

Mesenchymal Stem Cells for Bone Repair and Metabolic Bone Diseases

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Human mesenchymal stem cells offer a potential alternative to embryonic stem cells in clinical applications. The ability of these cells to self-renew and differentiate into multiple tissues, including bone, cartilage, fat, and other tissues of mesenchymal origin, makes them an attractive candidate for clinical applications. Patients who experience fracture nonunion and metabolic bone diseases, such as osteogenesis imperfecta and hypophosphatasia, have benefited from human mesenchymal stem cell therapy. Because of their ability to modulate immune responses, allogeneic transplant of these cells may be feasible without a substantial risk of immune rejection. The field of regenerative medicine is still facing considerable challenges; however, with the progress achieved thus far, the promise of stem cell therapy as a viable option for fracture nonunion and metabolic bone diseases is closer to reality. In this review, we update the biology and clinical applicability of human mesenchymal stem cells for bone repair and metabolic bone diseases.

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BMMNC = bone marrow mononuclear cell; BMP = bone morphogenic protein; BMT = bone marrow transplant; ESC = embryonic stem cell; FCS = fetal calf serum; iPSC = induced pluripotent stem cell; MSC = mesenchymal stem cell; OI = osteogenesis imperfecta; TNSALP = tissue nonspecific alkaline phosphatase

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Recent advances in stem cell research have prompted development of cell-based therapies for bone repair and treatment of metabolic bone diseases. Stem cells are defined by their ability to self-renew and their totipotency or potential to form cells derived from all 3 germ layers. In contrast, cells with self-renewal capacity but more restricted potential are called progenitor cells or tissue stem cells (eg, hematopoietic stem cells or mesenchymal stem cells [MSCs]). Finding an ideal stem cell for clinical applications with high self-renewal capacity and multipotent potential has been a challenge. In recent years, substantial advances have been made in examining the potential of stem cells, especially human embryonic stem cells (ESCs), in regenerative medicine. The ability of human ESCs to self-renew for prolonged periods without differentiation and, most importantly, their ability to differentiate into a large variety of tissues from all 3 germ layers were first characterized by Thomson et al.¹ These unique properties

of ESCs, specifically self-renewal and pluripotency, made human ESCs ideal candidates for regenerative medicine.

Initial enthusiasm for human ESCs has been tempered and limited by a number of issues, some of which were predicted on the basis of studies with murine ESCs, which were developed more than a decade earlier. Therapeutic use of human ESCs is complicated by immunologic incompatibility and possible development of malignant neoplasms or teratomas from administered cells.^{2,3} This complication is further hampered by the legal and ethical issues that surround derivation of ESCs from human embryos and their use in research. Thus, despite the ability of human ESCs to self-renew and to differentiate into many cell types, these controversies have restricted their use for therapeutic purposes and prompted scientists to seek other options, such as examining the potential of adult stem cells for regenerative medicine.

Adult stem cells are present in substantial numbers in many tissues throughout life; however, their frequency decreases with age. Tissues that harbor MSCs or MSC-like cells include blood,⁴ adipose tissue,⁵ skin,⁶ trabecular bone,⁷ and fetal blood, liver, and lung.^{8,9} The mesenchymal stem-like cells have also been identified in umbilical cord blood¹⁰ and placenta.¹¹ Despite sharing similar characteristics, these MSCs from different sources differ in their differentiation potential and gene expression profile.¹² Among the different types of adult stem cells, stem cells harbored in the bone marrow are considered to have the highest multilineage potential¹³ and have been studied for therapeutic purposes. Bone marrow is known to be a rich environment for many cell types. Among these cells are phenotypically and functionally diverse types of cells, collectively referred to as stromal cells. The MSCs comprise a small fraction (<0.01%) of stromal cells. We review the current literature on the biology and specific characteristics of human MSCs (Figure). We also describe recent advances in the use of systemic human MSC therapy in clinical studies related to fracture nonunion and metabolic bone diseases. We reviewed the PubMed literature using the keyword *stem cells*. The inclusion criteria were use of MSCs in animal models of bone repair and for clinical applications, especially in fracture nonunion, osteogenesis imperfecta (OI), and hypophosphatasia, as well as embryonic and induced

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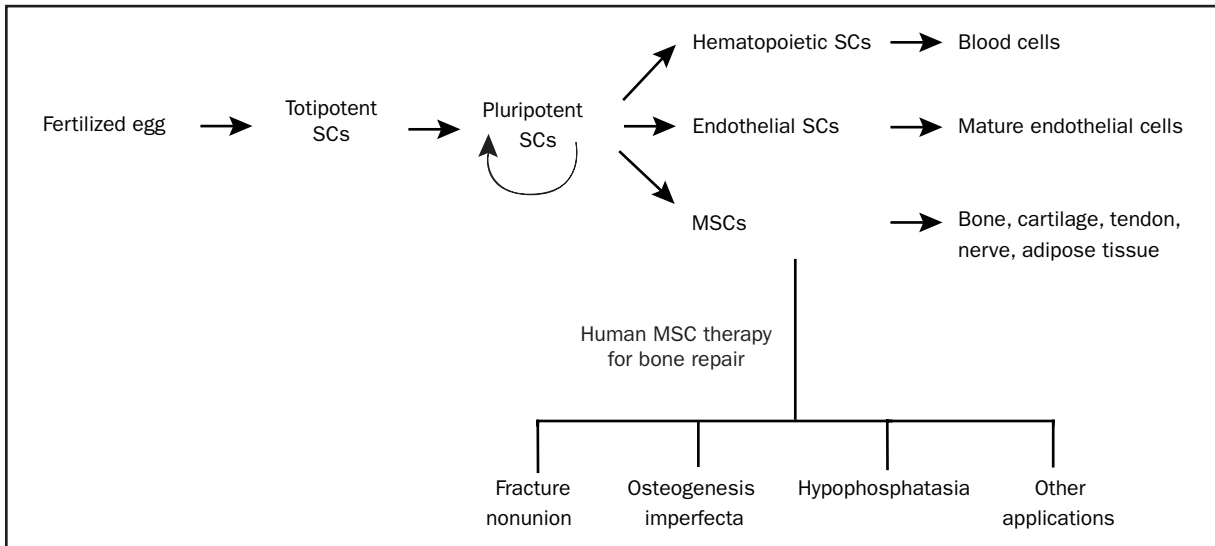


FIGURE. Developmental hierarchy of stem cells (SCs) and therapeutic potential of human mesenchymal stem cells (MSCs). On fertilization of an egg, a blastocyst forms. The inner cell mass of the blastocyst consists of the most primitive SC or totipotent SC. This totipotent SC can give rise to cells of embryonic and extraembryonic origin. Pluripotent SCs are multipotent SCs that can self-renew and differentiate into hematopoietic SCs, endothelial SCs, and MSCs. Hematopoietic SCs differentiate into blood cells, whereas endothelial progenitors give rise to mature endothelial cells. However, MSCs are characterized by their multilineage differentiation potential, including for bone, cartilage, and adipose tissue. Human MSCs have been tested in several clinical applications to repair bone in different types of bone disease, including fracture nonunion, osteogenesis imperfecta, and hypophosphatasia.

pluripotent stem cells (iPSCs) and their use in clinical applications. Additional articles were obtained by assessment of references in the published reviews.

BONE MARROW STEM CELLS

The pioneering work of Friedenstein et al¹³ in the 1960s led to the discovery of plastic-adherent MSCs, characterized as fibroblastoid cells. These observations were further extended by several other groups to show that these plastic-adherent MSCs were able to differentiate into a number of mesenchymal cell types, including osteoblasts, chondrocytes, and adipocytes,¹⁴⁻¹⁶ and according to recent reports also support hematopoiesis.¹⁷ In addition, the “criterion standard” assay for MSC stemness is based on the ability of the plastic-adherent bone marrow cells to form ectopic bone and a bone marrow microenvironment that supports hematopoiesis on subcutaneous implantation in immunodeficient mice.¹⁸

As described by Friedenstein et al,¹³ bone marrow MSCs are isolated on the basis of their plastic adherence in in vitro cultures. Murine bone marrow cells are typically obtained by flushing femurs and tibias, and the processed bone marrow mononuclear cells (BMMNCs) are isolated by density gradient centrifugation and then cultured. In contrast, human bone marrow is aspirated from the iliac crest of healthy volunteers and cultured directly on plastic

because density gradient centrifugation does not greatly improve the purification and human BMMNCs are often still contaminated with human hematopoietic stem cells. The method of isolation of human MSCs based on plastic adherence yields heterogeneous cultures, as suggested by the evidence of single-cell cloning of human MSCs in culture, which showed that only 30% of clonal human MSCs were multipotent.¹⁹ The plastic-adherent bone marrow MSCs, which account for 0.01% to 0.0001% of nucleated marrow cells,^{20,21} are characterized by a variety of cell surface markers. They are typically negative for CD34, CD45, CD14, CD11b, CD19, CD79a, and HLA-DR and have been shown to be positive for Stro-1, CD29, CD73, CD90, CD105, CD166, and CD44.^{18,22-25}

Researchers’ quest to develop more efficient methods of obtaining highly enriched multipotent MSC populations is ongoing. One successful approach is to isolate cells that express specific molecules on their cell surfaces using monoclonal antibodies and cell sorting technologies. Several groups have described homogenous populations of osteoprogenitor cells in the bone marrow in this way. Long et al²⁶⁻²⁸ identified the presence of nonadherent human bone marrow cells with osteogenic potential. These cells were identified by sorting for bone-related proteins, such as osteocalcin or alkaline phosphatase. The osteocalcin-positive cells were able to differentiate into osteoblastic cells when cultured in the presence of transforming growth factor β and as yet unde-

finer accessory bone marrow cells. Using similar methods, our group was able to detect the presence of nonadherent osteoprogenitors in human peripheral blood.^{29,30} Additional evidence for the existence of nonadherent bone marrow osteoprogenitors came from Falla et al.³¹ These investigators showed that a quiescent cell population in the murine bone marrow with fibroblastoid characteristics contributed to the formation of bonelike nodules *in vitro*.

Simmons and Torok-Storb³² were the first to use a monoclonal antibody, Stro-1, that recognizes an unknown epitope expressed on the surface of human MSCs and cells of the erythroblastoid lineage. Human BMMNCs sorted on the basis of Stro-1 expression were capable of establishing an adherent stromal layer *in vitro*, consisting of a number of phenotypically distinct stromal cell types, including fibroblasts, smooth muscle cells, and adipocytes.³² The Stro-1 antibody identified cells with osteogenic potential as assessed by the development of cells that exhibited 3 independent markers of differentiated osteoblastic cells: alkaline phosphatase expression, 1,25-dihydroxyvitamin D–dependent induction of the bone-specific protein osteocalcin, and production of a mineralized matrix (hydroxyapatite).³³ Recently, Gronthos et al³⁴ identified a novel monoclonal antibody, Stro-3, which was expressed on a high proportion of human bone marrow stromal cells that possess extensive proliferative and multilineage differentiating capacity. Using retroviral expression cloning, they determined that Stro-3 was in fact directed against tissue nonspecific alkaline phosphatase (TNSALP), a cell-surface glycoprotein usually associated with cells of osteoblast lineage. Their results suggested that, in addition to being expressed by osteoblasts, TNSALP might represent a marker of immature bone marrow stromal cells *in vivo*.

Recent studies have identified another marker, CD146, that characterizes an osteoprogenitor cell population contained within human bone marrow.^{17,35} In addition to functional hematopoietic support, CD146⁺ MSCs exhibited canonical stem cell properties, such as extended 12-week proliferation and multilineage potential toward chondrogenic, osteoblastic, and adipogenic differentiation.³⁶

CLINICAL APPLICATIONS OF MSCs

POTENTIAL ADVANTAGES COMPARED WITH ESCs

Several defining characteristics of MSCs make them a potentially more promising candidate for clinical applications than ESCs. The availability of autologous MSCs from patients offers easy accessibility to these cells for therapeutic applications. In terms of stemness, MSCs possess the ability to regenerate cell types specific for different tissues, including adipose tissue, bone, and cartilage.

One major advantage of using human MSCs for *in vivo* therapy is that they are nonimmunogenic. They are widely

described as major histocompatibility class I positive and major histocompatibility class II negative and are known to lack expression of costimulatory molecules such as CD40, CD80, and CD86, which renders these cells nonimmunogenic; therefore, allogeneic transplant of MSCs should not require immunosuppression of the host.³⁷ In addition to being immunoevasive, MSCs can suppress the immune system. The immunosuppressive effect of MSCs occurs through the modulation of T-cell responses, including suppression of T-cell proliferation and induction of T-cell anergy in the absence of costimulatory signals.^{38,39} Most of the evidence for the immunosuppressive effects of MSCs is from *in vitro* studies; thus, the exact mechanism by which allogeneic MSCs suppress and evade the immune system is unknown, and how they will behave *in vivo* is still unclear. Systemic administration of MSCs has been successful in several clinical applications. In the following discussion, we include examples of MSC therapy for bone repair in fracture nonunion and metabolic bone diseases, such as OI and hypophosphatasia (Table).⁴⁰⁻⁴⁹

FRACTURE NONUNION

Despite substantial advances in orthopedic surgery, fracture nonunion remains a clinically important problem. During normal fracture healing, undifferentiated MSCs, with the aid of bone morphogenetic proteins (BMPs) and regulatory cytokines, proliferate, differentiate into chondrocytes and osteoblasts, and form bone, thereby repairing the injury.⁵⁰ Nevertheless, some of the fractures fail to heal properly and result in either delayed union or nonunion, causing morbidity, prolonged hospitalization, and increased expenses. The diagnosis of nonunion is based on a combination of clinical symptoms and physical findings, including pain and motion at the fracture site with radiographic evidence of failure of union.⁵⁰ Typically, fractures that fail to heal even after 6 to 8 months of therapy are considered nonunions. The incidence of nonunion varies by fracture site but can be as high as 5% to 20%.⁵¹⁻⁵³

Cell-based strategies, including stem cell therapy, for fracture repair in cases of nonunion are currently receiving considerable attention. The use of MSCs for fracture repair has been tried successfully in animal models. Autologous bone marrow–derived MSCs were expanded in culture, loaded onto ceramic cylinders, and implanted into 8-mm segmental defects in rat femora with successful bone formation 8 weeks later.⁵⁴ This same group then successfully demonstrated bone formation at the segmental defect in adult athymic rats by implantation of human bone marrow–derived MSCs.⁵⁵ Several other animal studies performed with implantation of autologous bone marrow–derived MSCs using different scaffolds have resulted in bone regeneration.⁵⁶⁻⁶⁰

TABLE. Clinical Applications of Human Mesenchymal Stem Cells (MSCs) for Tissue Regeneration

Condition	Type of cells	Mode of administration	Outcome	Reference(s)
Fracture nonunion	Autologous bone marrow–derived pluripotent MSCs	100% hydroxyapatite macroporous ceramic scaffolds seeded with MSCs	Radiographic and computed tomographic evidence of bone formation; patients recovered limb function	40, 41
	Autologous bone marrow grafting	Subcutaneous	Positive correlation between volume of mineralized callus at 4 mo and number and concentration of fibroblast colony-forming units in graft	42
Osteogenesis imperfecta	Allogeneic bone marrow transplant	Intravenous	Histologic changes indicative of new bone formation; increases in total body bone mineral content	43
	Engraftment of donor osteoblasts	Intravenous	Improvement in linear growth, total body bone mineral content, and fracture rate in 3 of 5 children with severe osteogenesis imperfecta	44
	Gene-marked allogeneic MSCs	Two intravenous infusions	Of 6 patients, 5 showed engraftment in bone, skin, and marrow stroma and increase in growth velocity during 6 mo after infusion	45
	Allogeneic HLA-mismatched MSCs of fetal liver	Implantation into uterus at 32 wk of gestation	Presence of osteocalcin-, osteopontin-, and bone sialoprotein–positive cells of donor origin in bone more than 9 mo after transplant	46
Hypophosphatasia	Allogeneic cultured osteoblasts and bone fragments from crushed iliac	Intravenous	Clinical and radiologic evidence of full mineralization of patient bones; after transplant, patient began to walk and run	47
	Allogeneic HLA-matched T-cell–depleted marrow and second infusion of ex vivo expanded marrow cells	Intravenous	Clinical and radiographic improvement without correction of biochemical features of hypophosphatasia	48
	Allogeneic heterogeneous population of marrow cells and bone fragments	Intravenous, intraperitoneal, and subcutaneous	Radiographic evidence of improved skeletal mineralization; patient is active and growing 7 y after transplant and has clinical phenotype of mild hypophosphatasia	49

Clinical use of culture-expanded osteoprogenitor cells in conjunction with porous hydroxyapatite scaffolds has been reported in treatment of 4 patients with diaphyseal segmental defects that ranged from 3.0 to 28.3 cm³ in a tibia, a humerus, and 2 separate ulnar fractures.^{40,41} Autologous bone marrow–derived pluripotent MSCs were expanded in vitro and were loaded onto 100% hydroxyapatite macroporous ceramic scaffolds. The grafts seeded with MSCs and the fracture defects were stabilized with an external fixator. There was progressive integration of the implants with the surrounding bone, progressive new bone formation inside the bioceramic pores, and vascular ingrowth. Good integration of the implants with the preexisting bone was maintained during all follow-up periods, and no major adverse reactions were observed. Radiography and computed tomography showed that bone formation was far more prominent over the external surface and within the inner canal of the implants. This could be due to a higher density of loaded cells and/or a better survival of cells within the outermost portions of the bioceramics. All patients experienced recovery of limb function. With time, the implants revealed progressive appearance of cracks and fissures indicative of some bioceramic disintegration, whereas bone formation

progressed and the implants were completely integrated into the existing bone. In all patients at last follow-up (6–7 years after surgery), good integration of the implants was maintained.

The critical role of MSCs was further emphasized by the work of Hernigou et al.⁴² They demonstrated that percutaneous autologous bone marrow grafting is an effective and safe method for treating an atrophic tibial diaphyseal nonunion. Marrow was aspirated from both anterior iliac crests, concentrated on a cell separator, and then injected into 60 noninfected atrophic nonunions of the tibia. A positive correlation was noted between the volume of mineralized callus at 4 months and the number ($P=.04$) and concentration ($P=.01$) of fibroblast colony-forming units in the graft. In 7 patients in whom union was not achieved, both the concentration and the total number of stem cells injected were significantly lower than in patients with osseous union ($P=.001$ and $P<.01$). One potential weakness of the study was the absence of a cohort with a placebo treatment. However, the success of the treatment of nonunion with percutaneous bone marrow grafting appeared to depend on the number and concentration of stem cells available for injection.

OSTEOGENESIS IMPERFECTA

Osteogenesis imperfecta is a heterogeneous group of inherited disorders of connective tissue characterized by bone fragility and other evidence of connective tissue malfunction.⁶¹ The genetic defect responsible for OI results in abnormal type I collagen production by osteoblasts, which leads to osteopenia, multiple fractures, severe bony deformities, and considerably shortened stature. The clinical heterogeneity of OI is wide, ranging from death in the perinatal period, to marked short stature and severe bone deformities, to normal life expectancy with only mild osseous fragility and slightly decreased bone mass, and finally to mild forms that may elude clinical detection.⁶¹ Because the genetic defect cannot be corrected, treatment options for OI include nonsurgical and surgical management. Nonsurgical management includes drugs, such as bisphosphonates, to increase bone mass and strength and reduce fractures.

A number of studies have demonstrated the beneficial effects of oral or intravenous bisphosphonate therapy for children with severe OI. In particular, in a large, uncontrolled, observational study that involved 30 children (aged 3-16 years) with severe OI, Glorieux et al⁶² showed that cyclic administration (4- to 6-month intervals for 1.3-5 years) of intravenous pamidronate (mean \pm SD, 6.8 \pm 1.1 mg/kg yearly) improved clinical outcomes, reduced bone resorption, and increased bone density. Findings were a mean \pm SD annualized increase of 41.9% \pm 29.0% in spine bone mineral density, a mean \pm SD *z* score improvement from -5.3 \pm 1.2 to -3.4 \pm 1.5, and a mean \pm SD increase in the cortical width of the metacarpals of 27% \pm 20.2% per year.^{62,63} The mean incidence of radiologically confirmed fractures decreased markedly by 1.7 per year, whereas increases in the size of the vertebral bodies suggested that new bone had formed.⁶² The limitation of that study and earlier ones was that they were open trials, and double-blind, controlled studies need to be performed to confirm these results. Moreover, along with inhibition of bone remodeling, bisphosphonates have other potential adverse skeletal effects in children,⁶⁴ which has raised concerns about their long-term efficacy in the treatment of severe OI.

In principle, transplant of MSCs or marrow stromal cells would attenuate or possibly correct genetic disorders of bone, cartilage, muscle, and other connective tissues.^{65,66} When MSCs from wild-type mice were infused into transgenic mice that had a phenotype of fragile bones resembling OI, the MSCs served as a source for continual renewal of cells in a number of nonhematopoietic tissues.⁶⁷ A recent publication by Panaroni et al⁶⁸ evaluated intrauterine transplant of adult bone marrow into a knock-in murine model for classic, dominant OI. Adult bone marrow donor cells from enhanced green fluorescent protein transgenic mice engrafted into hematopoietic and nonhematopoietic tissues differentiated to

trabecular and cortical bone cells, synthesized up to 20% of all type I collagen in the host bone, and also eliminated the perinatal lethality of mice with dominant OI.⁶⁸

In their initial studies, Horwitz et al⁴³ performed allogeneic bone marrow transplant (BMT) in 3 children with OI. Three months after osteoblast engraftment (1.5%-2.0% donor cells), representative samples of trabecular bone showed histologic changes indicative of new bone formation. All patients had increases in total body bone mineral content that ranged from 21 to 29 g compared with predicted values of 0 to 4 g for healthy children with similar changes in weight. These improvements were associated with increases in growth velocity and reduced frequency of fractures. The authors concluded that allogeneic BMT can lead to engraftment of functional MSCs, indicating the feasibility of this strategy in the treatment of OI. This study also demonstrated that MSCs in transplanted marrow can migrate to bone in children with OI and then give rise to osteoblasts, whose presence correlated with an improvement in bone structure and function. However, this study was criticized because it had only 6 months of clinical follow-up and did not directly compare results with those for controls. Hence, this group undertook another pilot study.⁴⁴ A total of 5 children with OI were enrolled in a clinical trial during which they underwent engraftment of donor osteoblasts and 18 to 36 months of clinical follow-up; 2 children were excluded from the analysis because donor osteoblast engraftment after treatment could not be documented, rendering the mesenchymal engraftment status unknown. The investigators were able to demonstrate improvement in linear growth, total body bone mineral content, and fracture rate in 3 children with severe OI (2 children from the original report).⁴³ Although linear growth rate, total body bone mineral content, and fracture rate appeared to improve in some patients, the lack of reliable controls and relatively short-term follow-up prevented the authors from drawing firm conclusions about the efficacy of MSC therapy. In addition, with increasing time after transplant, growth rates slowed and eventually plateaued, whereas bone mineral content continued to increase.

These observations prompted the group to perform a subsequent study to demonstrate the feasibility of MSC therapy and to gain insight into the transplant biology of these cells. In this study, Horwitz et al⁴⁵ used gene-marked, donor marrow-derived MSCs to treat 6 children who had undergone standard BMT for severe OI. Each child received 2 infusions of the allogeneic cells. In 5 of 6 patients, engraftment was evident in 1 or more sites, including bone, skin, and marrow stroma, and these 5 patients had an acceleration of growth velocity during the first 6 months after infusion. This improvement ranged from 60% to 94% (median, 70%) of the predicted median values for age- and sex-matched unaffected children, compared with 0% to 40%

(median, 20%) during the 6 months immediately preceding the infusions. Encouraged by these studies, Le Blanc et al⁴⁶ transplanted allogeneic HLA-mismatched MSCs of fetal liver origin into the uterus at 32 weeks' gestation to treat a fetus with severe OI. Donor cell engraftment was 0.3% by analysis of the centromeric Y chromosome-specific probe. A considerably higher level of donor cell engraftment (7.4%) was estimated using the total Y chromosome painting probe, which recognized multiple parts of the Y chromosome. Because osteocytes are terminally differentiated cells, the presence of osteocalcin-, osteopontin-, and bone sialoprotein-positive cells of male origin in bone more than 9 months after transplant suggested that the transplanted cells participated in bone turnover and provided a continual source of osteoblastic progenitor cells. From the time of transplant until bone biopsy, the fetus grew appreciably. This report showed that the fetal liver MSCs were capable of engrafting and differentiating into bone in the human fetus, even when the recipient was immunocompetent and HLA incompatible, without provoking any graft-vs-host disease in the absence of immunosuppressive therapy.

HYPHOPHOSPHATASIA

Hypophosphatasia is a rare, heritable, metabolic bone disease due to deficient activity of TNSALP.⁴⁷ The infantile form features severe rickets, often causing death in the first year of life due to respiratory complications.⁴⁷ Currently, no established medical treatment exists for hypophosphatasia; however, a case report of an adult with hypophosphatasia who was treated with recombinant human parathyroid hormone 1-34 and demonstrated biochemical and clinical improvement suggests that this may represent a potential approach to therapy.⁶⁹ In addition, recent studies in mice show that targeting TNSALP to bone using a decaspargate sequence tag may represent an effective medical approach to therapy for this disorder.⁷⁰ An alternative strategy, used by Cahill et al,⁴⁷ is to perform transplant in patients with hypophosphatasia. They replaced the patient's osteoblasts with those of her father, who was a 4/6 HLA match. The patient was given cultured osteoblasts obtained from crushed iliac donor bone intravenously, and bone fragments were also inserted intraperitoneally. No engraftment of hematopoietic stem cells or increase in the patient's circulating alkaline phosphatase levels was evident, but the clinical and radiologic evidence of full mineralization of her bones was impressive. As the child grew, she began to walk and then later to run. A bone biopsy specimen from the patient's iliac crest revealed the presence of male donor cells by polymerase chain reaction amplification of male-specific sex-determining region sequences. The success of this transplant seemed to indicate a selective advantage

of the donor's healthy cells compared with the recipient's cells in sufficient quantities to ensure proper mineralization of bone.

In a subsequent study, an 8-month-old baby with infantile hypophosphatasia underwent, after full myeloablation, BMT using T-cell-depleted marrow from her 5/6 HLA-matched sibling.⁴⁸ During the first 6 months after BMT, the patient showed clinical and radiographic improvement without correction of the biochemical features of hypophosphatasia. However, clinical deterioration with skeletal demineralization occurred 13 months after BMT (21 months of age). Therefore, she then received, by intravenous infusion, bone marrow cells that had been expanded *ex vivo*. Six months later, considerable, lasting clinical and radiographic improvement ensued, still without correction of her biochemical abnormalities.

On the basis of the earlier study by Cahill et al⁴⁷ and reports obtained in mouse models that indicated that replacement of marrow stroma could be partially achieved using donor bone fragments placed intraperitoneally or subcutaneously in the recipient,^{71,72} an attempt was made to introduce TNSALP-replete osteoblasts to treat another severely affected girl with infantile hypophosphatasia.⁴⁹ The reasoning behind the strategy was that the donor stromal cells possibly migrated from the bone fragments to the recipient bone and other tissues, including the thymus.⁷³ Therefore, the investigators' strategy was to administer a heterogeneous population of cells, including use of bone fragments, by 3 different routes (intraperitoneal, subcutaneous, and intravenous) to enhance their migration and homing to the stroma.⁴⁹ The hope was that engraftment of these cells within the skeletal microenvironment would allow precursor cells to replicate and to differentiate into functional osteoprogenitor cells. Four months later, radiographs demonstrated improved skeletal mineralization. Twenty months later, polymerase chain reaction analysis of adherent cells cultured from recipient bone suggested the presence of small amounts of paternal (donor) DNA despite the absence of hematopoietic engraftment. At 8 years of age (7 years after transplant), the patient was active and growing and had the clinical phenotype of the more mild childhood form of hypophosphatasia. These findings suggest that, after immune tolerance, the marrow osteoprogenitors contained within the donor bone fragments migrated to the affected sites. This led to distribution and engraftment of the precursors in the skeletal microenvironment in patients with hypophosphatasia to form TNSALP-replete osteoblasts that could improve mineralization. This result also suggests that partially differentiated cells (toward osteoblasts) or bone-lining cells contained within the bone fragments may have played a role in bone repair.

FEASIBILITY OF HUMAN MSCs IN CLINICAL APPLICATIONS

The fact that MSCs possess the ability to differentiate into various tissues, including bone, that they can expand in vitro with relative ease, and that they have immunosuppressive and immunoevasive capabilities make them an attractive candidate for clinical applications. However, long-term cultures of human MSCs have limitations because over time the cells exhibit a reduced proliferation rate attributable to telomere shortening and undergo senescence.¹⁸ To combat the problem of human MSC senescence, several other approaches have been used to improve the ability of these cells to expand in vitro. For example, treatment with growth factors, such as fibroblast growth factor 2,^{74,75} or culturing these cells under low oxygen tension⁷⁶ or in a 3-dimensional environment⁷⁷ might prove effective in obtaining larger pools of human MSCs for clinical applications.

In addition, human MSCs need to be expanded in vitro in the presence of fetal calf serum (FCS) at a concentration of 10% to 20%.⁷⁸⁻⁸⁰ The use of FCS always raises concerns because of the fear of transmission of prions and still unidentified zoonoses, although FCS batches are routinely prescreened to meet the biosafety requirements of the cellular product.⁸¹ Moreover, proteins or peptides might incorporate into MSCs during culture, thus causing immune reactions in the host, especially if more than 1 infusion is required.⁸² One option is that Food and Drug Administration–approved products, such as Prochymal, Provacel, and Chondrogen, will help advance the prospects for human MSC therapy. These are proprietary formulations of adult MSCs designed to provide therapeutic benefit by controlling inflammation, promoting tissue regeneration, and preventing scar formation. These stem cells are obtained from the bone marrow of healthy adult donors.

Another issue not yet clarified is the mode of administration. In the clinical studies described herein, systemic infusion proved effective, which raises the issue about the ability of intravenously infused MSCs to repair distal tissues. Systemically administered MSCs may get trapped in the lungs⁸³ and secrete soluble factors into the peripheral bloodstream, thereby exerting effects on distal organs or tissues. A plausible hypothesis is that systemically administered MSCs might enhance repair of the distal tissues by recruitment and differentiation of tissue-specific stem cells either through secretion of soluble factors or by escaping the lungs to home to affected tissues.^{83,84} However, these soluble factors need to be secreted in greater amounts to exert effects distally, which might result in toxicity. Another problem with the proposed hypothesis is the fact that the number of MSCs that home to organs and tissues is extremely small. The presence of MSCs in the peripheral

organs creates another dilemma, and although the immunoregulatory properties of human MSCs have been demonstrated in several studies, questions still remain about the possibility of the long-term existence of administered stem cells in treated patients and the potential consequences that could arise. Thus far, the evidence for transformation of administered MSCs into tumors is lacking in human models, but extensive follow-up is required to determine the long-term effects of systemic MSC administration.

THE FUTURE OF STEM CELLS IN CLINICAL PRACTICE

GENE THERAPY AND STEM CELLS

There is considerable interest in combining gene therapy with stem cell therapy, which offers the prospect of molecular engineering of stem cells, either transiently or permanently. For example, in conditions such as fracture nonunion, OI, or hypophosphatasia (as described herein), individual lesions or fractures could be corrected by replacing mutated or defective osteoprogenitors with genetically engineered ones. Gene therapy can be performed in 2 settings: ex vivo gene transfer to a cell or tissue culture in vitro and in vivo gene transfer, where the gene is transferred directly to the host. An example of in vivo gene transfer includes an adenovirus vector that contains the BMP-2 growth factor, which was shown to induce healing of critical-sized bone defects in rat femurs.⁸⁵ However, in vivo gene transfer with viral vectors induces immune responses that limit the duration and effectiveness of the treatment.⁸⁶ One feasible option is to genetically modify autologous MSCs ex vivo to deliver the required genes or proteins to the affected sites. To date, this approach has been successful in an animal model. Thus, Park et al⁸⁷ showed that transplantation of BMP-2–producing primary rat bone marrow stromal cells, modified by either liposome-mediated or adenoviral BMP-2 gene transfer, into bone defects in vitro and in vivo healed critical-sized bone defects in rats. Ex vivo methods are also safer because no viral particles or DNA need to be inserted into the patient.⁸⁶ These preclinical studies show that gene therapy regimens can induce bone formation in vivo. Genetically engineered stem cells would eliminate the requirement of a large number of MSCs for implantation and possibly the need for in vitro culture and expansion. However, use of genetically engineered stem cells for clinical practice needs further development in terms of consistency and safety.

INDUCED PLURIPOTENT STEM CELLS

The iPSCs represent perhaps the future of stem cell therapy because they exhibit near-identical genetic and functional properties to human ESCs but without the ethical and

potential immunologic issues surrounding human ESCs. These cells are created from somatic cells by introducing 4 genes by transduction methods or by a combination of 2 genes with chemical induction of the others, thereby bypassing the need for destruction of embryos. Ectopic expression of pluripotency markers, such as Oct4, Sox2, c-myc, and Klf4 in human fibroblasts, is sufficient to yield iPSCs that resemble ESCs in their morphologic features, gene expression, and ability to form teratomas in immunodeficient mice.⁸⁸⁻⁹² Most instances of iPSC derivation involve integration of defined genes using viral vectors that can create unpredictable mutations and limit utility of these cells in clinical applications. Recent work in the iPSC field has focused on virus-free induction of pluripotency using a single transfection with oriP/EBNA1 (origin of latent viral DNA replication harboring plasmid/Epstein-Barr nuclear antigen 1)-based episomal vectors⁹³ and recombinant cell-penetrating reprogramming proteins.^{94,95} In the study by Zhou et al,⁹⁴ to improve reprogramming efficiency and to obtain iPSCs with long-term self-renewal capacity, murine somatic cells were also treated with valproic acid (a known histone deacetylase inhibitor). Generation of iPSCs using these current methods showed that reprogramming of somatic cells does not require genomic integration or the continued presence of exogenous reprogramming factors and removes one obstacle to the clinical application of human iPSCs. Therefore, iPSCs offer a technology that essentially can be tailor-made to treat individual conditions. However, even though iPSCs offer an attractive alternative to ESCs, the technology is not advanced enough to allow use in humans at this point.

CONCLUSION

The MSCs derived from the adult bone marrow provide an exciting and promising stem cell population for repair of bone in skeletal diseases. Their use in clinical applications still needs rigorous evaluation. Properly conducted clinical trials that include sufficient numbers of patients are warranted before claims regarding the therapeutic efficacy of MSCs can be made. However, use of adult stem cells, such as MSCs derived from bone marrow, is an innovative treatment for many disease conditions, including fracture non-union and a number of metabolic bone diseases.

REFERENCES

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts [published correction appears in *Science*. 1998;282(5395):1827]. *Science*. 1998;282(5391):1145-1147.
2. Tögel F, Westenfelder C. Adult bone marrow-derived stem cells for organ regeneration and repair. *Dev Dyn*. 2007;236(12):3321-3331.
3. Hentze H, Graichen R, Colman A. Cell therapy and the safety of embryonic stem cell-derived grafts. *Trends Biotechnol*. 2007 Jan;25(1):24-32. Epub 2006 Nov 3.
4. Zvaifler NJ, Marinova-Mutafchieva L, Adams G, et al. Mesenchymal precursor cells in the blood of normal individuals. *Arthritis Res*. 2000;2(6):477-488. Epub 2000 Aug 31.
5. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell based therapies. *Tissue Eng*. 2001;7(2):211-228.
6. Chunmeng S, Tianmin C. Effects of plastic-adherent dermal multipotent cells on peripheral blood leukocytes and CFU-GM in rats. *Transplant Proc*. 2004;36(5):1578-1581.
7. Sottile V, Halleux C, Bassilana F, Keller H, Seuwen K. Stem cell characteristics of human trabecular bone-derived cells. *Bone*. 2002;30(5):699-704.
8. Jackson L, Jones DR, Scotting P, Sottile V. Adult mesenchymal stem cells: differentiation potential and therapeutic applications. *J Postgrad Med*. 2007;53(2):121-127.
9. in 't Anker PS, Noort WA, Scherjon SA, et al. Mesenchymal stem cells in human second-trimester bone marrow, liver, lung and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica*. 2003;88(8):845-852.
10. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol*. 2000;109(1):235-242.
11. Igura K, Zhang X, Takahashi K, Mitsuru A, Yamaguchi S, Takashi TA. Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. *Cytotherapy*. 2004;6(6):543-553.
12. Wagner W, Wein F, Seckinger A, et al. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol*. 2005;33(11):1402-1416.
13. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow: analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation*. 1968;6(2):230-247.
14. Ashton BA, Allen TD, Howlett CR, Eaglesom CC, Hattori A, Owen M. Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. *Clin Orthop Relat Res*. 1980;151(3):294-307.
15. Bab I, Ashton BA, Gazit D, Marx G, Williamson MC, Owen ME. Kinetics and differentiation of marrow stromal cells in diffusion chambers in vivo. *J Cell Sci*. 1986;84(1):139-151.
16. Castro-Malaspina H, Gay RE, Resnick G, et al. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood*. 1980;56(2):289-301.
17. Sacchetti B, Funari A, Michienzi S, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment [published correction appears in *Cell*. 2008;133(5):928]. *Cell*. 2007;131(2):324-336.
18. Abdallah BM, Kassem M. Human mesenchymal stem cells: from basic biology to clinical applications. *Gene Ther*. 2008 Jan;15(2):109-116. Epub 2007 Nov 8.
19. Kuznetsov SA, Krebsbach PH, Satomura K, et al. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. *J Bone Miner Res*. 1997;12(9):1335-1347.
20. Dazzi F, Ramasamy R, Glennie S, Jones SP, Roberts I. The role of mesenchymal stem cells in haemopoiesis. *Blood Rev*. 2006 May;20(3):161-171. Epub 2005 Dec 20.
21. Sakaguchi Y, Sekiya I, Yagishita K, Muneta T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum*. 2005;52(8):2521-2529.
22. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells: the International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8(4):315-317.
23. Abdallah BM, Haack-Sørensen M, Burns JS, et al. Maintenance of differentiation potential of human bone marrow mesenchymal stem cells immortalized by human telomerase reverse transcriptase gene despite [corrected] extensive proliferation [published correction appears in *Biochem Biophys Res Commun*. 2005;329(4):1361]. *Biochem Biophys Res Commun*. 2005;326(3):527-538.
24. Foster LJ, Zeemann PA, Li C, Mann M, Jensen ON, Kassem M. Differential expression profiling of membrane proteins by quantitative proteomics in a human mesenchymal stem cell line undergoing osteoblast differentiation. *Stem Cells*. 2005;23(9):1367-1377.
25. Keating A. Mesenchymal stromal cells. *Curr Opin Hematol*. 2006;13(6):419-425.
26. Long MW, Williams JL, Mann KG. Expression of human bone-related proteins in the hematopoietic microenvironment. *J Clin Invest*. 1990;86(5):1387-1395.
27. Long MW, Robinson JA, Ashcraft EA, Mann KG. Regulation of human bone marrow-derived osteoprogenitor cells by osteogenic growth factors

- [published correction appears in *J Clin Invest*. 1995;96(5):2541]. *J Clin Invest*. 1995;95(2):881-887.
28. Long MW, Ashcraft EK, Normalle D, Mann KG. Age-related phenotypic alterations in populations of purified human bone precursor cells. *J Gerontol A Biol Sci Med Sci*. 1999;54(2A):B54-B62.
 29. Eghbali-Fatourehchi G, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S. Circulating osteoblast-lineage cells in humans. *N Engl J Med*. 2005;352(19):1959-1966.
 30. Eghbali-Fatourehchi GZ, Mödder UI, Charatcharoenwithaya N, et al. Characterization of circulating osteoblast lineage cells in humans [published correction appears in *Bone*. 2007;41(4):741]. *Bone*. 2007 May;40(5):1370-1377. Epub 2007 Jan 4.
 31. Falla N, Van Vlasselaer P, Bierkens J, Borremans B, Schoeters G, Van Gorp U. Characterization of a 5-fluorouracil-enriched osteoprogenitor population of the murine bone marrow. *Blood*. 1993;82(12):3580-3591.
 32. Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood*. 1991;78(1):55-62.
 33. Gronthos S, Graves SE, Ohta S, Simmons PJ. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood*. 1994;84(12):4164-4173.
 34. Gronthos S, Fitter S, Diamond P, Simmons PJ, Iteanu S, Zannettino ACW. A Novel monoclonal antibody (STRO-3) identifies an isoform of tissue nonspecific alkaline phosphatase expressed by multipotent bone marrow stromal stem cells. *Stem Cells Dev*. 2007;16(6):953-963.
 35. Baksh D, Yao R, Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells*. 2007 Jun;25(6):1384-1392. Epub 2007 Mar 1.
 36. Sorrentino A, Ferracin M, Castelli G, et al. Isolation and characterization of CD146+ multipotent mesenchymal stromal cells. *Exp Hematol*. 2008 Aug;36(8):1035-1046. Epub 2008 May 27.
 37. Javazon EH, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol*. 2004;32(5):414-425.
 38. Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or non-specific mitogenic stimuli. *Blood*. 2002;99(10):3838-3843.
 39. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol*. 2002;30(1):42-48.
 40. Quarto R, Mastrogiacomo M, Cancedda R, et al. Repair of large bone defects with the use of autologous bone marrow stromal cells [letter]. *N Engl J Med*. 2001;344(5):385-386.
 41. Maracci M, Kon E, Moukhachev V, et al. Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. *Tissue Eng*. 2007;13(6):947-955.
 42. Hernigou P, Poignard A, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions: influence of the number and concentration of progenitor cells. *J Bone Joint Surg Am*. 2005;87(7):1430-1437.
 43. Horwitz EM, Prockop DJ, Fitzpatrick LA, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med*. 1999;5(3):309-313.
 44. Horwitz EM, Prockop DJ, Gordon PL, et al. Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta. *Blood*. 2001;97(5):1227-1231.
 45. Horwitz EM, Gordon PL, Koo WK, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. *Proc Natl Acad Sci U S A*. 2002;99(13):8932-8937.
 46. Le Blanc K, Götherström C, Ringdén O, et al. Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta. *Transplantation*. 2005;79(11):1607-1614.
 47. Cahill RA, Klemperer MR, Steele A, El-Badri NS, Good RA. Successful transplantation to correct a metabolic bone disease (infantile hypophosphatasia) using bone fragments plus cultured osteoblasts with T-cell-depleted mismatched bone marrow without lymphohematopoietic engraftment [abstract 3314]. *Blood*. 2001;98:796A-797A.
 48. Whyte MP, Kurtzberg J, McAlister WH, et al. Marrow cell transplantation for infantile hypophosphatasia. *J Bone Miner Res*. 2003;18(4):624-636.
 49. Cahill RA, Wenkert D, Perlman SA, et al. Infantile hypophosphatasia: transplantation therapy trial using bone fragments and cultured osteoblasts. *J Clin Endocrinol Metab*. 2007 Aug;92(8):2923-2930. Epub 2007 May 22.
 50. Tseng SS, Lee MA, Reddi AH. Nonunions and the potential of stem cells in fracture-healing. *J Bone Joint Surg Am*. 2008;90(suppl 1):92-98.
 51. Einhorn TA. Enhancement of fracture-healing. *J Bone Joint Surg Am*. 1995;77(6):940-956.
 52. Hayda RA, Brighton CT, Esterhai JL Jr. Pathophysiology of delayed healing. *Clin Orthop Relat Res*. 1998;355(suppl):S31-S40.
 53. Marsh D. Concepts of fracture union, delayed union, and nonunion. *Clin Orthop Relat Res*. 1998;355(suppl):S22-S30.
 54. Kadiyala S, Jaiswal N, Bruder SP. Culture-expanded, bone marrow-derived mesenchymal stem cells regenerate a critical-sized segmental bone defect. *Tissue Eng*. 1997;3(2):173-185.
 55. Bruder SP, Kurth AA, Shea M, Hayes WC, Jaiswal N, Kadiyala S. Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. *J Orthop Res*. 1998;16(2):155-162.
 56. Arinzech TL, Peter SJ, Archambault MP, et al. Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. *J Bone Joint Surg Am*. 2003;85-A(10):1927-1935.
 57. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am*. 1998;80(7):985-996.
 58. Kon E, Muraglia A, Corsi A, et al. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. *J Biomed Mater Res*. 2000;49(3):328-337.
 59. Petite H, Viateau V, Bensaïd W, et al. Tissue-engineered bone regeneration. *Nat Biotechnol*. 2000;18(9):959-963.
 60. Viateau V, Guillemain G, Bousson V, et al. Long-bone critical-size defects treated with tissue-engineered grafts: a study on sheep. *J Orthop Res*. 2007;25(6):741-749.
 61. Byers PH. Osteogenesis imperfecta. In: Royce PM, Steinmann B, eds. *Connective Tissue and Its Heritable Disorders: Molecular, Genetic and Medical Aspects*. New York, NY: Wiley-Liss Inc; 1993:137-350.
 62. Glorieux FH, Bishop NJ, Plotkin H, Chabot G, Lanoue G, Travers R. Cyclic administration of pamidronate in children with severe osteogenesis imperfecta. *N Engl J Med*. 1998;339(14):947-952.
 63. Antoniazzi F, Mottes M, Frascini P, Brunelli PC, Tatò L. Osteogenesis imperfecta practical treatment guidelines. *Paediatr Drugs*. 2000;2(6):465-488.
 64. Roldán EJA, Pasqualini T, Plantalech L. Bisphosphonates in children with osteogenesis imperfecta may improve bone mineralization but not bone strength: report of two patients. *J Pediatr Endocrinol Metab*. 1999;12(4):555-559.
 65. Mashiba T, Hirano T, Turner CH, Forwood MR, Johnston CC, Burr DB. Suppressed bone turnover by bisphosphonates increases microdamage accumulation and reduces some biomechanical properties in dog rib. *J Bone Miner Res*. 2000;15(4):613-620.
 66. Prockop DJ. What holds us together? Why do some of us fall apart? What can we do about it? *Matrix Biol*. 1998;16(7):519-528.
 67. Pereira RF, O'Hara MD, Laptev AV, et al. Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. *Proc Natl Acad Sci U S A*. 1998;95(3):1142-1147.
 68. Panaroni C, Gioia R, Lupi A, et al. In utero transplantation of adult bone marrow decreases perinatal lethality and rescues the bone phenotype in the knockin murine model for classical, dominant osteogenesis imperfecta. *Blood*. 2009 Jul;114(2):459-468. Epub 2009 May 4.
 69. Whyte MP, Mumm S, Deal C. Adult hypophosphatasia treated with teriparatide. *J Clin Endocrinol Metab*. 2007 Apr;92(4):1203-1208. Epub 2007 Jan 9.
 70. Millán JL, Narisawa S, Lemire I, et al. Enzyme replacement therapy for murine hypophosphatasia. *J Bone Miner Res*. 2008;23(6):777-787.
 71. Ishida T, Inaba M, Hisha H, et al. Requirement of donor-derived stromal cells in the bone marrow for successful allogeneic bone marrow transplantation: complete prevention of recurrence of autoimmune diseases in MRL/MP-lpr/lpr mice by transplantation of bone marrow plus bones (stromal cells) from the same donor. *J Immunol*. 1994;152(6):3119-3127.
 72. Hisha H, Nishino T, Kawamura M, Adachi S, Ikehara S. Successful bone marrow transplantation by bone grafts in chimeric-resistant combination. *Exp Hematol*. 1995;23(4):347-352.
 73. Li Y, Hisha H, Inaba M, et al. Evidence for migration of donor bone marrow stromal cells into recipient thymus after bone marrow transplantation plus bone grafts: a role of stromal cells in positive selection. *Exp Hematol*. 2000;28(8):950-960.
 74. Bianchi G, Banfi A, Mastrogiacomo M, et al. Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp Cell Res*. 2003;287(1):98-105.

75. Ito T, Sawada R, Fujiwara Y, Seyama Y, Tsuchiya T. FGF-2 suppresses cellular senescence of human mesenchymal stem cells by down-regulation of TGF- β 2. *Biochem Biophys Res Commun*. 2007 Jul 20;359(1):108-114. Epub 2007 May 21.
76. Ren H, Cao Y, Zhao Q, et al. Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions. *Biochem Biophys Res Commun*. 2006 Aug 18;347(1):12-21. Epub 2006 Jun 6.
77. Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T. Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. *J Cell Physiol*. 2006;207(2):331-339.
78. Sotiropoulou PA, Perez SA, Salagianni M, Baxevanis CN, Papamichail M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells*. 2006 Feb;24(2):462-471. Epub 2005 Aug 18.
79. O'Flaherty E, Sparrow R, Szer J. Bone marrow stromal function from patients after bone marrow transplantation. *Bone Marrow Transplant*. 1995; 15(2):207-212.
80. Lukomska B, Janczewska S, Durlak M, Olszewski WL. Kinetics of bone marrow repopulation in lethally irradiated rats after transplantation of vascularized bone marrow is syngeneic hind limb. *Ann Transplant*. 2000;5(1):14-20.
81. Pelagiadis I, Dimitriou H, Kalmanti M. Biologic characteristics of mesenchymal stromal cells and their clinical applications in pediatric patients. *J Pediatr Hematol Oncol*. 2008;30(4):301-309.
82. Locatelli F, Maccario R, Frassoni F. Mesenchymal stromal cells, from indifferent spectators to principal actors: are we going to witness a revolution in the scenario of allograft and immune-mediated disorders [editorial]? *Hematologica*. 2007;92(7):872-877.
83. Prockop DJ. Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. *Mol Ther*. 2009 Jun;17(6):939-946. Epub 2009 Mar 31.
84. Munoz JR, Stoutenger BR, Robinson AP, Spees JL, Prockop DJ. Human stem/progenitor cells from bone marrow promote neurogenesis of endogenous neural stem cells in the hippocampus of mice [published correction appears in *Proc Natl Acad Sci U S A*. 2006;103(6):2000-2002]. *Proc Natl Acad Sci U S A*. 2005 Dec 13;102(50):18171-18176. Epub 2005 Dec 5.
85. Baltzer AW, Lattermann C, Whalen JD, et al. Genetic enhancement of fracture repair: healing of an experimental segmental defect by adenoviral transfer of the BMP-2 gene. *Gene Ther*. 2000;7(9):734-739.
86. Lind M, Bünger C. Orthopaedic applications of gene therapy. *Int Orthop*. 2005 Aug;29(4):205-209. Epub 2005 May 18.
87. Park J, Ries J, Gelse K, et al. Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes. *Gene Ther*. 2003;10(13):1089-1098.
88. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131(5):861-872.
89. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007 Dec 21;318(5858):1917-1920. Epub 2007 Nov 20.
90. Park IH, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature*. 2008 Jan 10;451(7175):141-146. Epub 2007 Dec 23.
91. Nakagawa M, Koyanagi M, Tanabe K, et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol*. 2008 Jan;26(1):101-106. Epub 2007 Nov 30.
92. Lowry WE, Richter L, Yachechko R, et al. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci U S A*. 2008 Feb;105(8):2883-2888. Epub 2008 Feb 15.
93. Yu J, Hu K, Smuga-Otto K, et al. Human induced pluripotent stem cells free of vector and transgene sequences. *Science*. 2009 May;324(5928):797-801. Epub 2009 Mar 26.
94. Zhou H, Wu S, Joo JY, et al. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell*. 2009 May 8;4(6):381-384. Epub 2009 Apr 23.
95. Kim D, Kim CH, Moon JI, et al. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell*. 2009 Jun 5;4(6):472-476. Epub 2009 May 28.