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Suitability and limitations of mesenchymal stem cells to elucidate human bone illness

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Abstract

Functional impairment of mesenchymal stem cells (MSCs), osteoblast progenitor cells, has been proposed to be a pathological mechanism contributing to bone disorders, such as osteoporosis (the most common bone disease) and other rare inherited skeletal dysplasias. Pathological bone loss can be caused not only by an enhanced bone resorption activity but also by hampered osteogenic differentiation of MSCs. The majority of the current treatment options counteract bone loss, and therefore bone fragility by blocking bone resorption. These so-called antiresorptive treatments, in spite of being effective at reducing fracture risk, cannot be administered for extended periods due to security concerns. Therefore, there is a real need to develop osteoanabolic therapies to promote bone formation. Human MSCs emerge as a suitable tool to study the etiology of bone disorders at the cellular level as well as to be used for cell therapy purposes for bone diseases. This review will focus on the most relevant findings using human MSCs as an *in vitro* cell model to unravel pathological bone mechanisms and the application and outcomes of human MSCs in cell therapy clinical trials for bone disease.

Key words: Mesenchymal stem cells; Bone illness; Osteoporosis; Osteogenesis; Osteoanabolic therapies; *In vitro* cell models; Cell therapy

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Core tip: Human mesenchymal stem cells (hMSCs) have emerged as an encouraging therapeutic strategy for the treatment of bone diseases. Moreover, certain limitations of

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animal models for the study of bone disorders highlight the suitability and benefits of hMSCs for the elucidation of these pathologies. The current review explains the available strategies based on hMSCs for bone illness, new treatment development, and future directions in the field for more accurate knowledge of the cause underlying these human pathologies.

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INTRODUCTION

In humans, the structural maintenance of the skeleton during adulthood is ensured by the continuous self-regeneration of bone tissue in a process called bone remodeling. The entire skeleton is renewed approximately every 10 years^[1] by a sequentially coordinated action of two coupled processes performed in bone remodeling units at distinct locations all throughout the skeleton: Bone resorption and bone formation. Bone resorption, in which old and damaged bone is removed by osteoclasts, is a relatively fast process that can last 4-6 wk; whereas, new bone formation orchestrated by osteoblasts, which produce collagen and mineralized bone matrix, takes approximately 4-5 mo^[2]. Osteoclasts and osteoblasts are differentiated cells originating from two separate cell lineages: Osteoclasts differentiate from hematopoietic precursors, and osteoblasts are cells of mesenchymal origin. Thus, osteogenic differentiation and the generation of new osteoblasts are driven by a sequential cascade of processes performed by mesenchymal stem cells (MSCs). First by the recruitment of MSCs to bone remodeling sites and subsequent proliferation, then lineage commitment with expression of lineage-specific markers, and finally with collagen secretion and extracellular matrix (ECM) mineralization^[3].

Bone remodeling is a continuous process throughout life; however, the balance between bone formation and bone resorption is age-dependent. Thus, bone formation predominates for the first three decades until peak bone mass occurs^[4]. Thereafter, when the growth period is complete in adulthood, there is a remodeling balance in which the previously achieved bone mass is maintained, and the amount of resorbed bone equals that which is subsequently formed. Later, in aging, the bone loss common to this period of life is due to an imbalance between bone resorption and bone formation: Accelerated osteoclastic bone resorption occurs compared to the amount of new bone formed by osteoblasts. Moreover, aged MSCs show a shift of lineage commitment to adipogenesis at the expense of osteogenesis^[5] and a concomitant reduction in self-renewal capacity^[6]. This dysfunction of MSCs, which contributes to the remodeling imbalance, lies at the root of bone loss due to aging. As a consequence, bone aging is the leading risk factor for primary osteoporosis, a progressive systemic skeletal disease characterized by a reduction in bone mineral density, predisposing the elderly population to an increased risk of fractures. In this scenario, the use of MSCs (osteoblast progenitor cells) for bone disease modeling emerges as a suitable approach to perform mechanistic studies, devise drug discovery by high throughput screenings, and test cell-based therapies. This review will focus on the current benefits and limitations of MSCs for two different goals related to bone illness: As *in vitro* disease models to study the pathogenic mechanisms of bone disease in order to screen and/or develop new therapeutic drugs, and as treatments based on cell therapies.

THE SOCIO-ECONOMIC IMPACT OF BONE DISEASES

Age-related osteoporosis is the most prevalent bone disease, especially among postmenopausal women and older men, affecting over 200 million worldwide and causing more than 9 million fractures per year^[7]. Improvements in socioeconomic and health-related factors have resulted in an increase in population life expectancy making osteoporosis a global and growing public health challenge. Osteoporotic fractures cause a 20% increase in mortality within 1 year of the broken bone and also result in poor quality of life, functional impairment, and loss of independence leading

to an increased financial burden in health care systems^[8]. In addition to osteoporosis, more than 450 skeletal dysplasias have been described that affect primarily bone and cartilage, most of them with limited treatment options^[9]. Abnormal bone formation directed by osteoblasts, abnormal bone resorption by osteoclasts, or both may be among the underlying pathological cellular mechanisms of these heritable diseases. Studying these rare genetic bone disorders is clinically highly relevant, and although individually they affect a small percentage of the population, their overall frequency is high: Two to five per 10000 live births^[10]. Importantly, many of these diseases become apparent early in life and are present throughout the patient's entire life implying tremendous burdens in disability and suffering and requiring extensive medical and surgical treatments. Research focusing on these genetic skeletal disorders is not only beneficial for future treatment of patients but significantly contributes to the knowledge of key concepts of bone biology.

Pharmacologic therapies for osteoporosis can be categorized as either antiresorptive or anabolic; both strategies focus on reducing the risk of fractures^[11]. Current treatments are mainly based on antiresorptive agents, including estrogen, selective estrogen receptor modulator, bisphosphonates, and a monoclonal antibody to receptor activator of NF- κ B ligand (RANKL) (denosumab)^[12]. These therapies decrease the generation, function, and survival of osteoclasts thereby reducing the rate of bone resorption. However, because bone resorption and formation are coupled processes, this inhibition in bone resorption also results in lower bone formation. Although antiresorptive drugs are effective in reducing fracture risk^[13], there are concerns about side effects accompanying their continued use, such as increased cardiovascular events, increased breast cancer risk due to estrogen use^[14], and more rare side effects, such as atypical femur fractures^[15] and osteonecrosis of the jaw^[16].

Moreover, bisphosphonates are known to accumulate in the skeleton and continue to be released for long periods of time following treatment^[17]. Given that osteoporosis is a chronic disease, treatments for osteoporosis should theoretically be administered throughout the patient's life. However, due to the aforementioned side effects of antiresorptive drugs, they are generally not administered for more than 5 years. Taking into account both increased life expectancies and these limitations regarding the continued use of antiresorptive agents, there is an urgent need to develop new drugs for osteoporosis focused on osteoanabolic goals (to increase bone formation).

Currently, the two available anabolic drugs are teriparatide and abaloparatide, both recombinant human parathyroid hormone (PTH) analogs, which have been demonstrated to increase bone formation when given intermittently in small doses^[18]. However, there were initial concerns regarding the long-term administration of these therapies as well because extended exposure to analogs of PTH in preclinical (animal) studies was associated with a higher risk of osteosarcoma^[19]. However, a later long-term surveillance study of adult cases of osteosarcoma did not show an increased risk of osteosarcoma associated with teriparatide treatment^[20]. Overall, these observations evidence that the range of current anabolic treatment is quite limited, making it imperative to identify, characterize, and develop novel effective and safe osteoanabolic therapies.

ADVANTAGES AND FLAWS OF ANIMAL MODELS OF BONE DISEASES

Several animal models have been developed in order to study the different molecular mechanisms underlying bone-related diseases and to serve as fundamental tools in which to test and develop new therapeutic strategies. The biggest challenge when choosing the appropriate animal model is not knowing the exact cause of the disease. Rodents are the most commonly used animal model for research, despite the fact that large animals show bone development resembling the human process more accurately than rodents^[21]. We will briefly summarize the advantages and disadvantages of different animal models used for the study of various bone diseases, and we will focus here on the success and failures of murine models to mimic different types of the bone disorder called osteogenesis imperfecta (OI).

Some rodent models successfully resemble the human characteristics of several bone-related diseases. A mouse model of Paget's disease in which the normal bone recycling process is affected shows increased bone resorption and bone formation and increased numbers of osteoclasts that are larger and multinucleated, a finding similar to human patients suffering from this disease^[22,23].

Osteoporosis is distinguished by low bone mass and structural deterioration of bone tissue, occasioning bone fragility, and increased risk of fractures^[24]. Osteoporosis has been studied in different animal models; however, none of these models

satisfactorily resembles the characteristics of the disease in humans^[25]. The most extensively used model is the ovariectomized rodents (mouse or rat). This process induces a loss of bone mass and strength due to the reduction of estrogen, similar to the loss of estrogen in postmenopausal women. Despite the low costs and easy handling, rodents lack the Haversian canal system of the cortical bone present in humans^[25]. This is the initial animal system for identifying possible therapies. Potential drugs or treatments are subsequently replicated and tested on larger animals, such as primates, rabbits, sheep, and pigs^[25,26].

Hypophosphatasia (HPP), or deficiency of the alkaline phosphatase (ALP) enzyme^[27], has been investigated in various murine models. ALP knock-out mice have been largely used to identify mechanisms underlying the disease since affected mice adequately mimic the phenotype of children with HPP^[28-31].

OI is a genetic disease with high heterogeneity at both the genotypic and phenotypic levels^[32-34]. OI patients are classified into different OI types according to their phenotype and genetic mutation causing the disease. The majority of the mutations are autosomal dominant and are located in the *COL1A1* and *COL1A2* genes (Type I-IV), while some less frequent mutations are recessive and located in different genes involved in the osteogenic process (*IFITM5*, *CRTAP*, *LEPRE1*, *SERPINF1*, *PPIB*, and *FKBP10* among others)^[32-34]. As the genetic causes of the OI phenotype are so diverse, several different models have been described for the study of the different OI types. Various models have been useful for the elucidation of OI pathology, while some models have shown effects opposite to those observed in OI patients. Here we present some of the murine models and their effectiveness in reproducing human OI phenotypes/symptoms (Table 1).

The low prevalence of certain types of OI (IX^[35,36], XII^[37,38], XIII^[39], XIV^[40,41], XV^[42], and XVI^[43]) makes it difficult to develop an exact diagnosis of symptoms and causes of these types of OI. Therefore, it is difficult to assess the suitability of the models even though such models could be a useful tool for gaining basic knowledge of these OI types. In contrast, several OI types have been successfully described for which the suitability of the animal models can be evaluated. Murine models for OI types I^[44-46], II^[44,47], III^[44,48-51], IV^[52-56], VI^[57], VII^[58-61], and XI^[62,63] positively mimic human phenotypes. Models developed for OI type V^[64-66], VI atypical^[67], VIII^[60,68,69], and X^[70,71] show differences in the mechanisms underlying those mutations with diverse grades of severity when compared to humans and different signaling pathways involved in the process.

Despite murine models being the most utilized animal models for the study of human bone-related diseases, mice and humans diverged at some stages of the skeletal regulatory process^[72]. More than half of the signaling pathways and bone development-related genes are expressed in both species. These include *BMP*, *Hedgehog*, *FGF*, and *Notch* and transcriptional regulators of osteogenesis like *RUNX2* and *SOX9*^[72]. On the other hand, divergent genes comprise various members of the WNT signaling pathway, such as *SOST*, *CXXC4*, and deoxyribonucleic acid (*DNA*)/*JB6*^[72]. This fact should be kept in mind when trying to extrapolate results from murine models to patients.

In summary, animal models are a useful and necessary tool when elucidating the molecular mechanisms underlying disease with low prevalence, but are not sufficient to properly understand the human pathophysiology of the disease.

MSCs AS EXPERIMENTAL HUMAN DISEASE MODELS

In vitro bone disease modeling by primary MSCs

The failure of some animal models to resemble the features of many human diseases led to the development of a field focused on the creation of *in vitro* cell models using primary cells isolated from patients and healthy cohorts. These disease-relevant cell types recapitulate the majority of the pathological phenotypes observed in patients, providing new opportunities to study the cell biology and pathophysiology of the disease.

An example of such models focusing on prematurely aging cells is based on either human MSCs (hMSCs)^[73] or induced pluripotent stem cells (iPSCs)^[74]. MSCs are characterized by multipotency, self-renewal capacity, and the ability to differentiate into different cell lineages, *e.g.*, an osteogenic lineage^[75-78]. The osteogenic potential of MSCs has been demonstrated in MSCs expanded culture^[79] making them a perfect cell type for the study of molecular mechanisms regulating bone disorders, especially those disorders caused by osteoblast alterations^[76-78,80]. Thus, MSCs, which are the context-related cell type for modeling diseases with mesenchymal defects, have emerged as an essential tool to unravel the molecular and cellular mechanisms

Table 1 Mouse models developed for OI

OI type	Mutations at gene	Human phenotype	Ref.	Mouse model	Mouse phenotype	Effectiveness	Ref.
I	COL1A1/2	α 1 chain collagen haplo-insufficiency; vertebral compression fractures; short height; low lumbar spine bone mineral density	[32,33]	Col1a1+/Mov13	Decreased type I collagen in mineralized tissue, weakened bone strength; abnormal shape of long bones; alterations of the mechanical properties of long bones	+	[44-46]
II	COL1A1/2	Perinatal lethal	[32,33]	BrtlII; Aga2/b	Perinatal lethal	+	[44,47]
III	COL1A1/2	High bone turnover; decreased mineralization; increased osteoclastic activity; small size; fractures; osteopenia; bone deformities	[32,33]	COL1A2 KO	Increased bone formation rate; fractures; reduced size; osteopenia; decreased mineralization; abnormal bone shape	+	[44,48-51]
IV	COL1A1/2	Increased bone fragility; growth deficiency; weak bone geometry; impaired bone remodeling; decreased bone volume	[32,33]	349G->C COL1A1	Decreases in severity with age; increased bone brittleness; reduced bone size; abnormal bone shape; impaired bone remodeling	+	[52-56]
V	IFITM5	Increased mineralization; increased osteoblast markers; decreased COL1A1 expression, secretion and deposition in the matrix; hyperplastic callus; calcification of the forearm interosseous membrane; radial-head dislocation; subphyseal metaphyseal radiodense band	[32,33]	14C->T IFITM5	Severe skeletal defects; perinatal lethality; decreased mineralization; reduced expression of osteoblast markers	-	[64-66]
VI Atypical	IFITM5	Decreased levels of PEDE; decreased mineralization	[32,33]	IFITM5 Knock-Down	Reduced skeletal size less extreme in adults; no abnormal osteoclastogenesis; no abnormal osteoblastogenesis	-	[67]
VI	SERPINF1	Decreased mineralization; decreased trabecular bone	[32,33]	PEDF KO	Decreased ECM mineralization; reduced trabecular bone volume	+	[57]

VII	CRTAP	Growth delay; osteopenia; decreased bone formation; decreased mineralization; multiple fractures	[32,33]	CRTAP KO	Growth underdevelopment; osteopenia; decreased osteoblastogenesis; decreased mineralization; no spontaneous fractures	+	[58-61]
VIII	LEPRE1	Lethal; severe growth deficiency; bone fragility; poorly mineralized skull; scoliosis; decreased mineralization	[32,33]	LEPRE1 Knock-Down	No lethality; abnormal collagen fibril ultrastructure in bone, tendon and skin	-	[60,68,69]
IX	PPIB	Lethality; severe bone mass reduction; extreme bone strength reduction	[32,33]	PPIB KO	Bone mass reduction; bone strength reduction	No enough information	[35,36]
X	SERPINH1	Embryonic lethality; delayed type I collagen secretion; collagen accumulation in Golgi apparatus; osteopenia; dentinogenesis imperfecta; thin bones	[32,33]	HSP47 KO	Delayed type I collagen secretion; collagen accumulation in the endoplasmic reticulum	-	[70,71]
XI	FKBP10	Growth delay; neonatal lethality; bone fragility	[32,33]	FKBP10 KO	Bone brittleness; underdeveloped growth; lethality	+	[62,63]
XII	OSX	Skeletal deformities; fractures; osteoporosis	[32,33]	Osx KO	No bone formation; decreased mineralization	No enough information	[37,38]
XIII	BMP1	Skull defects; reduced bone mass; reduced bone strength	[32,33]	BMP1 KO	Reduced ossification of certain skull bones	No enough information	[39]
XIV	Tric-b	Reduced bone mass	[32,33]	Tric-b	No incorporation of collagen in the matrix; matrix insufficiency	No enough information	[40,41]
XV	WNT1	Reduced bone mass; reduced bone strength; fractures; increased ductility	[32,33]	sw/sw	Bone fragility; low bone mass	No enough information	[42]
XVI	CREB3L1	Reduced bone mass and fractures	[32,33]	CREB3L1 KO	Severe osteopenia; reduced type I collagen	No enough information	[43]

+/- stand for positive mimicry of the OI type symptoms in humans (+) or negative mimicry of OI type symptoms in humans (-). OI: Osteogenesis Imperfecta; KO: Knock-out; ECM: Extracellular matrix.

involved in normal and pathological bone biology. Physiological aging is known to be accompanied by a switch of MSCs differentiation to the adipogenic lineage at the expense of osteogenesis, which leads to osteoporosis^[81]. MSCs used as *in vitro* disease models of aging have been essential to elucidate various mechanisms that account for the osteogenic differentiation impairment exhibited in the context of aging, such as dysregulation of transcription factors and microRNAs, autophagy impairment, alterations of the nuclear lamina, and epigenetic modifications of DNA^[82].

MSCs isolated from patients with particular bone disorders have also been essential in deciphering the underlying molecular mechanisms of the associated bone diseases.

HPP: MSCs isolated from pediatric patients suffering from HPP showed a premature entry into senescence and a differentiation switch to adipogenesis at the expense of osteogenesis, both of which are typical features of aging MSCs. These results indicated that the *ALPL* gene contributes to controlling MSC lineage differentiation and prevents cell senescence^[83].

Hutchinson-Gilford progeria syndrome (HGPS): Also known as progeria, is a devastating rare genetic disorder characterized by dramatic premature aging in children, and the disease primarily affects tissues of mesenchymal origin^[84]. Skeletal defects are among the HGPS phenotypes, including abnormalities in bone morphology and alterations in bone structure, which result in a unique skeletal dysplasia^[85]. MSCs differentiated from patients iPSCs recapitulate some aspects of the syndrome, including abnormal nuclear architecture, progerin expression, defects in the DNA repair process, and premature differentiation into the osteoblastic lineage^[74].

Recently, two simultaneous works based on a high throughput drug screening in progeria-MSCs showed the usefulness of this cell model to decipher the functional effects of drugs that are currently used in HGPS patients and to identify new potential pharmacological drugs to treat the disease^[86]. Both works evaluated the capacity of already known and new screened drugs to restore the impaired osteoblastic differentiation exhibited by progeria-MSCs. Moreover, paracrine signaling appears to be impaired in aged MSCs, a hypothesis supported by results in which an *in vitro* aged hMSCs model has a secretome enriched in osteogenesis-related proteins that can trigger accelerated early osteogenesis in normal MSCs^[87]. Among the increased secreted factors, insulin-like growth factor binding protein 7 (IGFBP7) was identified. Subsequent experiments silencing IGFBP7 expression revealed an essential and unknown role for IGFBP7 to maintain the viability of MSCs during the first steps of osteogenesis in which MSCs and pre-osteoblasts proliferate actively. Moreover, sheets of hMSCs overexpressing IGFBP7 improved bone healing in a rat tibial osteotomy model^[88].

2D versus 3D culture of MSCs

Although experimental modeling of human bone disorders represents a breakthrough to provide outstanding insight into the cellular and molecular mechanisms involved in bone pathologies, there are several drawbacks regarding the use of MSCs models that must be taken into account. The limited availability and extreme heterogeneity of MSCs from patients as well as limited proliferation capacity and loss of functionality are among the most common pitfalls when using MSCs *in vitro*.

In addition, the main cell culture approach used in research is 2D cell cultures in which cells adhere to the culture dish forming monolayers, a situation that does not reflect the *in vivo* cellular display where cells grow in a complex 3D disposition^[89]. The conditions of the natural environment *in vivo* are poorly mimicked by 2D cell cultures since they do not preserve normal physiological shape and function. In other words, the morphology and physiology of 2D cultured cells highly diverge from *in vivo* grown cells^[89,90]. Due to the complex architecture of bone tissue, the use of 2D cell cultures does not adequately mimic the actual mechanisms involved in bone tissue development and repair^[90] making it a limited approach to the study of bone-related diseases. Furthermore, bone remodeling consists of a highly regulated balance between bone resorption and bone formation mediated by osteoclasts and osteoblasts, respectively. Osteoclasts are phagocytic cells derived from circulating macrophages in charge of bone degradation while osteoblasts differentiate from MSCs and are in charge of bone formation^[91,92]. Osteoblast and osteoclast activity is tightly coupled positively influencing the osteogenic differentiation and matrix deposition in the same way as osteoclast development^[91,92]. Therefore, osteoblast and osteoclast activity is directly regulated by the crosstalk between both cell types leading to an increased matrix deposition in osteoblast and osteoclast co-culture experiments^[91,92]. Moreover, conventional 2D cultures have limited cell-cell and cell-matrix interactions, which are especially relevant in bone tissue such as the direct crosstalk between osteoblasts and osteoclasts, highlighting the need for more realistic 3D *in vitro* models of bone disease^[90]. 3D cultures have been proposed as a bridge between 2D cell cultures and *in vivo* models, and therefore have been used in the study of bone diseases^[93] as a consequence of their higher structural complexity and cellular homeostasis, which is more closely comparable to that of tissues and organs^[89].

Due to the importance of the ECM in bone microarchitecture, a wide range of scaffolds have been developed for 3D culture of bone tissue^[94]. The purpose of these scaffolds is to serve as extracellular support for adhesion of growing cells in a 3D structure^[89]. Scaffolds used for bone tissue culture can be formed by different materials, such as collagen, bioceramics, titanium, gelatin, chitosan, polymers, hydrogels, and others^[94-96]. The ideal scaffold should have similar mechanical

properties to bone; therefore, hydrophobicity and porosity are two essential features to keep in mind when engineering the scaffold^[94,96,97]. Scaffolds have been used for different purposes such as basic research tools for *in vitro* and *in vivo* studies. Certain bone pathologies require therapeutic grafts due to the necessity of extensive bone regeneration^[96]. Autografts are the best choice when compared to allografts; however, both have certain disadvantages. Autografts are size restricted and could create infections or morbidity in the healthy tissue from which the graft is taken. On the other hand, allografts lack the cellular content to assist tissue regeneration and could carry diseases^[96]. Nevertheless, engineered scaffolds are considered a promising solution for bone grafts.

Several studies on animal models have been performed showing positive results for bone regeneration using engineered scaffolds and MSCs^[80,98-100]. 3D scaffolds could also be used for drug delivery into bone tissue^[93,101]. However, several disadvantages have been described when using scaffolds, such as cell adhesion, degradability of the scaffold, appropriate communication between cell types, and the simple 3D architecture of scaffolds^[93]. Given these challenges, bioprinting has emerged as a potential solution to develop more sophisticated, complex, and accurate architectures of bone tissue *in vitro*^[93].

Bioprinting is the latest tool in tissue engineering. This technology is based on a computer-aided design to create a 3D construct assembling biocomposite materials and living cells^[93,102,103]. This strategy has the advantage of more accurate control of cell distribution, higher resolution, ability of cell deposition, spatial complexity in cell types and tissue organization, scalability, and lower cost when compared to 3D cultures using scaffolds. In addition, bioprinting provides a better cell-cell interconnection, oxygen diffusion, nutrient transportation, appropriate attachment, proliferation, and tissue formation factors^[102-105]. Several studies have described the possibility of 3D-bioprinted bone substitutes for tissue regeneration^[102,103,106]. In these studies, osteogenic differentiation of MSCs is possible allowing successful bone repair processes *in vitro* and *in vivo*^[102,106,107]. Vascularization of the tissue is a crucial limitation^[108]. Bioprinting of MSCs along with a functionalized vascular endothelial growth factor allows vascularization of the tissue leading to a successful proliferation, differentiation and generation of the mineralized ECM *in vitro*^[108].

The classical methods for osteogenic differentiation of MSCs in culture are based on the addition of chemical and growth factors although environmental properties influence the *in vivo* osteogenic differentiation of MSCs. Osteogenic differentiation of 3D-bioprinted MSCs could be performed by the classical addition of chemical and growth factors or by the use of the appropriate bioink containing these stimuli. In fact, environmental effects can be mimicked through 3D bioprinting by the addition of soluble factors and additives into the chosen bioink^[109]. Accessibility of nutrients and osteogenic stimuli are problems recognized in 3D cultures on scaffolds. Thus, stimulation of the cells through components of the bioink allows for a homogeneous distribution of the osteogenic stimulus reaching all the seeded cells^[109].

On the other hand, 3D co-cultures of osteoblasts and osteoclasts have been described in which cells are able to deposit mineral matrix^[91,92,110]. Most of the literature describing 3D co-cultures of bone cells is based on human and murine cell lines, which are barely exportable to human primary cells lines. However, recently a 3D co-culture system has been successfully described using patient MSCs for the study of jawbone osteonecrosis^[110], which would be exportable to other bone-related diseases. This system means advancement in the elucidation of the pathogenic mechanisms and the discovery of novel therapeutic strategies for the treatment of bone-related diseases^[110].

MSCs AS THERAPEUTIC TOOLS FOR BONE DISEASES

MSCs are fibroblast-like cells that exist in almost all tissues, including bone marrow, fat, and the umbilical cord among others. They comprise a heterogeneous population of cells with differentiation and self-renewal ability ensuring a replacement mechanism for cells that die due to normal aging, injury, or disease^[111]. Three criteria were proposed by the International Society for Cellular Therapy to define hMSCs as a cell type: (1) Plastic adherence when grown in standard culture conditions; (2) Expression of the cell surface molecules CD73, CD90, and CD105 and lack expression of hematopoietic markers CD34, CD45, CD14, CD19, CD11b, and HLA-DR; and (3) Multilineage differentiation potential into osteoblasts, adipocytes, and chondrocytes^[112]. MSCs also exhibit immunosuppressive properties and express a broad range of chemokine receptors and therefore can migrate in response to many chemotactic factors^[113].

On account of the aforementioned features of MSCs, they are thought to be ideal candidates for cell therapy purposes. However, from a clinical point of view, it must be considered that MSCs show huge variability in terms of functional capacities depending on different factors: Donors, tissue sources, clonal subpopulations, and even at the single-cell level^[114]. When focusing on bone diseases, it would be recommended to screen those MSCs with a higher osteogenic potential to enhance the efficacy of cell therapy applications. With this regard, a striking paper recently described the identification of a human skeletal stem cell population that gave rise to the progenitors of bone and cartilage by the differential expression of four surface markers: PDPN, CD146, CD73 and CD164^[72]. Importantly, these human skeletal stem cells were also shown to be locally amplified in response to skeletal injury. We anticipate that further characterization, isolation and amplification of human skeletal stem cells would be of special interest to obtain better outcomes in the treatment of skeletal disorders by future cell therapy approaches^[72].

For most clinical indications, hMSCs are administered intravenously despite a post-infusion febrile reaction, which is a unique adverse effect associated with their use^[115]. It was initially thought that, upon administration, the cells would home to the sites of injury, engraft, and differentiate into functional cells and then replace affected tissues. However, after administration, especially if they are systemically infused, MSCs engraftment levels are low, and their numbers decrease rapidly with time. The greater cell size of MSCs relative to the pulmonary microvasculature causes the vast majority of infused MSCs to be transiently trapped in the lungs upon the first pass through the circulation; the cells then become undetectable within hours^[116]. This low survival and homing capacity of exogenous MSCs after administration raised the question of the underlying mechanisms responsible for the reported therapeutic benefits of MSCs therapy. Currently, there is growing evidence suggesting that the beneficial effects of MSCs come mainly from their paracrine properties. MSCs are known to secrete a wide range of bioactive factors and extracellular vesicles (exosomes and/or microvesicles) containing proteins, microRNAs, and hormones in response to the local environment, which affects the biology of nearby and distant responder cells and tissues^[117]. Whether the observed beneficial effects of MSCs infusions are directly induced by their secreted factors, or if these factors initiate a cascade of signaling in the resident cell population, which then perform tissue repair, is currently under intense investigation.

MSCs-based therapies for skeletal dysplasias

MSCs infusion has already been tested in clinical trials for two types of skeletal dysplasias, OI and HPP.

OI, or brittle bone disease, is a highly heterogeneous group of genetic disorders mainly caused by autosomal dominant mutations in one of the two genes (*COL1A1* or *COL1A2*) that encode type I collagen. These mutations can affect collagen structure (more severe phenotypes) or collagen quantity (milder phenotypes)^[118]. In addition, severe additional non-collagenous genes have been described recently that cause severe forms of OI, including genes involved in post-translational modification, bone matrix mineralization, and osteoblast differentiation and function^[32]. At this time, there is no cure for OI, and current treatments are focused on inhibiting bone resorption in these patients thus preventing bone loss.

The first proof of principle with allogeneic MSCs infusions in the context of human OI was performed in 2002 by Horwitz *et al.*^[119]. They based their approach on a previous preclinical study that showed successful MSC engraftment into a murine model of OI, which produced a small but appreciable improvement in the disease phenotype^[120]. Horwitz's study included six children, who had received bone marrow transplantation in a previous clinical trial that were given two infusions of adult MSCs. Although MSC engraftment was minimal (< 1% in osteoblasts), an increase in linear growth velocities was observed. Thus, it was established that allogeneic MSC infusion was not only safe in those pediatric patients affected by OI but also resulted in an increase in growth velocity albeit for a limited period of time^[119]. A later investigation from this group of children indicated that the observed benefits could not be attributed to the direct differentiation of surviving infused MSCs into osteoblasts. The authors showed that infusion of MSCs conditioned medium in a mouse model stimulated chondrocyte proliferation suggesting that the secreted factors from MSCs could be responsible for the observed benefits in patients^[121].

Gotherstrom and collaborators demonstrated the safety and efficacy of prenatal transplantation of human fetal MSCs in two fetuses affected by OI, with the premise that the administration of MSCs before birth would be more effective in alleviating OI symptoms^[122]. However, both studies showed that the benefits from a single transplant of MSCs, regardless of the stage of life at administration, are transient, and subsequent infusions with the same donor-MSCs are needed to maintain the

beneficial effects.

HPP is a rare metabolic disorder resulting from a loss-of-function mutation in the *ALPL* gene that codes for the tissue-nonspecific ALP (TNSALP). There is no curative therapy for the disorder^[123]. Impaired function of TNSALP leads to increased concentration of inorganic pyrophosphate in bone ECM; the deposition of this pyrophosphate hampers mineralization of bone and teeth and leads to pathological fractures. Due to the fact that current therapies for HPP have shown limited clinical improvements, hMSCs transplantation offers an attractive therapeutic option for these patients since MSCs, as well as osteoblasts, express high levels of TNSALP in their cell membrane, where it functions as an ectoenzyme^[124].

Two studies have been carried out in which hMSC therapy has been administered to children suffering HPP showing improvements in bone mineralization in patients. In both of these studies, an hMSC infusion was given after previous transplantation of allogeneic bone marrow^[125]. Moreover, chimerism analysis of the *ALPL* gene in the latest study revealed both the expression of wild type and mutant *ALPL* gene products suggesting that donor-derived MSCs were engrafted^[126].

MSCs-based therapies for delayed fracture healing

Non-unions are complications that imply a permanent failure of healing 6 mo after the fracture^[127]. *In vitro* studies showed a decreased functionality of the pool of hMSCs in patients affected by nonunions likely due to a decreased serum expression level of chemokines and growth factors required for their recruitment and proliferation^[128]. However, there was no impairment in the osteogenic capacity of these hMSCs once they were committed to osteogenic differentiation. Taking into account these previous results, a very recent prospective study described the treatment of fracture non-unions in patients with autologous culture expanded bone marrow-derived MSCs. A total of 35 patients received cell therapy, and fracture union was observed in 21 patients. Interestingly MSCs doubling time as well as age, diabetes, and multiple surgeries arose as significant predictors for the outcome of fracture unions^[129].

Cell-free therapies based on the secretome of MSCs

A concentrated secretome of MSCs, *i.e.* the paracrine factors secreted by MSCs mixed with beta-tricalcium phosphate scaffold have been used as a treatment in a recent clinical study for alveolar bone regeneration with encouraging outcomes. In this clinical study, authors showed an enhancement in vascularization, and early bone formation in patients treated with grafts impregnated with MSCs conditioned medium when compared to control patients, which were treated only with beta-tricalcium phosphate scaffolds. Moreover, the presence of MSCs conditioned medium shortened the time needed for degradation and replacement of beta-tricalcium phosphate scaffolds^[130].

CONCLUSION

In summary, primary MSCs isolated from patients in comparison with established cell lines efficiently resemble the pathological mechanisms of bone disease *in vivo*. Secondly, co-cultures offer a greater opportunity to mimic the *in vivo* intercellular crosstalk occurring in patients affected by bone diseases. Lastly, 2D cultures are easier to handle but are quite limited in mimicking the 3D architecture of bone *in vivo*; therefore, 3D cultures are more appropriate to resemble the *in vivo* cellular phenotype in the pathological conditions.

Moreover, MSCs are demonstrating their potential as human experimental models, as essential tools to develop new pharmacological and cell-based treatment strategies, and specifically as a therapeutic modality for bone disorders. Still, there are many questions to be elucidated regarding MSCs therapeutic effects and action mode on human pathologies. A better characterization of the pro-osteogenic MSCs will enable the development of more efficient cell therapies focused on the skeletal disorder.

The advances in using MSCs for therapeutic purposes indicate the extreme relevance of MSC in addressing bone disorders, and the unanswered challenges also suggest many opportunities for further research in this intensive field.

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