The authors showed that CXCR4 expression increased the homing and retention of cells, which alone led to the recovery of bone mass. However, RUNX2 coexpression with CXCR4 was necessary for recovery of bone stiffness and strength [70]. Using a similar approach, Cho et al. engineered human MSCs to express CXCR4, as well as RANK-Fc on the cell surface. RANK-Fc is a nonfunctional fusion protein consisting of the RANK receptor with Fc region of human IgG. This antagonist of normal RANK-RANK ligand (RANKL) signaling between osteoblasts and preosteoclasts functionally inhibits osteoclastogenesis. The authors concluded that the expression of CXCR4 increased the retention of MSCs within the BM, and alone could be responsible for the observed maintenance of bone mass after ovariectomy. However, CXCR4 could also increase the effect of RANK-Fc mediated antagonism of osteoclastogenesis by attracting more MSCs to the bone surface, thus increasing the interaction of RANK-Fc with RANKL [69].
Using an ex vivo enzymatic process, Sackstein and colleagues successfully modified the CD44 glycoform present on MSCs into the glycoform present on HSCs, capable of binding E/L-selectin on the vessel wall to initiate vascular rolling, thus increasing their engraftment capabilities. When injected into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, MSCs infiltrated BM within hours of injection. Rare foci of human cells associated with human osteoid were documented [73]. In a complementary fashion, Guan et al. generated a peptidomimetic ligand (LLP2A) that bound with high affinity to the α4β1 integrin on the surface of MSCs. When LLP2A was targeted to the bone surface with the bisphosphonate alendronate (LLP2A-Ale), it led to donor MSC accumulation on the trabecular surface. It was also found that the donor MSCs differentiated into osteoblasts and mature into osteocytes, resulting in increased bone formation. Importantly, LLP2A-Ale injection alone was able to recruit endogenous MSCs to the bone surface, leading to increased trabecular and cortical bone formation and bone volume in both OVX and the age-related C57BL/6 mouse models of osteoporosis. LLP2A-Ale was also able to initiate the restoration of trabecular strut features, normally only achieved through anabolic PTH treatment [74].

**Clinical MSC Transplant-Based Interventions to Treat Bone Diseases**

As of December 2016, over 500 clinical trials using MSCs have been registered with clinicaltrials.gov, with a small number of these targeting bone including nonunion fracture healing [77], distraction osteogenesis [78, 79], high tibial osteotomy [80], avascular necrosis of the hip [81, 82], and spinal fusion [83]. Such site specific clinical trials are informative, as they support the hypothesis that transplanted MSCs can increase bone formation in vivo. However, the systemic nature of osteoporosis, affecting many boney sites, reduces the utility of site-specific MSC delivery as a treatment option.

Osteogenesis imperfecta (OI) is a spectrum of disease phenotypes associated with dysfunction of the collagen type I gene product [84]. Clinically, OI results in connective tissue malfunction disorders such as increased susceptibility to fractures, fragile deformed bones, slow or stunted growth, and low bone mass. Severity ranges from in-utero death or severe morbidity due to multiple fractures, to a relatively normal life with mild fracture tendency. Most children are restricted in play activity due to the high fracture risk [85]. OI was chosen as the first disease to investigate the utility of MSC therapy. This was due to a successful preclinical study that documented successful MSC engraftment into a murine model of OI produced a small but appreciable improvement in the disease phenotype [86]. The pilot clinical study undertaken by Horwitz and colleagues used allogeneic WBM transplantation into three children with OI, and assessed engraftment and phenotypic relief. Low-level osteoblast engraftment was documented, and trabecular bone showed histologic changes indicative of new dense bone formation. All patients had increased bone mineral content, increases in growth velocity, and reduced frequencies of bone fracture that lasted for 6 months [56]. A second study that included two of the original patients documented increases in body length in the 6 months following the transplant, which then plateaued, but bone mineral content continued to increase at a rate similar to that for weight-matched healthy children, even as growth rates declined [57]. The subsequent trial used culture expanded MSCs instead of WBM on the six children who had previously undergone BM transplants to treat OI. Five of six patients showed engraftment in one or more sites, including bone, skin, and marrow stroma, and had an acceleration of growth velocity during the first 6-months post-infusion. No adverse events were observed, demonstrating safety, and the authors attributed the long-term graft loss to a host immune response against the neomycin transgene that was introduced into MSCs for tracking purposes [58].

A recent study by the same group suggested that nonadherent BM cells and MSCs contribute differently to healing in OI, and surprisingly, the nonadherent BM cells may be the cells directly differentiating to osteoblasts, while the MSCs function by secreting soluble mediators to stimulate growth [60]. One interesting anecdotal study documented the transplant of allogeneic fetal liver derived MSCs into a fetus diagnosed with severe OI. At nine months of age, 7.4% (range 6.8%–16.6%) of osteoblasts were of donor origin, with no signs of rejection, and histology showed normal trabecular arrangement. By 2 years of age, the child had sustained only three fractures, and growth was assessed as normal [87]. At age 8, the patient was re-transplanted, and assessed two years later. The patient showed no lymphocyte proliferation, anti-FCS abs, anti-HLA I and II abs, anti-IgG, or anti-IgM against MSC donor cells. Donor cell engraftment was low (0.003%) and limited to bone. In the two years since re-transplantation, her linear growth improved, as has her ability to walk, and she participates in modified physical activity [88].

Anecdotal evidence suggests MSC therapy as a viable option for another systemic bone disease, hypophosphatasia. This rare inherited metabolic bone disease is characterized by an electrolyte imbalance where low blood phosphate levels result from the loss of tissue nonspecific ALP activity. Bone mineralization is affected, and rickets and osteomalacia may occur [85]. Currently, there is no medical treatment for this disorder, although anecdotal evidence showed PTH administration may be beneficial [85]. MSCs have been shown to be potentially beneficial for the treatment of hypophosphatasia. To date, three cases of MSC (or BM in one case) transplantation from HLA mismatched parents or siblings mediated a clinical improvement in disease presentation [89, 90]. Further clinical trials would be required to assess the use of MSCs to treat this disease; however, the orphan disease status of hypophosphatasia makes large trials unlikely.

**TRANSLATION OF SSCs: FUTURE DIRECTIONS TOWARD TARGETED BONE REGENERATION**

Before the full benefit of SSC therapy can be leveraged toward bone regeneration, certain basic, translational, and clinical scientific questions will need to be answered. First, SSCs have only recently been characterized in murine models [22, 23, 49], and aside from one study documenting a BM stromal cell possessing some properties of SSCs (the generation of the hematopoietic microenvironment), this cell remains unidentified. As such, the human SSC still needs to be located, and fully characterized for phenotypic characteristics and cell surface antigen profile. Second, once identified, methods need to be developed to ensure the cell can be harvested and expanded to clinically relevant quantities. Stem cells often lose their multipotent and self-renewal capabilities soon after removal from their native environment; therefore, mitogens and physical parameters (i.e., 3D scaffolds, substrate stiffness, and oxygen tension) will need to be optimized to enable...
large scale culture. Finally, although the safety and tumorigenic profile of MSCs has been fully evaluated and deemed safe [91], necessary due diligence will need to be performed on SSCs. Standard preclinical and subsequent phase I clinical trials will need to be undertaken to assess the overall safety of SSC transplants.

**Precedence for an Ancillary Clinical Trial**

Ancillary studies are carried out in conjunction with larger, ongoing parent clinical trials. Such studies enhance the scientific impact of clinical trials by maximizing resources and patient samples. Ancillary studies can be related to the primary trial, or, in some cases, address a fundamentally different question. For example, the VTam D and OmegA-3 Trial (VITAL) is a double-blind, placebo-controlled trial assessing the utility of vitamin D3 and omega-3 FA to reduce the risk of cancer and cardiovascular disease (CVD) in men aged ≥50 and women aged ≥55 [92]; however, an ancillary study has been initiated to assess the effects of vitamin D and/or omega-3 fatty acid on incident fractures and bone health outcomes in this same cohort [93].

Conceptually, the ancillary study should be of high quality and take advantage of the primary trial design and add to its scientific merit. Arrangement of an ancillary arm during the parent trial design phase is critical, as feasibility and careful planning of patient resources must be considered. The aforementioned VITAL-Bone Health trial leverages the power of the nearly 26,000 patients enrolled in the double blind, placebo controlled primary VITAL trial for Cancer and CVD. The Vital-Bone Health trial was funded by the NIH-National Institute of Arthritis and Musculoskeletal and Skin Disease alongside numerous other NIH-Institutes that sponsored the primary trial. More ancillary studies are underway, all funded under NIH initiatives investigating a myriad of maladies including: Diabetes, Hypertension, respiratory diseases, autoimmune disorders, depression/mood disorders, infections, diabetic nephropathy, atrial fibrillation, anemia, macular degeneration, dry eye syndrome, mammographic density, magnesium and vitamin D, racial/ethnic differences, and vitamin D/adiposity [94]. Similar coordination of forthcoming large cell therapy clinical trials, potentially initiated by the funding agencies and/or major stakeholders, could enable substantial ancillary studies. Ancillary funding could be competed for in the trial initiation phase, and subsequently included in the trial design. The primary agency could entirely fund the ancillary study, or share costs with disease specific not-for-profit groups, other funding agencies, or private industrial partners generating the cell products, as is done in the NIH model.

Importantly, an ancillary study must not interfere with the aims of the primary study. Consent sought in the patient recruit-ment should indicate the ancillary use of specimens [95]—and at the very least, indicate that a portion of the sample may be stored for future, yet to be determined research use. Care must be taken to minimize patient burden (number of extra blood draws, number of extra questionnaires, number of extra clinic visits etc.), to not detrimentally impact the recruitment, compliance, conduct, and aims of the primary study. In the VITAL-Bone Health trial, patients would experience little to no increased burden. Blood samples drawn as part of the primary trial will be used to assess markers of bone formation, and self-reporting will be used to monitor incidence of fracture over the observation period. A subset of 1054 VITAL patients will have slightly more burden in the form of two clinic visits to undergo bone density, structure, and architecture measurements [93]. Added patient burden for VITAL associated studies are also minimal and include: basic measurements (body measurements, blood pressure, echocardiogram, spirometry, and cognitive function), outcome monitoring (death, fracture, physical activity, heart failure, and hypertension related nephropathy), and specimen assessment (blood, urine).

With over 500 clinical trials using MSC registered with clinical-trials.gov and numerous others being conceived, there presents a tremendous opportunity to maximize the scientific value of these expensive, laborious studies. The ability to co-operate, and leverage the availability of large, well-characterized cohorts of patients receiving MSCs (or other cell therapy/regenerative medicine agents) will maximize resource utilization. The ubiquitous nature of age-related bone loss in humans makes it an ideal candidate for regenerative medicine. Thus, we propose an ancillary study for osteoporosis to assess bone formation gains after systemic MSC transplant. Furthermore, being a chronic disease that causes acute debilitating injury (hip fracture, vertebral fracture), and mortality, a large cohort would be necessary to address the clinical relevance of any bone formation gains. By teaming up with other, even multiple, clinical trials the necessary number of patients could be more readily reached. This innovative model could be used to assess stem cell effects on various diseases in patients with existing comorbidities and chronic disease from trials that would normally only focus on one of the patient disease states. Significant savings can be achieved using an ancillary model in cell therapy due to cost sharing of expensive cell isolation, manipulation, and patient delivery. This leaves only the specific sample isolation and analysis tasks to the individual investigators (Fig. 2).

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AUTHOR CONTRIBUTIONS

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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