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A Therapeutic Role for Hematopoietic Stem Cells in Osteogenesis Imperfecta

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1. Introduction

1.1. Osteogenesis Imperfecta (OI)

“Fragile bones” have been described in medical literature for centuries. Osteogenesis imperfecta (OI), whose name means “imperfect birth of bones”, is one such fragile bone syndrome. A generalized disorder of the body’s connective tissue, it is most obvious in its effects on the bone, but also involves the body’s ligaments, tendons, fascia, eyes, skin, teeth and ears. It is a highly variable heritable disorder characterized by recurring bone fractures, low bone mass and bone fragility [1]. Bone fragility has led to the common name “brittle bone disease” for OI. Its overall incidence is approximately one in 10,000 births. The incidence of forms of OI recognizable at birth is 1/16-20,000, with about equal incidence of mild forms that are not recognizable until later in life. The clinical range of this condition is extremely broad, ranging from cases that are lethal in the perinatal period to cases that maybe difficult to detect and can present as early osteoporosis [2]. Individuals with OI may have varying combinations of growth deficiency, defective tooth formation (dentinogenesis imperfecta), hearing loss, macrocephaly, blue coloration of sclerae, scoliosis, barrel chest and ligamentous laxity. In more severe cases, people are susceptible to fracture from mild trauma and even from acts of daily living.

1.2. Classification and types of OI

Classical OI is an autosomal dominant condition caused by defects in type I collagen, the major structural component of the extracellular matrix of bone, skin and tendon. This deficiency arises from an amino acid substitution of glycine to bulkier amino acids in the collagen triple helix structure. The larger amino acid side-chains create steric hindrance that

creates a bulge in the collagen complex, which in turn influences both the molecular nano-mechanics as well as the interaction between molecules, which are both compromised [3]. As a result, the body may respond by hydrolyzing the improper collagen structure. If the body does not destroy the improper collagen, the relationship between the collagen fibrils and hydroxyapatite crystals to form bone is altered, causing brittleness. Another suggested disease mechanism is that the stress state within collagen fibrils is altered at the locations of mutations, where locally, larger shear forces lead to rapid failure of fibrils even at moderate loads as the homogeneous stress state found in healthy collagen fibrils is lost [3]. In the past several years, autosomal recessive forms of OI have been identified. Although recessive OI is not due to defects in collagen, its etiology in a collagen-modification complex is collagen-related. Hence, in ~10% of cases, genes encoding proteins involved in type I collagen's complex posttranslational modifications and intracellular trafficking can also be involved in the causation of OI [4]. Autosomal recessive OI is caused by defects in two of the components of the prolyl 3-hydroxylation complex, which modifies the $\alpha 1(I)$ chain of collagen in the endoplasmic reticulum, cartilage-associated protein (CRTAP) [5, 6] and prolyl 3-hydroxylase (P3H1) [7]. OI can also occur as a consequence of mutations in key osteoblast genes that code for proteins involved in matrix homeostasis [8, 9] and are not directly related to collagen metabolism and matrix structure. About 5% of OI cases are not caused by defects of type I collagen or the P3H1 hydroxylation complex and their etiology is presently unknown. Most people with OI receive it from a parent but in 35% of cases it is an individual (*de novo* or "sporadic") mutation.

1.3. Sillence classification

Classical OI is generally described using the Sillence classification, a nomenclature based on clinical and radiographic features, which was first proposed in 1979 [10]. This classification subdivides patients into four types based on disease severity and progression:

Type I OI, the most common and mildest form of the disease and is caused by a quantitative defect with synthesis of structurally normal type I procollagen at about half the normal amount [11]. Type I OI is non-deforming and results in patients attaining close to normal height, however vertebral fractures are common and can lead to mild scoliosis. Joint hyperextensibility is also a common feature. Growth deficiency and long bone deformities are generally mild. In these patients, fractures are rare at birth but begin with ambulation [1]. Patients also present with blue sclera and 50% may have hearing loss. Type I has been divided into A and B subtypes based on the absence or presence of dentinogenesis imperfecta, a genetic disorder of tooth development also known as hereditary opalescent dentin. This condition causes teeth to be discolored (most often a blue-gray or yellow-brown color) and translucent. Teeth are also weaker than normal, making them prone to rapid wear, breakage, and loss. These problems can affect both primary teeth and permanent teeth.

Type II OI is the most severe OI form, generally resulting in death in the perinatal period, although survival up to one year has been noted. These patients exhibit multiple intrauterine rib and long bone fractures and severe skeletal deformities, which eventually result in respiratory failure. Legs are usually held in a frog leg position with hips abducted and knees

flexed. The skull is severely under mineralized with wide open fontanelles. The bones of these infants are predominantly composed woven bone without haversian canals and organized lamellae. In 1984 a radiological sub classification of type II OI was proposed [12]: OI II A—broad ribs with multiple fractures, continuous beaded ribs, severe undermodeling of femur; OI II B—normal/thin ribs with some fractures, discontinuous beaded ribs, some undermodeling of femur; OI II C—varying thickness of ribs, discontinuous beading of ribs, malformed scapulae and ischia, slender and twisted long bones.

Type III OI is a severe form of the disease, characterized by severe progressive skeletal deformities. This is due to the synthesis of mutated collagen. Fractures may be present *in utero* and are very common during the growth period as the bones are extremely fragile. The incidence of fracture remains high even in the adult life. The long bones are soft and deformed from normal muscle tension. Individuals are severely short statured and scoliosis can lead to respiratory problems. These patients also present with dentinogenesis imperfecta. Radiographically, metaphyseal flaring and “popcorn” formation at growth plate is seen. These patients require intensive physical rehabilitation to attain assisted ambulation.

Type IV OI is the most clinically diverse group of patients of OI. The phenotype can vary from mild to severe. Scleral hue is also variable. Typically, these patients suffer several fractures in a year and present with bowing of long bones. While fractures decrease after puberty, individuals have a short final stature. Radiographically, the patients have osteoporosis and mild modeling abnormalities. Like type I OI, this group can also be divided into two subgroups on the basis of the presence or absence of dentinogenesis imperfecta [13]. It is from this heterogeneous group that types V, VI and VII have been identified based on distinct clinical and histological features [14-16].

Although type V to VIII continues the Sillence classification, they are based on different criteria than other types. Type V and VI are defined using bone histology and have a phenotype that would be included in type IV. However, these individuals do not have defects in type I collagen. Type VII and VIII are recessive forms whose phenotype overlaps type II and III. These patients have deficiencies of components of collagen modification complex in endoplasmic reticulum.

Type V OI is moderately deforming and there are three distinctive features: the frequent development of hypertrophic calluses at the fracture site, the calcification of interosseous membranes between the bones of the forearm and the presence of radio-opaque metaphyseal band immediately adjacent to the growth plate on X-rays [14]. The calcified interosseous membrane severely limits the pronation/supination of the hand and may lead to secondary dislocation of the radial head. These patients have normal teeth and white sclerae. Patients with type V OI represent 4-5 % of the OI population seen in the hospital.

Type VI OI also presents with moderate to severe deformities and do not have blue sclera and dentinogenesis imperfecta. Distinctive histological features are the fish-scale like appearance of the bone lamellae and presence of excessive osteoid accumulation on bone forming surfaces. Inheritance is autosomal dominant and may represent approximately 4% of moderately to severely affected patients [15].

Type VII OI is an autosomal recessive form caused by defects in *CRTAP*, cartilage-associated protein [16]. Patients have moderate to severe skeletal deformities, bone fragility, lack of blue sclera and no dentinogenesis imperfecta. The distinctive clinical feature is the rhizomelic shortening of the humerus and femur. To date, this disorder has only been observed in a community of Native Americans in northern Quebec [16].

Type VIII OI is an autosomal recessive form caused by defects in prolyl 3-hydroxylase 1 (P3H1, encoded by *LEPRE1*). P3H1 forms a complex in the endoplasmic reticulum with *CRTAP*. This causes a phenotype which overlaps types II and III OI but has distinct features, including white sclera, extreme growth deficiency and under mineralization [7].

1.4. Dominantly inherited OI

Most patients with OI (~ 90%) have mutations in one of the type I collagen genes, *COL1A1* or *COL1A2*. These mutations are dominantly inherited and the phenotype can vary from the very mild to lethal. There are two general cases of mutation in Type I collagen genes that result in OI: those that cause a quantitative defect with synthesis of structurally normal type I procollagen at about half the normal amount and those that result in synthesis of structurally abnormal collagen. The former is usually due to premature termination codons in one *COL1A1* molecule that initiates decay of the mRNA from the affected allele. This generally results in mild nondeforming phenotype with blue sclera (Type I OI) [11]. The most prevalent mutation results in substitution of one of the invariant glycine residues that have a critical role in helix formation. A collagen type I molecule comprises a triple helix made up of two alpha 1 and one alpha 2 polypeptide chains. In the center of each helical turn, i.e. every third amino acid is a glycine residue, which is essential for the structure of the molecule. Any substitution of the residues can result in structural abnormalities and produce a mixture of normal and abnormal collagen strands. Depending on the substitution type and location, the phenotype can vary from mild to very severe. Usually, patients with the more severe type of the disease have a mutation at one essential glycine residue site [17]. Alterations in collagen type I molecules lead to structural changes in the bone and the abnormal collagen has lower tensile strength. This leads to the brittleness of the bones in OI. OI not only results in low trabecular bone mineral density and thin cortices, but also in small, slender bones. Together, these factors contribute to the fragility of the bones.

1.5. Recessively inherited OI

In the past decade, the genetic basis of 10 new OI variants has been discovered, seven of which result from mutations in genes encoding proteins involved in the post translational modifications of type I procollagen [6, 7, 18-22]. In 2007, mutations in *CRTAP* were identified in patients without mutations in *COL1A1* and *COL1A2* but with excess posttranslational modification of type I collagen indicative of delayed folding of the triple helix [6, 7]. Patients with similar phenotype with mutations in genes such as *LEPRE1* (prolyl 3-hydroxylase, P3H) and *PP1B* (Peptidyl-prolylcis-trans isomerase B; cyclophilin B) have also been identified [19, 20]. FK binding protein 10 (FKBP10) mutations present as a milder phenotype in late childhood or adolescence with long bone fracture, acetabular protrusion and scoliosis

[22]. Among the most recent discoveries are the association of mutations in the gene *SERPINF1* (serpin peptidase inhibitor, clade F) with type VI OI [23]. The latter two are proteins responsible for chaperoning collagen through the endoplasmic reticulum. *SERPINF1* encodes pigment epithelium derived factor, a secreted glycoprotein with uncertain function in bone. Mutations in *LRP5*, a key regulator of osteoblast function, affects bone accrual during growth [8]. More recently, a child with moderate OI phenotype has been identified with homozygous mutation in *SP7*, which codes for osterix, a transcription factor specifically expressed in osteoblasts in the developing skeleton [9].

2. Animal models used for the study of OI

The many different types and subtypes of OI highlight the importance of developing animal models to study the disease. Canine, feline, bovine and ovine models of OI have been described (reviewed in [24]). However, the majority of animal studies have been conducted using engineered and spontaneously occurring murine models.

2.1. Mov-13 mouse: A model for OI type I

In Mov-13 mice, transcription of the $\text{pro}\alpha 1(\text{I})$ gene was completely blocked as a result of Moloney leukemia virus integration at the 5' end of the gene [25]. No functional $\alpha 2(\text{I})$ was detected in embryos [26], likely as a result of rapid degradation of $\text{pro}\alpha 2(\text{I})$ procollagen chains which are unable to form stable triple helices. Mice homozygous for the null mutation produced no type I collagen and died at mid-gestation while heterozygotes survived to young adulthood [27]. Heterozygotes produced 50% less type I collagen which causes progressive hearing loss and alterations in the mechanical properties of long bones [28]. The heterozygous Mov-13 mouse therefore serves as a model for type I OI. As many as 5% of osteoblasts from long bones were shown to produce normal amounts of type I collagen, thus implying that a small set of osteoblasts did not express the mutant phenotype [29]. This bone tissue mosaicism for expression of the mutant allele may explain why Mov-13 heterozygotes do not display an obvious bone fragility phenotype.

2.2. Brittle II mouse: A model for type II OI

The cre/lox recombination system was used to develop a lethal murine knock in model of OI type II [30]. A 3.2 kbp transcription/translation stop cassette was introduced in intron 22 and flanked by directly repeating lox recombination sites. After homologous recombination in ES cells, two male chimeras were obtained. A knock in mouse carrying and intronic inclusion was generated by mating chimeras with wild-type females. Alternatively splicing involving the stop cassette resulted in retention of non-collagenous sequences. This mouse had the lethal phenotype of the similar human mutation and was designated BrtIII. Skeletal staining showed rib fractures, poor skeletal mineralization and shorter vertebral bodies. The mice die a few hours after birth from apparent respiratory distress.

2.3. Oim/Oim mouse: A model for type III OI

Chipman et al. [31] described a strain of mice with a nonlethal recessively inherited mutation that resulted in phenotypic and biochemical features that simulate moderate to severe human OI. This *oim* mutation arose spontaneously in 1985 in the Mutant Mouse Resource of The Jackson Laboratory. It has since been determined that the underlying defect in the *oim* mouse is a mutation in COL1A2 [32]. This mutation changes the reading frame at the 3' end of the mRNA causing synthesis of an altered C-propeptide that ultimately inhibits the association of these molecules into heterotrimeric type I procollagen bundles in the bone matrix. Instead, homotrimers are formed which interferes with the integrity and quantity of the osteoid that accumulates in the bone. This mouse model has been shown to have a phenotype similar to that seen in human type III OI, including a decreased body size, abnormal bone mineralization (contributing to the brittleness of the bones), decreased bone density and a fragile skeleton susceptible to fractures. While the skeleton becomes progressively deformed with age, homozygous mice can live a normal life span. The heterozygous mice simulate the mild form of the disease in which the bones show abnormal cortical morphology and reduced bone mechanical strength even though no fractures are seen. Heterozygote *oim/+* mice have subtle skeletal fragility whereas homozygous *oim/oim* mice have marked skeletal fragility. The dental phenotype in *oim/oim* is more severe in incisors than in molars and includes changes in pulp chamber size, tooth shape, and dentin ultrastructure. Teeth in *oim/oim* animals are clinically fragile. Although *oim/+* teeth are grossly normal, ultrastructural abnormalities such as reduction in the number and regularity of spacing of the dentinal tubules, less mineralization, and blurring of the boundary between peritubular and intertubular dentin can be found in *oim/+* teeth [33].

Breeding studies showed that the *oim* mutation was inherited in most crosses as a single recessive gene on chromosome 6, near the murine COL1A2 gene. Biochemical analyses of skin and bone, as well as isolated dermal fibroblast cultures, demonstrated that $\alpha 1(I)$ homotrimer collagen accumulated in these tissues. Short labeling studies in fibroblasts demonstrated an absence of pro $\alpha 2(I)$ collagen chains. Nucleotide sequencing of cDNA encoding the COOH-propeptide revealed a G deletion at pro $\alpha 2(I)$ nucleotide 3983; this results in an alteration of the sequence of the last 48 aminoacids. Normal-sized mRNA is transcribed, but no secreted protein has been identified in *oim/oim* fibroblasts and osteoblasts. Collagen from the *oim/oim* mouse showed reduced resistance to tensile stress [34]. Neutron activation analyses demonstrated that *oim/oim* femurs had significant differences in magnesium, fluoride, and sodium content compared to wild type mouse femurs [35]. These and other studies suggest that the known decreased biochemical properties of *oim/oim* bone reflect both altered mineral composition and decreased bone mineral density, which further suggests that the presence of $\alpha 2(I)$ chains plays an important role in bone mineralization [36].

2.4. Brittle IV mouse: A model for type IV OI

The cre/lox recombination system was used to develop a nonlethal knock-in murine model for OI [30]. A moderately severe OI phenotype was obtained from an $\alpha 1(I)$ 349 Gly \rightarrow Cys substitution in type I collagen, which is the same mutation in a type IV OI child. These mice,

designated as Brittle IV (Brtl IV), have phenotypic variability ranging from perinatal lethality to long-term survival with reproductive success. The size of Brtl IV mice was about 50% that of normal littermates at 6 weeks of age, after which their size increased to about 80% of normal. Deformity of the rib cage was apparent and both forelegs and hindlegs were bowed and thinner than those of control littermates. The Brtl IV mouse has the molecular, biochemical, and radiographic features of human OI type IV. Heterozygous mutant mice have the undermineralization of the skeleton, the bone fragility, and the deformity characteristic of human patients. Their growth pattern, with normal size at birth followed by growth deficiency until 4–5 weeks of age, resembles the early childhood growth pattern reported for moderately severe OI patients. However, no significant deformities in long bones were evident in mutant mice after puberty and long bone fractures were also infrequent in adult mice.

3. Therapies for OI

At present, there is no cure for OI; however, some ‘symptomatic’ treatment options are available. The management of OI includes multidisciplinary input with experienced medical, orthopedic, physiotherapy and rehabilitation specialties. The current goals of therapy for OI are: to decrease the incidence of fractures; to increase growth velocity; to decrease pain; to have a positive effect on bone metabolic markers, bone histomorphometry and bone mineral density; and finally, to increase mobility and independence.

During past decades, various pharmacological agents have been administered to patients with OI and the majority of them initially claimed beneficial results, although none proved effective in controlled trials [37]. Among these were anabolic steroids, vitamin D, vitamin C, sodium fluoride, magnesium oxide, flavonoids (catechin) and calcitonin. Until 18 years ago, calcitonin was the most common therapy for OI, although its beneficial effects during the clinical course of the disease were disputed in the literature [38]; however it is no longer used. Thus, the search for effective treatments for OI remains ongoing.

3.1. Drug therapies

3.1.1. Bisphosphonates

In the last decade, the potential of bisphosphonate (BP) treatment has caused great excitement in the OI patient community and has generated new therapeutic options. BPs have been accepted as the standard of care for children with OI and in particular with moderate to severe forms of OI. The BP compounds are analogs of pyrophosphate which, when administered either orally or parentally, are characterized by a rapid and strong binding to hydroxyapatite crystals in the bone mineral. Once BPs are buried in the skeleton they are released only when bone is destroyed in the course of bone turnover. The success of BP appears to be related to the unremitting osteoclastic activity. These agents are potent inhibitors of bone resorption, decreasing osteoclast activity and number, although some effect on bone

formation also occurs [39]. The potent anti-resorptive properties of BP inhibit the normal remodeling activity that acts to renew and repair bone. This activity results in improved vertebral shape and mass, higher cortical width, increased cancellous bone volume and suppressed bone turnover as shown by histomorphometric studies [40]. The net effect is to promote bone mineral accretion and at the same time to reduce bone turnover. Although the quality of the new bone that is formed remains unchanged, the bones benefit from greater mechanical strength due to overall increased bone mass [17].

A number of prospective studies have now shown that BPs can reduce fracture frequency, increase bone mineral content and improve the radiographic assessment of bone shape in growing children [41, 42]. In addition, linear growth is not impaired and fractures heal at their expected rate. Increase mobility was reported in the two largest studies conducted [42]. Muscle force measured by maximal isometric grip force of the non-dominant hand showed significant increases with BP therapy which was maintained for two years [43]. Patients with OI types I, II and IV showed significant improvement in height after four years of BP therapy [44]. It is difficult to assess the fracture rates as with increased mobility there might be a transient increase in fractures. However, overall decrease in fracture rate has been demonstrated after therapy when compared to historical controls [42]. Bone mineral density in the lumbar vertebrae also shows a rapid increase [45]. Radiographically, cycles of BP therapy leave dense sclerotic bands at the metaphysis of long bones which may contribute to the increased strength of the bones [46]. However, questions remain as to the selection of patients for treatment, which BPs to use, the minimum effective dose, the minimum effective treatment interval, appropriate duration of treatment and the role of oral BPs.

Concerns also remain regarding the potential buildup of microcracks and calcified cartilage which could lead to poor bone healing and increase fragility [47]. Osteonecrosis of the jaw is a complication of poor soft tissue and bone healing associated with BP therapy. While this is mainly reported in elderly patients with cancer who have been given very high doses of BP [48], there are concerns whether this complication could arise with long-term use of BP in children. However, the greatest concern in children with OI is over suppression of bone modeling and remodeling and worsening of bone quality. Long-term treatment, even at standard doses, interferes with bone remodeling and can be detected as metaphyseal under-tubulation [49, 50]. Reports from surgeons describe treated bone as “rock-hard” and “crumbly”, providing insight into paradoxical increases in fractures in some treated patients. Long-term suppression of bone turnover leads to accumulated micro-damage (microcracks) in bone [51] that may underlie the decrease in material strength. The equivocal improvement in fractures in children is illuminated by data from BP treatment of the *Brtl* mouse [52]. Treatment increases bone volume and load to fracture of murine femora, but concomitantly decreases material strength and elastic modulus. Femurs become, ironically, more brittle after prolonged treatment and bands of mineralized cartilage create matrix discontinuities that decrease bone quality. Prolonged treatment also alters osteoblast morphology. BP are also buried in the skeleton where they have a half-life of many years, so long term side effects may still surface. Thus, long-term use of BPs may not be beneficial as they decrease material properties and have detrimental effects on osteoblasts and bone formation.

3.1.2. Growth hormone

In mild forms of OI, agents increasing the production of type I collagen may have a therapeutic role. Growth hormone (GH) action positively affects bone growth and bone turnover by stimulating osteoblasts, collagen synthesis and longitudinal bone growth [53]. GH has a positive action on collagen metabolism, stimulating the expression in osteoblast cultures of insulin like-growth factor-1 (IGF-1) and IGF binding protein-3, which in turn regulate the synthesis of type I collagen [54, 55]. Osteoblasts from various species have IGF-I receptors and respond to both endogenous and exogenous IGF by accelerating the proliferation and increasing DNA and collagen synthesis [56, 57]. Animal studies in the *oim/oim* mouse model of OI, with bone phenotype comparable to a mild form of OI in humans, showed that systemic GH injections [58] or GH transgene expression in marrow [59] increased spine and femur length, produced significant changes in densitometric parameters and ameliorated biomechanical structural properties of bone. There is limited literature regarding GH in OI as only a few human studies have been performed using GH in patients with OI [60, 61]. One of the first attempts to treat OI with GH was more than 20 years ago by Kruse and Kuhlencordt[62], who treated two patients affected by OI with GH. The patients had an increase in periosteal new bone formation and in intracortical bone resorption with enhanced osteoblastic activity [62]. Following these results, no further study was reported in the literature until Marini and colleagues published their preliminary results from a limited number of patients treated with GH or clonidine (a pituitary GH secretagogue) [60]. In a further study by this group, the authors concluded that there is a group of type IV OI children who would benefit from GH treatment in terms of linear growth, bone matrix synthesis and bone histomorphometric parameters [63]. During GH therapy, patients have an improvement in general wellbeing, muscular performance and motor ability, which increases physical activity and, consequently, fracture risk in some cases [64]. In a study examining the efficacy of one year of GH treatment in patients affected by type I OI with an ascertained quantitative defect in type I collagen synthesis, GH treatment showed a positive effect on bone turnover, markers of bone apposition (i.e., osteocalcin and procollagen type I carboxy terminal propeptide levels) and bone mineral density, while the fracture risk did not change [65]. Thus, the results indicate that this is a useful therapy in patients with moderate forms of OI (the majority of type I and a good proportion of type IV). Patients with pre-existing scoliosis or bone deformities must be treated with particular caution because of the potential risk of worsening of these problems. Therefore, the selection of patients for GH treatment should be done carefully. It is possible that GH may be of benefit in combination with BP therapy [65, 66], but this has still to be adequately investigated [63].

3.1.3. Parathyroid hormone

Parathyroid hormone (PTH) also has anabolic effects on the bone and has been shown to have a positive effect for treatment of osteoporosis. Animal studies have shown that daily injection of recombinant human PTH results in increased bone mass, substantial new bone formation and altered bone architecture [67]. Based on this, daily injections of PTH should be beneficial in OI. However, these animal studies have demonstrated that sustained deliv-

ery in young rats resulted in development of bone lesions and tumors [67]. Due to this proposed increased risk for development of osteosarcoma, PTH is currently not recommended for children.

3.1.4. RANKL inhibitors

The potential therapeutic effects of receptor activator of nuclear factor kappa B ligand (RANKL) inhibitors in OI are currently under investigation. A recent study in a mouse model of OI (*oim/oim*) compared the effects of BP and RANKL inhibition. They found that although there were subtle differences between the two treatments, one was not superior to the other. There were similar decreases in fracture incidence with increases in metaphyseal bone volume via increase number of thinner trabeculae. BPs have the disadvantage of persisting in the bone for decades. Therefore RANKL inhibition is a newer, though more expensive treatment option, but may be preferred by some families and doctors as it is not deposited in the bone matrix. However, studies are needed to optimize the age of onset of therapy and the dose in children [68].

3.1.5. Bortezomib

The proteasome inhibitor Bortezomib is widely used in the treatment of multiple myeloma [69] and has been demonstrated to have an osteoblastogenic affect on adult murine and human mesenchymal stem cells by stabilizing RUNX-2 and acting directly on type I collagen [70]. It enhances osteoblast activity, differentiation [71] and also number [72]. Using the *Brtl* mouse model for OI, impairment in the differentiation of the progenitor cells towards osteoblasts has been demonstrated [73]. Treatment of the *Brtl* mice with Bortezomib rescued the osteoblastogenic capacity *in vitro* and ameliorated the bone properties *in vivo*, thus potentially identifying a new target for OI pharmacological treatment [73].

3.1.6. Sclerostin

A very recent study has investigated the potential of treating OI with antibodies to sclerostin, an anabolic bone agent produced by osteocytes that negatively regulated bone formation [74]. Antibodies to sclerostin are thought to stimulate osteoblasts and this agent is currently in clinical trials for treatment of osteoporosis [75]. Using the *Brtl/+* mouse model, Sinder et al [74] demonstrated that treatment of OI mice for two weeks with antibodies to sclerostin stimulated bone formation, improved bone mass and increased bone load and stiffness to those of wildtype mice. These studies suggest short-term treatment of OI patients with sclerostin antibody may lead reduced fractures and improved bone quality.

3.2. Cell-based therapies

Normal bone responds to fracture or loading by increasing bone resorption and formation [76]. In a similar way, the OI bone initiates a cycle of bone remodeling in an attempt to form a stronger matrix. However, in OI, mutant collagen is synthesized, secreted from the cell and incorporated into matrix, where it actively participates in weakening the structure. Giv-

en the high turnover of bone seen in OI [77, 78], it is feasible that the deleterious effects in OI could be reduced or neutralized by the presence of normal osteoprogenitor cells. Thus, the potential to correct OI may lie in replicating the natural example of carriers, who have a substantial proportion of cells heterozygous for the collagen mutation, but are clinically normal. Studies of osteoblasts from carriers of type III and IV OI have shown that 40-75% of cells are mutant, setting the threshold for minimal symptoms at 30-40% normal cells [79]. Based on these findings, approaches that either target cells to suppress expression of mutant collagen or replace mutant cells with donated bone cell progenitors have potential to serve as long-term treatment for OI.

3.2.1. Gene-targeting therapy

While drug-based therapies may result in a more functional life for patients with moderate to severe OI, gene therapies aimed at correcting or replacing the defective gene may potentially provide long-term reversal of symptoms. Antisense technologies to inactivate mutant mRNA have been proposed as a method for mutation suppression [80]. In fibroblasts derived from a patient with type IV OI, antisense oligonucleotides were shown to suppress mutant protein $\alpha 2(I)$ mRNA to 50% and mutant $\alpha 2(I)$ mRNA to 40% [80]. While promising, these oligonucleotides also targeted the normal allele mRNA, suppressing it to 80% of its level in control cells, rendering this therapy ineffective. Similar studies have tested the ability of allele-specific suppression of mutant collagen expression by hammerhead ribozymes (short RNA molecules with catalytic potential) to biochemically transform the recipient from type II, III or IV OI into type I OI, in which individuals have a null allele, make half the normal amount of collagen and have mild disease [81]. These findings show that this suppression was complete and specific *in vitro* and substantial (50%) and highly selective (90%) in cells. However, the successful application to animal models is still to be tested.

Another approach involves gene targeting of mutant COL1A1 and COL1A2 using adeno-viral vectors in adult mesenchymal stem cells (MSCs). Two studies have shown successful production of normal collagen cells targeted with a COL1A1 or COL1A2 mutation [82, 83]. In a recent study by Deyle et al [84], MSCs were isolated from OI patients and mutant collagen genes were inactivated by adeno-virus-mediated gene targeting. Induced pluripotent stem cells (iPSC) were then derived from these gene-targeted cells with a floxed, polycistronic reprogramming vector, all vector-encoded transgenes were deleted with Cre recombinase. These iPSCs were then differentiated into mesenchymal and osteogenic cells *in vitro*, which produce bone *in vivo* after transplantation into the subrenal capsule of immunodeficient mice. These approaches could be potentially valuable for individuals with OI who are past early childhood. However, issues with low targeting success and random integration need to be solved before these approaches can be validated in humans.

3.2.2. Cellular replacement therapy

A number of reports in literature using animal models have suggested that bone marrow (BM) cells could be transplanted via the circulatory system and that the transplanted cells contribute to skeletal tissues including bone [85, 86]. Also encouraging have been transplan-

tation studies of adult BM into Brtl pups *in utero* [87]. Despite low engraftment in bone (~2%), transplantation eliminated the perinatal lethality of Brtl mice and improved the biomechanical properties of femora in two-month old treated Brtl mice [87]. Current dogma suggests that BM contains two types of stem cells, mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs), and that their repertoire of differentiation/reconstituting potentials are distinct and separate from each other. MSCs are defined by their adherence to plastic and potential to differentiate into mesenchymal tissue cells such as bone, fat, muscle, cartilage and fibroblasts [88-91]. The term “MSC” has been applied to define both mesenchymal stem cells and mesenchymal stromal cells [89]. HSCs are defined by their capability of hematopoietic reconstitution *in vivo* and have also been shown to give rise to a few tissue cell types including mast cells and osteoclasts. However, recent studies have begun to question the distinction between the potentials of MSCs and HSCs, particularly with regard to osteo-chondrogenic tissues.

4. MSC-based therapy

Transplantation studies using murine models have evaluated the potential of MSCs to directly differentiate into osteogenic cells to treat OI [92, 93]. Studies in a mouse model of OI showed that infusion of marrow stromal cells (MSCs) resulted in a significant increase in collagen production [85]. The data presented by Wang et al [92] demonstrated that murine MSCs migrate and incorporate into the developing neonatal heterozygous and homozygous OI mice, differentiate into osteoblasts and appear to participate in the bone formation of the recipient mouse *in vivo*. A recent study from the same group evaluated green fluorescent protein (GFP)-expressing single cell expanded, marrow-derived progenitors for engraftment in a neonatal model of OI following systemic transplantation [93]. Tissues from the recipient mice were examined at two and four weeks post transplantation. Their study shows that the progenitors infused in the neonatal OI mice engraft in the various tissues including bones, undergo differentiation, deposit matrix and form bone *in vivo*. The authors also state that the progenitor cell transplantation is more efficient in developing OI mice than adult mice [93].

Cell therapy protocols also involve direct delivery of cells into target tissues with the hope that the cells will differentiate into cells of the target tissues and repair or regenerate host tissues. Li et al (2010) have demonstrated that MSCs infused into femurs of the *oim/oim* mouse model contribute to bone formation *in vivo*. Improvement in mechanical properties of the recipient bones seen may be the result of bone deposition by both endogenous and donor cells or paracrine actions of donor cells. The recipient mice were followed for six weeks following cell infusion into femurs. It still remains to be investigated if this positive effect lasts beyond this time period.

Intrauterine transplantation of fetal human MSCs was shown to markedly reduced fracture rates and skeletal abnormalities in an *oim/oim* mouse model [94]. In a similar model, Vanleene et al [95] showed that human fetal MSCs isolated based on their adherence to plastic, transplanted *in utero* in *oim/oim* mice migrated to bone, differentiated into mature osteo-

blasts, and expressed the missing protein COL1a2, altering the apatite mineral structure and increasing bone matrix stiffness. The changes in microscopic material properties and micro-architecture contribute to the mechanical integrity of the bone, making the bone less brittle and resulting in a decreased incidence of fracture. However, further work needs to be done to investigate strategies to maximize donor cell homing to bone, differentiation, and collagen expression to maximize the therapeutic effects of transplantation.

While these studies suggest a role for MSCs in generation of osteogenic cells, the difficulty in defining and isolating MSCs as well as the sometimes complex history of manipulation before being tested for differentiation potentials *in vivo*, makes it difficult to determine the mechanism by which these cells have an effect [89, 96, 97].

5. HSC-based therapy

Recent studies have identified a population of circulating human osteoblastic cells which express osteocalcin or alkaline phosphatase and increase during pubertal growth and during fracture repair [98]. Studies showed that these osteocalcin positive cells were able to form mineralized nodules *in vitro* and bone *in vivo*. This population was subsequently shown to be CD34⁺ [99], suggesting that it is derived from the HSC. In support of this, Chen et al [100] have shown that the frequency of osteoblast progenitor cells is higher in CD34⁺ cells (approximately 1/5000) than in CD34⁻ population (1/33,000) of human BM. Murine transplantation studies have demonstrated that transplantation of 3000 side population (SP) cells that are highly enriched for HSCs generated osteoblasts *in vivo* [101]. In another study, Dominici et al [102] transplanted marrow cells that had been transduced with GFP-expressing retroviral vector and observed a common retroviral integration site in clonogenic hematopoietic cells and osteoprogenitors from each of the recipient mice. This is consistent with observations that non-adherent BM cells, the fraction enriched for HSCs, give rise to bone in culture [101, 103]. A study of the kinetics and histological/anatomic pattern of osteopoietic engraftment after transplantation of ~ two million GFP-expressing non-adherent BM cells in 6-8 week old FVB/N OI mice revealed that osteopoietic engraftment was maximum two weeks after transplantation [104]. However, this osteogenic engraftment decreased to negligible levels after six months to one year while the hematopoietic reconstitution remained stable over the entire period of observation [104]. The authors explain the lack of durable donor derived osteopoiesis by the intrinsic genetic program or the external environmental signals that suppress the differentiation capacity of the donor stem cells. Studies in both an animal model and patients (CT NCT00187018) demonstrated that the non-adherent bone marrow population was able to significantly and robustly provide osteoprogenitors for treatment of OI [105]. Together, these studies provide compelling evidence for the existence of a common progenitor cell with both hematopoietic and osteocytic differentiation potentials in the non-adherent or CD34-expressing, HSC-enriched, fraction of BM cells.

In the last decade, many conflicting reports have been published regarding tissue-reconstituting ability of HSCs. To determine the tissue reconstituting potential of HSCs, we have

carried out a series of studies based on BM reconstitution by a single HSC (reviewed in [106-108]). These studies have shown that transplantation of a clonal population derived from a single HSC expressing transgenic enhanced GFP (EGFP) results in efficient generation of mice exhibiting high-level, multi-lineage engraftment from a single HSC. In this model, putative HSCs are sorted based on surface marker expression and Hoechst dye efflux (side population, SP), identified by combining single cell deposition with short-term culture and functionally defined *in vivo* by the ability to reconstitute the BM when a single cell is transplanted into lethally irradiated mice [109-112]. It is important to note that there is no equivalent test for defining a MSC, making it difficult to isolate and characterize MSCs [96, 97]. Findings from studies using a single cell/clonal cell transplantation method have shown that HSCs can give rise to non-hematopoietic cells such as fibroblasts and fibrocytes [109], tumor-associated fibroblasts/myofibroblasts [110], valve interstitial cells [113], glomerular mesangial cells [111], brain microglial cells and perivascular cells [112], inner ear fibrocytes [114], retinal endothelial cells [115] and epithelial cells in multiple organs [116]. Most recently, our lab has demonstrated that HSCs give rise to adipocytes [117], a cell also thought to be of mesenchymal origin, and osteo-chondrogenic cells [118].

Based on these findings, we hypothesized that the primary defect in OI may lie in the HSC. As the bone turnover is high in OI, introduction of the normal progenitor cells would quickly populate the bone with cells making normal matrix and therefore ameliorate and/or prevent the occurrence of associated pathologies. To test this hypothesis, we conducted HSC transplantation in a mouse model of OI (*oim/oim*) [119]. In these studies, recipient *oim/oim* mice were first scanned by micro-computed tomography (micro-CT) before transplantation to obtain baseline images and information on bone histomorphometrics. The BM of lethally irradiated *oim/oim* mice was then reconstituted with EGFP⁺ non-adherent mononuclear cells or purified HSCs from EGFP mice. Transgenic EGFP⁺ mice (C57BL/6) [120] which ubiquitously express EGFP under the control of the actin promoter were used as BM donors for transplantation into homozygous OI mice (*oim/oim*; B6C3Fe a/a-Col1a2^{oim}/J; Jackson Labs). Either 2×10^5 mononuclear cells or 50 Lin⁻ Sca-1⁺ c-kit⁺ CD34⁻ SP cells (putative HSCs) prepared from the EGFP mice were injected via tail vein into irradiated *oim* mice. The mice transplanted with 50 Lin⁻ Sca-1⁺ c-kit⁺ CD34⁻ SP cells also received injection of 2×10^5 un-manipulated BM cells from an *oim/oim* mouse which served as radio-protective cells during the post radiation pancytopenia period. *Oim/oim* mice with no engraftment and irradiated *oim/oim* mice transplanted with 2×10^5 MNCs from another *oim/oim* mouse were used as controls. Changes in bone parameters were analyzed using longitudinal micro-CT. To confirm participation of HSC-derived osteoblasts and osteocytes in *oim/oim* bones, the EGFP⁺ cells were analyzed in paraffin sections.

Dramatic improvements in bone architecture were observed in the 3D micro-CT images of bones of HSC-engrafted *oim* mice at three, six and nine months post-transplantation which correlated with high levels of hematopoietic engraftment. These improvements corresponded to improvements in histomorphometric parameters including an increase in bone volume, trabecular number, thickness and density and a decrease in trabecular spacing. Decrease in trabecular pattern factor indicated an improvement in the connectivity and

structure of the trabeculae. In addition to quantifiable improvements in the bone architecture, we also observed clinical improvements in the engrafted *oim/oim* mice. The weight of the mice increased over the course of the experiment, perhaps in part due to the dramatic improvements in the bone architecture and density. The mice also became more active and were less prone to fractures during routine bedding changes and animal husbandry. In contrast to the mice engrafted with normal HSCs, the bone architecture and clinical parameters in the control mice continued to deteriorate over the course of the experiment. Analysis of paraffin sections showed the presence of numerous EGFP⁺ cells within the bone (unpublished data) that expressed Runx-2 and osteocalcin, demonstrating that they were osteoblasts and osteocytes as well as their origin from the HSCs. Studies are under way to determine the mechanisms by which HSCs affect structural and clinical improvements in the OI model.

6. Clinical bone marrow transplantations

Together, these preclinical studies suggest a potential for bone marrow transplantation in treating osteopoietic disorders. Findings from these studies are consistent with clinical transplantation of whole BM or fractionated MSCs in children with severe form of OI. In the first trial, three children with OI were transplanted with un-manipulated BM from a sibling donor [121]. Three months after osteoblast engraftment, specimens from trabecular bone show evidence of new dense bone formation. There was an increase in the total body bone mineral content associated with increase in growth velocity and reduced frequency of fracture [121]. Similar results were seen in an additional study with five children with severe OI [122]. With extended follow-up, the patients' growth rates either slowed down or plateaued, but bone mineral content continued to increase. These findings suggest a durable engraftment of osteogenic donor cells, which could potentially convert a severe clinical phenotype to a less severe one. Due to the promising results obtained with the previous trials, a study was conducted where gene marked, donor marrow derived mesenchymal cells were used to treat six children with severe OI. The cells engrafted in the bone, marrow stroma and skin and produced clinically measurable benefits in the form of increase growth velocities. But surprisingly, no increase was observed in the total body bone mineral content [123]. An additional study of a single human fetus receiving *in utero* transplantation of fetal MSCs reported that very low engraftment (0.3%) could still be demonstrated in bone at nine months of age, however evaluation of clinical outcome was complicated by treatment in infancy with bisphosphonate [124]. While statistical significance in these studies was often lacking because of the small number of patients in each study, these findings nonetheless suggest beneficial clinical effects of BM transplantation in OI.

7. Conclusions and future perspectives

There is significant interest in the use of BM transplantation to repair various tissues as illustrated by many ongoing clinical trials (reviewed in [89, 125]). Several preclinical studies

have suggested that transplantation of BM cells may lead to improvements in other genetic diseases that involve collagen synthesis such as Alport syndrome [126, 127] and Epidermolysis bullosa [128]. As detailed above, preclinical studies and those in patients also demonstrate a therapeutic role for BM transplant for OI. Despite these studies, the mechanism by which marrow transplant ameliorates the genetic disorder remains unclear. Given that the BM is thought to contain two stem cell populations, MSCs and HSCs, elucidation of the stem cell with osteogenic potential would potentially drive therapies for OI. Our studies demonstrate that the HSC has this potential [118, 119] and can correct the osteogenic defect in an animal model of OI [119]. Our findings are supported by a recent study that compared the mechanisms of action for non-adherent mononuclear cells and MSCs in OI [105]. In this study, it was shown that both non-adherent BM cells enriched for HSCs and MSCs are clinically effective agents for cell therapy of bone, but that the two populations function by distinct mechanisms. Non-adherent BM cells were found to directly differentiate into osteoblasts and secrete normal collagen to the bone matrix. In contrast, MSCs did not engraft in the bone, but secreted soluble mediators that indirectly stimulated growth. Together, these studies demonstrate the potential for stem cell-based therapies for long-term treatment of OI. However, several issues remain to be elucidated including: what is the optimal delivery schedule, which type of cell to deliver for greatest efficacy (MSC, HSC or combination), and how to expand their potential with adjunct drug therapy.

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