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Review Article

Stem cell-based bone repair

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Abstract: To accelerate bone repair, one strategy is to deliver the cells that make bone. The current review focuses on stem cell-based bone repair. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can self-renew unlimitedly and differentiate into the bone forming cells – osteoblasts. Scientists have been actively investigating culture conditions to stably and efficiently induce differentiation of these stem cells into osteoblasts. However, ESCs have the issues of ethnics, immune response and both ESCs and iPSCs have tumorigenic potential. In contrast, bone marrow stromal/stem cells (BMSCs) hold great potential to enhance bone formation. Use of BMSCs can avoid the ethical issues and can obviate the immune response problem. However, BMSCs are a rare population with limited self-renewal ability and their differentiation ability decreases in elderly individuals. Considering the unlimited self-renewal ability, it is promising to develop protocols to differentiate ESCs into osteoblasts faithfully and efficiently. It is important to eliminate undifferentiated ESCs or iPSCs because of their tumorigenic potential. Therefore, future studies need to identify BMSCs specific cell surface markers since the cell surface markers utilized currently are not specific to BMSCs. Future studies also need to enhance the osteogenic potential without using viral vectors for transgene delivery to eliminate the risk of tumor generation.

Keywords: Bone repair, embryonic stem cells, and bone marrow stromal/stem cells

Introduction

Skeletal defects can result from trauma, tumor resection, surgical correction of hereditary defects or from diseases and congenital anomalies [1]. Fracture is one of the most common skeletal defects, yet one in five fractures is unable to heal satisfactorily [2]. Thus, the skeletal defects markedly compromise mobility and quality of life. Further, the reconstruction of skeletal defects poses an enormous healthcare burden, which is complicated by the increasing cost and an aging population. Therefore, more effective approaches to treat these bone defects are needed.

Current available materials to reconstruct skeletal defects include autologous bone (from the patient), allogeneic bone (from a donor), and inorganic or alloplastic materials [1, 3]. The traditional gold standard for bone repair and regeneration has been the autologous bone grafts [4]. Autologous bone grafts, usually obtained from the patient's iliac crest, do not cause immune rejection. Besides containing patient's own bone-forming cells, autologous bone grafts might also recruit mesenchymal cells and direct them to differentiation into bone forming cells [3]. Although there are a number of advantages, the major drawbacks of autologous grafts are donor site pathology and limited graft supply [1, 3]. One alternative source for bone repair and regeneration is the allogenic graft, usually demineralized bone from cadaver. However, compared to autografts, these allografts have poor quality, with fewer cells, higher bone resorption and lower new bone formation. In addition, allografts carry high potential for immune rejection and disease transmission [1, 3]. Another source utilized are demineralised bone matrices and synthetic biomaterials such as metals, ceramics and polymers. These materials also carry high risk of infection and structural failure [1].

Bone tissue engineering will probably overcome the drawbacks of traditional bone graft materials and offer an effective approach for bone

repair and regeneration. Instead of implanting materials to bridge the defects, bone tissue engineering regenerates new bone [5]. One promising approach in bone tissue engineering uses both stem cells and scaffold capable of promoting robust bone formation. The current review will focus on stem cells studied for bone repair and regeneration. Embryonic stem cells (ESCs) and adult stem cells particularly bone marrow mesenchymal/stem cells (BMSC) are promising cell sources for bone repair and regeneration. Before discussing this topic, we will first introduce some basics about bone formation.

Basics in bone formation

Bones can be thought as an organ of the skeletal system. They provide mechanical support to soft tissues, and support muscle action and hematopoiesis. They also contribute to mineral homeostasis. These functions are accomplished by continuous bone remodeling which is characterized by bone resorption coupled to bone formation [6, 7]. Bone resorption is carried out by osteoclasts, which are derived from hematopoietic lineage. Bone formation is carried by osteoblasts, which are mesenchyme-derived. Differentiation of mesenchymal stem cells towards osteoblasts is governed by a series of transcription factors, including runt-related transcription factor 2 (Runx2), osterix and activating transcription factor 4 [8]. Osteoblasts will lay down bone matrix proteins that will slowly mineralize. An early marker of osteoblast differentiation is type I collagen. Mature osteoblasts secrete osteocalcin, which is considered to be a late stage osteoblast marker. Most osteoblasts die through the process of apoptosis, but some osteoblasts become osteocytes embedded in bone matrix and some osteoblasts become bone-lining cells [9].

During development, two processes give rise to the skeletal elements [10]. Flat bones that comprise the cranium and medial clavicles are formed by intramembranous ossification. Long bones that comprise the appendicular skeleton, facial bones, vertebrae, and the lateral medial clavicles are formed by endochondral ossification. In the intramembranous ossification, a group of mesenchymal cells forms a condensation by proliferating and differentiating directly into osteoblast progenitors [10]. In the endochondral ossification, mesenchymal cells first differentiate into hypertrophic chondrocytes, which then form hypertrophic cartilage [10]. The hypertrophic cartilage provides a template for trabecular bone formation. The major difference between the endochondral and intramembranous ossification is the presence of the cartilage phase in the former but not the latter.

Scientists have been actively investigating what is the ideal cell source to regenerate and repair bone. Pluripotent stem cells have gained a lot of attention because of the remarkable ability of self-renewal and the ability to become tissue- or organ specific cells with specific function. ESCs and BMSCs have been actively studied for bone repair.

Bone generation using pluripotent stem cells

ESCs are harvested from the inner cell mass of the blastocyst. Their pluripotent characteristic enables them to self-renew unlimitedly and differentiate into all cell types in the body [11-13]. The unique properties of pluripotent cells to generate large amounts of osteoblasts make ESCs an attractive source for bone engineering. Both *in vitro* and *in vivo* experiments have demonstrated the ability of ESCs to differentiate into osteoblasts that form bone.

Some approaches to drive ESC differentiation into osteoblasts lead to the formation of cell aggregates in non-adherent spheroids, called embryoid bodies. Embryoid bodies recapitulate many aspects of the embryo development including cellular signals and events, which will lead to differentiation of cells of the three germ layers: endoderm, mesoderm and ectoderm. This is similar to the process of gastrulation of an epiblast-stage embryo *in vivo* [14].

Through the initiation of embryoid bodies, Buttery *et al* demonstrated in 2001 that murine ESCs are able to differentiate into osteoblasts and form bone *in vitro* [15]. Later in 2004, Buttery's group reported *in vitro* differentiation and *in vivo* mineralization of osteogenic cells from human ESCs [16]. To selectively direct ES cells to differentiation towards osteoblast lineage, they used a differentiation medium containing ascorbate 2-phosphate, beta-glycerophosphate and dexamethasone. This differentiation method has been established to differentiate rodent and human primary osteoblasts. The authors particularly investigated the effect of the timing of dexamethasone supplementation on osteogenic differentiation. They observed that dexamethasone supplementation from day

14 until day 28 of the culture led to the largest amount of bone nodule formation. They assessed *in vitro* differentiation by assaying Alizarin red staining of mineralized bone nodules and Runx2 expression in the differentiated cultures. They further showed that these *in vitro* differentiated osteoblasts are viable and functional *in vivo* by seeding them onto a polymer scaffold and implanting them in severe combined immunodeficiency (SCID) mice. But other studies demonstrated that ESCs are able to form bone which includes bone lining cells and osteocyte [17, 18].

In order to produce a large source of multipotent progenitor cells that are able to differentiate into bone lineage, investigators have been trying to derive MSCs from ESCs before ESCs differentiate into lineage specific cell types [18-21]. These MSCs from ESCs share similar properties with BMSCs in term of their immunophenotype CD73+, STRO-1+ and CD45- [18]. These ESCs derived MSCs are able to differentiate towards osteoblast lineage *in vitro* and are capable of regenerating bone in calvarial defects [18]. In addition, ESCs can efficiently generate bone in an orthotopic bone defect model [17, 18].

Challenges for ESC-derived bone formation

While multiple lines of evidence including those discussed above suggest that ESCs can form bone *in vivo* [17, 18, 22], one report argued that ESCs failed to form functional bone *in vivo* [23]. The authors demonstrated [23] that, although human or mouse ES cells can form bone and osteoid via teratoma formation in SCID mice, they cannot do so via the MSC intermediate step as reported [22]. Some studies show that ESCs are capable of endochondral ossification if the cells receive chondrogenic stimulation before implantation [17]. Therefore, a reproducible protocol to ensure ESC differentiation into functional bone is needed.

The clinical application of ESC-derived tissues faces two major hurdles. One is the ethical debate over the use and destruction of human embryos for human ESC derivation [24]. Another is the concern of immune response to tissues generated by ESCs as they are usually allogenic to recipient patients [24]. The discovery of induced pluripotent stem (iPS) cells [25-27] has opened a potential avenue to autologous therapy by overcoming both hurdles. iPS cells are morphologically and functionally like ES cells, but derived via viral vector-mediated reprogramming of somatic cells such as patient's skin fibroblasts. New strategies have been reported to derive iPS cells by using virus- or DNA-free methods [28, 29].

Another challenges to apply ES or iPS cells for bone formation in patients is their tumorigenic potential. Both ES or iPS cells can form teratomas *in vivo* [30], because of their pluripotent characteristic. Therefore, it is critical to remove any remaining undifferentiated ES or iPS cells from their derivatives before implantation into patients [30]. Molecular imaging has been used to track implanted ESCs and detect early tumor formation in animals [30, 31]. Meanwhile, further research is needed to develop efficient methods to direct ES/iPS cells into therapeutically desired cell lineages such as osteoblast while eliminating any remaining pluripotent cells.

Bone regeneration using mesenchymal stem/stromal cells (MSCs)

Although pluripotent stem cells including ESCs and iPS cells have remarkable ability of self-renewal and potential to become a specific functional cell type, the issues regarding the ethics, immune response and tumorigenic potential need to be addressed before their application for bone regeneration in humans. In contrast, adult stem cells including mesenchymal stem/stromal cells (MSCs) can avoid the ethical issue and they can be autologous. MSCs are capable of differentiating into multiple cell lineages, including osteoblasts [32]. For bone regeneration, the most studied MSCs are bone marrow-derived mesenchymal stem/stromal cells (BMSCs) [5].

As early as 1987, Friedestein *et al.* [33] demonstrated the existence of fibroblast colony-forming cells or MSCs in the bone marrow. Later, Pittenger, *et al.* described in detail that BMSCs can differentiate into osteoblasts and other cell types [32]. In 2001, Quarto *et al.* reported the first clinical trial to repair large bone defects using autologous BMSCs [34].

Isolation and characterization of BMSCs

The traditional method to isolate BMSCs is to collect the low-density mononuclear cell fraction from bone marrow aspirates based on the

higher adherence of the mononuclear cells to the plastic surface than the hematopoietic cells [35]. BMSCs isolated via this method are heterogeneous, containing both stem cells and progenitor cells. The current standard for the stemness of BMSCs is to examine their ability to form ectopic bone and bone marrow in SCID mice [36]. Around 30% of the colonies isolated by the adherence method are truly stem cells and the rest are other cells with varying proliferation and differentiation potentials [37].

To better purify and identify MSCs, investigators have searched for a cell-surface antigen profile. However, so far there is no definitive and exclusive marker for MSCs, although there is a myriad of reported positive markers. Researchers often use different sets of markers to identify MSCs [38]. Currently, the best-known human MSC marker is Stro-1 [38]. Stro-1 positive cells are capable of differentiating into multiple mesenchymal lineages including osteoblasts, adipocyte, chondryocytes, smooth muscle cells and hematopoetic-supporting fibroblasts [39]. Consistent with this finding, Stro-1 negative cells are not capable to form colony forming units [38]. However, we cannot use Stro-1 as an exclusive marker for MSCs as expression of stro-1 was identified in some adipose tissue and blood vessels [40]. In addition, MSCs lose Stro-1 expression during culture expansion [38], and Stro -1 does not have its mouse counterpart. Future studies are needed to address whether Stro-1 negative MSCs have already lost the multipotential of differentiation. Researchers now often define and isolate MSCs by using Stro-1 together with some other positive markers, e.g., CD106 (vascular cell adhesion molecule-1), CD73 (lymphocyte-vascular adhesion protein 2) [38] and negative markers, e.g., CD11b (an immune cell marker), glycophorin-A (an erythroid lineage marker), and CD45 (a marker for all hematopoietic cells) [38].

Favorable characteristics of human BMSCs for bone generation

First, BMSCs can be easily collected from human body by using a relatively simple aspiration procedure [3]. Second, BMSCs can be easily expanded *in vitro* while maintaining genetic stability. Third, human BMSCs are less immunogenic as they do not express co-stimulatory molecules, express low levels of major histocompatibility complex (MHC) class II molecules and intermediate levels of MHC class I molecules [41, 42]. Thus, BMSCs may be feasible for allogenic transplantation.

Animal studies on bone generation from BMSCs

Many studies have proved that BMSCs are capable of differentiating into osteoblasts, and produce bone matrix, and repair bone defects in rodent models [22, 43-50]. For example, Mendes, *et al.* demonstrated *in vivo* bone formation using human BMSCs in nude mice [45]. They loaded human BMSCs into porous particles of calcium phosphate material. After a period of one week of *in vitro* culture, they implanted the constructs subcutaneously into nude mice. After 4 weeks, they found new bone formation using histology analysis [45]. In the newly formed bone, they found osteocytes embedded in the bone matrix, and bone marrow containing blood vessels, hematopoietic cells, and fat [45].

The majority of studies have shown that BMSCs can generate bone tissue by direct osteoblastic differentiation, a process resembling intramembranous ossification. However, most bones are formed through endochondral ossification by remodeling hypertrophic cartilaginous templates. Scientists reported endochondral bone formation using chick embryonic mesenchymal cells [51] and human adult BMSCs [46]. Scotti, *et al.* also recapitulated endochondral bone formation using human BMSCs [46]. Only when the BMSCs develop into hypertrophic cartilaginous tissue structures, can they form endochondral bone tissue [46]. The authors also compared bone-forming capacity of the early and advanced maturation of hypertrophic cartilage tissues formed by human BMSCs. They found that late hypertrophic cartilage tissues result in accelerated bone formation as revealed by quantitative microtomography analysis. Furthermore, the investigators demonstrated the presence of the delivered human BMSCs within the newly formed bone tissue by testing human Alu repeat sequences. However, future studies need to address whether the implanted BMSCs or the recruited host cells induce the endochondral ossifications of the new bone.

Clinical studies on bone generation from BMSCs

To date, more than 300 articles [3] on bone generation using stem cells in animal models have been published. As early as 2001, Quarto

et al reported the first clinical trial of repair of large bone defects using autologous bone marrow stromal/stem cells [34]. However, as of today, there are only a few studies (around 10 papers) in humans [3, 5, 35].

Challenges and future directions for bone generation using BMSCs

Despite the great potential of BMSCs in bone generation, there are limitations for their clinical application. First, in human bone marrow, BMSCs are a rare population of clonogenic non-hematopoietic stem cells, approximately 1 in 100,000 of the bone marrow mononuclear cells [35]. Second, bone marrow aspiration causes pain in the donor sites. Therefore, searching for MSCs from other sources are necessary. Adipose tissue stem cells represent an attractive source because of their osteogenic potential [52, 53] and relative abundance (1% to 5% of isolated nucleated cells) [54]. Third, the osteogenic potential of BMSCs declines substantially in the elderly people [55-57]. Lastly, BMSCs have senescent features including limited self-renewal and gradual loss of the osteogenic potential [58]. It has been shown that ectopic expression of telomerase in BMSCs can extend their life-span and maintain their osteogenic potential [59, 60]. However, it is important to address how to efficiently express the transgene without using viral vectors and assure the elimination of the tumorigenic potential of BMSCs [28, 61, 62].

Summary and future directions

Overall, great progress has been made in the field of bone regeneration. ESCs, iPS cells and BMSCs are promising cell sources for bone regeneration. ESCs and iPS cells have a great advantage of unlimited division and pluripotency. Multiple lines of evidence support that ESCs and iPS can form bone *in vivo*. However, a reproducible protocol to ensure ESCs to differentiate into functional bone needs to be developed. In addition, ESCs studies need to overcome the ethical issues, immune response and tumorgenic potential. In contrast, BMSCs can avoid the ethical issues and they can be autologous. Further, scientists have developed protocols to differentiate BMSCs into osteoblasts faithfully. The disadvantage of BMSCs is their limited available cell number for therapy. In addition, BMSCs from elderly patient lose osteogenic potential substantially. Several strategies have been proposed to overcome the disadvantage of ESCs and BMSCs. One strategy is to develop a protocol to differentiate ESCs into osteoblasts faithfully. Since non-differentiated ESCs have the tumorigenic potential, it is critical to eliminate the non-differentiated ESCs from the differentiated MSCs. One study enriched MSCs differentiated from ESCs by fluorescent-activated cell sorting for alkaline phosphatase expressing cells [18]. However, alkaline phosphatase is not specific to MSCs, as ESCs also express alkaline phosphatase. Although many cell surface markers are utilized to isolate BMSCs, these markers are not specific to BMSCs. Therefore specific markers for BMSCs need to be identified. One strategy to increase the osteogenic potential of BMSCs is to express genes including telemorase, and future strategies need to accomplish this goal without using viral vectors to eliminate tumorigenic potential.

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