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Cell Sources for Bone Tissue Engineering: Insights from Basic Science

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One of the goals of bone tissue engineering is to design delivery methods for skeletal stem/progenitor cells to repair or replace bone. Although the materials used to retain cells play a central role in the quality of the constructs, the source of cells is key for bone regeneration. Bone marrow is the most common cell source, but other tissues are now being explored, such as the periosteum, fat, muscle, cord blood, and embryonic or induced pluripotent stem cells. The therapeutic effect of exogenous stem/progenitor cells is accepted, yet their contribution to bone repair is not well defined. The *in vitro* osteo- and/or chondrogenic potential of these skeletal progenitors do not necessarily predict their differentiation potential *in vivo* and their function may be affected by their ability to home correctly to bone. This review provides an overview of animal models used to test the efficacy of cell-based approaches. We examine the mechanisms of endogenous cell recruitment during bone repair and compare the role of local versus systemic cell recruitment. We discuss how the normal repair process can help define efficacious cell sources for bone tissue engineering and improve their methods of delivery.

Introduction

Bone repair is a dynamic process beginning with the recruitment of skeletal stem/progenitor cells during the inflammatory phase of repair, followed by cell differentiation, extracellular matrix deposition, and remodeling. In human, bone repair occurs spontaneously, providing that the fractures are properly reduced. Surgical methods employed to realign and stabilize bone ends are the central component of orthopedic interventions. In 10% of all fractures, however, delayed or impaired healing requires additional treatment.[1] Electrical stimulation and ultrasound can be beneficial, but more robust stimulation of bone formation is necessary when facing trauma or fractures associated with age or other disease conditions such as diabetes.[2–6] Bone morphogenetic proteins (BMPs) are strong bone inducers that were discovered based on the osteoinduction of bone and were approved to augment bone formation in spine fusion and tibial nonunion in 2001.[7–9] Other treatments are now in use or in trial, such as WNT pathway regulators, parathyroid hormone, statins, and prostaglandin agonists.[10–14] In parallel, the demand for new cell-based therapies is growing. The need for additional sources of cells is particularly evident for severe trauma cases, cancer treatment, and maxillofacial reconstructive surgery when large bone defects cannot be filled solely with artificial scaffolds or autografts. Skeletal developmental diseases, such as osteogenesis imperfecta, and degenerative diseases, such as osteoporosis, are associated with poor bone quality and could also benefit from cell-based therapy.

The majority of bone tissue engineering approaches take advantage of bone marrow-derived cells that are easily accessible and have been extensively described in the literature. These cells can differentiate into chondrocytes and osteoblasts *in vitro* and appear as an ideal autologous cell type.[15–19] Other autologous cell types are similarly attractive, such as adipose-derived cells, which are also very accessible, and exhibit osteogenic and chondrogenic potential *in vitro*. Less is known about the *in vivo* potential of these cells in an orthopedic setting. This review describes the origins of skeletal progenitors during bone repair and highlights a number of animal models that have been developed to test the therapeutic effects of skeletal stem/progenitor cells with the emphasis on the fate of cells once transplanted at the bone repair site.

Systemic Recruitment of Cells During Bone Repair

Cell-based therapies target primarily the early stages of bone repair when the recruitment of skeletal progenitors may be impaired. The challenge in making these therapies more efficient is to identify the cell sources that can be implanted or attracted to the bone injury site and will differentiate into osteoblasts and chondrocytes. It is generally accepted that bone repair relies on endogenous skeletal stem/progenitor cells derived from multiple sources, both local and systemic. These cells may come from the bone marrow, periosteum, and surrounding soft tissues, as well as from distant sites, and carried to the fracture site by blood vessels that invade the callus. In the absence of molecular markers to trace skeletal stem cells *in vivo*, several strategies

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have been used to elucidate the origins of skeletal progenitors that support the formation of the fracture callus.

Many efforts have concentrated on the identification of systemic cell sources. The existence of circulating osteoblast precursors suggested a possible recruitment of these cells in response to bone injury.[20,21] In the study by Kumagai *et al.*, the parabiosis mouse model was used to show that circulating cells were mobilized to the fracture site.[22] Although cells brought by blood vessels expressed the osteoblast marker alkaline phosphate, they were found as bone-lining cells, but did not integrate within new bone as osteocytes. Whether these circulating cells can produce new bone matrix or support repair via producing osteoinductive factors is not clear. Blood vessels carry various progenitor cells including endothelial progenitors that are found at higher numbers in the circulation following an injury and that can stimulate repair, but there is no *in vivo* evidence yet for their direct contribution to repair as skeletal progenitors.[23] Other cell types associated with blood vessels, such as pericytes, may play an important role in bone repair. As pericytes are closely associated with vessels in every tissue, they could either be brought from distant organs to the site of injury or be activated locally.[24]

Bone marrow is recognized as a source of skeletal progenitors that can be brought systemically to the injury site via blood vessels. To trace bone marrow-derived cells during bone repair, Taguchi *et al.* transplanted GFP bone marrow into wild-type mice and identified GFP-expressing cells at the bone surface in the fracture callus. Similar to the parabiosis model, donor cells did not incorporate within the new bone as osteocytes.[25] When combining parabiosis and bone marrow transplantation, circulating bone marrow-derived cells were also recruited at sites of ectopic bone formation, where they line the new bone.[26] The exact role of these bone-lining cells remains to be determined. Another lineage-tracing study using Rosa26 donor mice for bone marrow transplantation did not reveal a contribution of donor bone marrow to cartilage and bone within the callus.[27] Donor bone marrow gave rise to inflammatory cells and osteoclasts at the fracture site, pointing out the role of bone marrow as a source of cells within the hematopoietic lineage.[28,29] Consequently, bone marrow transplantation can compensate for defects in inflammation and bone remodeling during bone repair, but cannot compensate for defects that are intrinsic to cartilage and/or bone.[30,31]

Nonetheless, bone marrow also contains nonhematopoietic cells that have been characterized as mesenchymal stem cells (MSCs), which are now widely used for tissue engineering approaches.[32] After bone marrow transplantation, MSCs remain of host origin and are not maintained long term, which explains the poor osteogenic potential of bone marrow transplants for bone repair.[33,34] Granero-Molto *et al.* carried out systemic transplantation of bone marrow-derived MSCs (BMSCs) in a mouse model by injecting the cells intravenously.[35] Via bioluminescence imaging, transplanted BMSCs were detected at the fracture site by 3 days postinjury. BMSCs were marked genetically and located within bone marrow and at the endosteal surface of bone by histological analyses. BMSCs that did not express C-X-C chemokine receptor type 4 (CXCR4) at their surface were not found within the callus, demonstrating the role of CXCR4 in BMSCs homing to the injury site. Although BMSCs can differentiate into osteoblasts and chondrocytes *in vitro*,[15,36–38] they do not participate in cartilage and bone formation in the fracture callus.[35] Thus, BMSCs do not spontaneously stimulate repair by providing a source of skeletal progenitors when recruited systemically.

Other reports have shown that when BMSCs were manipulated *in vitro* before transplantation, they could be recruited systemically and integrate into the bone matrix. In the study by Shirley *et al.*, bone marrow cells were cultured in osteogenic conditions and transplanted into a remote bone marrow site. Following osteotomy, donor cells were mobilized from the distant bone marrow site and localized to the callus. Some of these cells integrated in the new bone as osteocytes.[39] Another study used genetically labeled stromal cells that were injected into the blood circulation.[40] Following fracture, stromal cells were recruited systemically to the callus where they integrated in bone matrix and localized mostly at the bone surface.

The characterization of skeletal stem/progenitor cell populations within bone marrow is the object of active research. However, given the heterogeneity of the bone marrow and the multiple molecular markers identified so far, it is unclear whether bone marrow actually contains an endogenous group of skeletal stem cells that can be recruited systemically during bone repair to form cartilage and/or bone.[41–43] The difficulty comes from the small percentage of MSCs within the bone marrow and their close association with cells from the hematopoietic lineage.[44] Further *in vivo* analyses are required to better understand BMSCs' normal physiological functions. The work by Sacchetti *et al.* identified a molecular marker for BMSCs that can reconstitute hematopoietic and skeletal lineages both *in vitro* and *in vivo*.[45] The role of these stem cells in bone repair remains to be characterized.

Local Recruitment of Cells During Bone Repair

As systemic recruitment of skeletal progenitors during normal bone repair appears to be minimal, the recruitment of skeletal progenitors within the local environment of bone is presumably predominant. These skeletal progenitors may come from the bone marrow within the injured bone, from the surrounding periosteum, and from soft tissues in close proximity with the bone. All these tissues are closely linked, which makes it difficult to distinguish their participation during bone regeneration. In addition, once osteoblast precursors start differentiating, they express key osteogenic factors that are lineage specific, but not tissue specific, making it even more difficult to distinguish the specific roles of the various local cell sources. The periosteum is known for its key role in the endogenous repair process.[46–51] Cauterizing the periosteum from the surface of the bone delays healing, suggesting the presence of a key cell source.[52–54] Periosteum activation following injury is very localized and coincides with a local increase in cell proliferation.[55] Simultaneously, fundamental changes can be observed in the periosteal vasculature characterized by increased populations of endothelial cells and pericytes and transformed mesenchymal cells.[56] Using an Osterix-GFP mouse line, Maes *et al.* showed that osteoblast precursors within the periosteum were also found in close association with invading blood vessels within the fracture callus, suggesting that osteoblast precursors within the periosteum locally

migrate along with blood vessels to contribute to new bone within the callus.[57]

To circumvent the lack of tissue-specific Cre lines for skeletal lineage analyses, bone transplantation approaches have been developed. Live bone grafts from Rosa26 mice containing periosteum were transplanted at the fracture site.[47] During repair, periosteum gave rise to chondrocytes, osteoblasts, and osteocytes adjacent to the graft, but not at a distance from the graft. Therefore, skeletal stem/progenitor cells derived from the periosteum did not migrate from distant sites to form the fracture callus. Using the same approach, endosteum was shown to contribute locally to osteoblasts and osteocytes, but not chondrocytes. Bone marrow including cells of the endosteal surface primarily gave rise to osteoblasts and osteocytes adjacent to the graft. These results showed that the local periosteum is a major contributor to cartilage and bone in the fracture callus and that endosteum and bone marrow also contribute to bone within the bone marrow cavity. The role of the local periosteum as a cell source was also illustrated in a murine segmental graft model.[49,58] This model showed that bone graft devitalization decreased graft survival and integration because of the absence of local periosteal cells within the transplanted bone.[48] Besides bone marrow and periosteum, the role of other local sources of cells has not been yet addressed *in vivo*. Skeletal stem/progenitors cells have been isolated from fat, muscle, and tendon.[59–61] These cells can differentiate into osteoblasts and chondrocytes *in vitro*, but their participation in the normal repair process has not been demonstrated. Nevertheless, they have been tested as exogenous sources of cells for bone tissue engineering.

Main Cell Sources for Bone Tissue Engineering

Various sources of stem cells are now being employed for bone tissue engineering (Table 1). Some of these cell sources normally contribute to bone repair (bone marrow, periosteum), whereas others may or may not participate in repair (fat and muscle). Stem cells that normally would not participate in adult tissue repair, such as embryonic stem cells (ESCs), induced-pluripotent stem cells (iPSCs), and cord blood cells, have also been investigated.[62–65] The therapeutic potential of skeletal stem cells is usually defined by their ability to differentiate into osteoblasts and/or chondrocytes *in vitro*. However, these *in vitro* assays as well as *in vivo* assays such as subcutaneous transplantation or ectopic bone formation in muscle may not reflect the fate of the cells during bone repair (Table 1). The difficulty is to prompt skeletal stem/progenitor cells to integrate into the fracture callus or the bone defect and to differentiate toward the chondrogenic and osteogenic pathways *in situ*. For cell-based therapies, a number of animal models have been developed, including stabilized and nonstabilized fractures, distraction osteogenesis, segmental defects, cortical defects and calvarial defects, implant osseointegration, bone grafting, and bone transplantation.[39,47,58,66–72]

BMSCs are the most used both clinically and experimentally with various degrees of success depending on the method of delivery as discussed in the next section. BMSCs alone are not very effective as exogenous osteoblast progenitors, and their capacities decrease with age.[73,74] BMSCs are easy to collect compared with other adult stem cells, but there are risks associated with the collection of autologous BMSCs because of donor site morbidity.[75] The regenerative potential of periosteum-derived cells is high and these cells directly contribute to cartilage and bone.[48,76–78] However, periosteum-derived cells cannot be easily harvested. Methods to purify and expand skeletal stem cells from the periosteum would be valuable. Approaches to reconstitute a live periosteum *in vitro* by combining a structural bone allograft with BMSCs have been proposed, but the ability of BMSCs to home to a periosteal niche has not been yet demonstrated.[79]

Table 1. Contribution of Endogenous and Exogenous Cell Sources to Orthotopic and Heterotopic Bone Induction *In Vivo*

	Systemic recruitment		*Local recruitment*	
Endogenous cell sources	*Orthotopic*	*Heterotopic*	*Orthotopic*	*Heterotopic*
Bone marrow	Bone-lining cells[22,25] Osteoclasts[22,27,31] Inflammatory cells[27,30]	Bone-lining cells[26]	Osteoblasts/osteocytes within bone marrow[47] Chondrocytes (weak)[47]	N/A
Periosteum	ND	ND	Osteoblasts, osteocytes, chondrocytes within the callus[46–49,57]	ND

	Systemic delivery		*Local delivery*	
Exogenous cell sources	*Orthotopic*	*Heterotopic*	*Orthotopic*	*Heterotopic*
Bone marrow	Bone-lining cells[35] Oseoblasts, osteocytes[39,40,98]	Bone-lining cells[45] Osteoblasts, osteocytes[45]	Bone-lining cells[77,79] Osteoblasts, osteocytes[60,77,79,110,111]	Bone-lining cells[60] Osteoblasts, osteocytes[60]
Periosteum	ND	ND	Osteoblasts, osteocytes[47,48,77]	Osteoblasts, osteocytes[78]
Fat	ND	ND	ND	Osteoblasts, chondrocytes[61]
Muscle	ND	ND	Osteoblasts, osteocytes[82–84]	Osteoblasts, chondrocytes[60]
Embryonic stem cells	ND	ND	Osteoblasts, osteocytes[89]	Osteoblasts[65]

ND, not determined; N/A, not applicable.

Cells derived from adipose tissue and muscle are more accessible than periosteum and can potentially serve for autologous transplants. Adipose-derived stem cells (ADSCs) have been expanded *in vitro* and tested *in vivo* for cartilage and bone formation.[80] When transplanted in muscle, ADSCs induce ectopic bone.[61] In a canine defect model, ADSCs did not have a significant effect on repair when transplanted locally even after osteogenic differentiation. ADSCs can augment bone regeneration after genetic modification to overexpress BMP2, but their contribution to bone is unclear.[81] Similarly, muscle-derived stem cells (MDSCs) gave better results when expressing BMP4.[72,82] Shen *et al.* used genetic markers to follow transplanted cells in a critical-size defect. MDSCs could still be found in the repair site at 3 weeks postsurgery, but were mostly gone by 4 weeks, although some of the cells appeared to differentiate into osteoblasts in the new bone.[83,84] Thus, MDSCs and ADSCs act mainly as carriers, producing osteogenic factors to recruit endogenous cells. Although MDSCs and ADSCs can differentiate into osteoblasts and chondrocytes *in vitro*, their *in vivo* osteogenic potential is weak. Are these adipose- or muscle-derived MSCs with osteogenic potential real stem cells or tissue-specific progenitors, which can be driven toward skeletal lineages *in vitro*? MSCs have been described in many adult tissues and may have distinct origins. Local pericytes associated with blood vessels could serve as a local reservoir of cells for tissue repair.[85,86] Whether any tissue-specific MSCs can be efficiently exploited to repair bone will require further investigation.

Instead of adult stem/progenitor cells, less mature cells isolated from fetal bone marrow, cord blood cells, ESCs, and even iPSCs have been considered more recently.[64,87] These cells can all be induced into osteoblasts or chondrocytes *in vitro*. In the study by Zhang *et al.*, human fetal MSCs (hfMSCs) were shown to augment healing of rat critical-sized defects via stimulating vascularization.[88] Although more primitive than adult MSCs, hfMSCs did not exhibit higher osteogenic capacities *in vivo* even after *in vitro* osteogenic priming. Cells filled the defect by 4 days, but vanished after 4 weeks. New bone was produced by endogenous cells in the defects as shown by the lack of human-specific osteopontin expression. Human ESCs are osteogenic in subcutaneous bone formation assays and calvaria defects.[65,89] iPSCs have been tested for periodontal tissue regeneration, but the origin of the bone-forming cells was not verified.[90] Further *in vivo* assays will need to prove the effectiveness of cord blood cells, ESCs, and iPSCs in bone repair in correlation with their cellular contribution.[91]

Systemic Versus Local Delivery of Cells for Bone Repair

Regardless of the source of cells, the method of delivery can affect the regenerative potential of transplanted cells. As discussed earlier, systemic recruitment of skeletal progenitors is minimal compared with local recruitment in the course of endogenous repair. Therefore, systemic delivery may not be the ideal route to direct exogenous skeletal progenitors toward osteogenesis and chondrogenesis in a bone repair site. The study by Granero-Molto *et al.* showed that BMSCs injected intravenously do not give rise to osteoblasts within the callus. BMSCs stimulated repair via expression of BMP2 and by decreasing the expression of key inflammatory factors such as TNFalpha, IL1beta, and other interleukins, suggesting that BMSCs may have systemic anti-inflammatory effects.[35] MSCs were previously known for their immunosuppressive effects.[92,93] This immunomodulatory effect of BMSCs correlates with the previously reported role of bone marrow stromal cells and osteoblasts in regulating hematopoietic stem cells.[94,95] Future research may find ways to control homing of stem cells to the correct niche and allow their long-term integration.[34,96] One major advantage of systemic delivery of cells is that it is more practical, no surgical intervention is needed, and cells could potentially be injected at multiple time points after injury. Moreover, systemic delivery could also benefit other bone disorders affecting the whole body.[97,98]

A number of strategies have been used to deliver cells locally and support bone formation. Percutaneous injection of autogenous bone marrow can be beneficial for the treatment of tibial nonunions and congenital pseudoarthrosis.[99–101] A clinical trial showed successful treatment of nonunion fractures with direct injection of concentrated bone marrow cells.[102] Interestingly, there was a positive correlation between the volume of mineralized callus at 4 months and the number and concentration of fibroblast colony-forming units in the bone marrow graft, suggesting that the number of stem cells was proportional to the extent of osteogenic stimulation. Compared with bone marrow aspirates, ilial crests contain more osteogenic progenitors and are the gold standard for many orthopedic trauma applications.[75] The presence of trabecular osteoblasts may also increase the osteogenic potential of these iliac crest autografts. It is assumed that autografts provide osteoblasts and/or osteoblast precursors, but their direct contribution to repair is difficult to assess in human. The identification of molecular markers for clonogenic skeletal progenitors within the bone marrow stroma may help further enrich these cell populations before transplantation.[45]

To augment the therapeutic effects of bone marrow cells delivered locally, many efforts are focused on the design of scaffolds to create a biocompatible environment and to provide a surface for cell adhesion and migration. The classical orthopedic carriers include allogeneic bone, demineralized bone matrix, and various bone graft substitutes such as hydroxyapatite and calcium phosphate. New scaffolds are now being developed using nanotechnologies to combine nanofiber mesh with biocompatible carriers such as hydrogels.[103–105] The design of these new scaffolds aims to protect cells from the inflammatory environment and cell death. *In vivo*, cells need blood vessels to receive oxygen, nutrients, and the proper signals to proliferate and differentiate. Following transplantation, cells are exposed to hypoxia until the construct can be properly vascularized by the host; therefore, methods to protect cells from apoptosis are essential.[106,107] Preconditioning in bioreactors may reinforce cell integration within artificial scaffolds before transplantation.[108,109] Predifferentiation in the osteogenic pathway can also be employed, but it decreases the capacity for expansion. Thus far, the most robust donor contribution to bone has been reported in calvaria defect models, wherein inflammatory and mechanical signals may be reduced and cell retention may be favored.[110,111] In long bones, the challenge is to provide early structural support, while allowing cell survival, proliferation,

and osteogenic differentiation followed by the timely resorption of the scaffold to support matrix deposition. Another approach aimed at supporting cell survival and stimulating MSC-induced bone is coculture of MSCs with endothelial cells. Although healing was improved, an increase in the vascularisation of the construct or in the osteogenic differentiation of MSCs was not determined.[112]

Growth factors added exogenously or through genetic manipulation can stimulate angiogenesis and osteogenic differentiation. Indeed, in most animal models, purified BMSCs loaded onto various materials give better results when combined with exogenous growth factors. Burastero *et al.* showed that bone repair was enhanced in a rat critical-sized defect when BMP7 was added to hBMSCs.[69] In another animal study, BMSCs were transfected with an adenovirus vector to overexpress BMP2, leading to improved union of a mouse critical-sized defect.[70] Genetic modification can be a powerful method to drive osteogenesis via either expression of BMPs, key transcription factors, and/or angiogenic factors.[113] Although these *ex vivo* genetic manipulation aim to further enhance the regenerative potential of cells, they may also induce major alterations of their inherent osteogenic capacity. These approaches are still in their infancy as new vectors are required to prevent tumorigenesis that may be due to insertional mutations.[64] New vectors have been developed and nonviral approaches, such as gene activated matrices, are also promising.[114]

Conclusions

When considering biological methods to stimulate bone formation and repair, several strategies may be envisioned. The ideal treatment for skeletal diseases or trauma would be minimally invasive and would stimulate the endogenous machinery, such as utilization of growth factors or small molecules. However, when endogenous cell sources are compromised or cannot be activated because of disease conditions, cell-based therapies may be necessary. The source of cells and route of delivery are two considerations that are still being addressed experimentally. Autologous cells can prevent immune rejection but require time to collect and expand. Although allogeneic cells allow the preparation of off-the-shelf products, the downside is the likelihood of immune rejection. So far, BMSCs are the most appealing, but other adult stem cells and ESCs are being explored. All exhibit *in vitro* osteogenic and chondrogenic potential, but need to be more effectively osteogenic *in vivo*. When delivered systemically, MSCs home to the injury site but contribute modestly to bone-forming osteoblasts and osteocytes. MSCs stimulate repair mostly by modulating inflammation and by creating a microenvironment to recruit endogenous stem/progenitor cells, via their "trophic" activity (i.e., expression of BMPs and other factors).[93] Future research may help control homing of MSCs to allow their integration within osteogenic niches that are mobilized for bone repair.[115] Local delivery of cells may be more efficient to deliver skeletal progenitors for bone repair. However, in many approaches, proof of the origins of the cells is often lacking and it is generally assumed that the ectopic bone is derived from the transplanted cells. Several studies have illustrated the weak osteogenic potential of BMSCs, MDSCs, or ADSCs seeded on specific scaffolds to concentrate the cells at the repair site. Even in these conditions, cells are more effective when combined with growth factors or used as a vehicle for growth factor delivery. To augment the direct contribution of exogenous cells to bone formation, a great deal of work is still required to define the ideal cell source(s) and identify methods of purification and ways to direct/stimulate stem cell differentiation *in vivo*. For this purpose, the development of new scaffolds is essential. Tissue engineering will also largely benefit from new ways to track cells *in vivo* either experimentally or for clinical applications using genetic methods or nanotechnologies.[116] Bringing together tissue engineering, stem cell biology, and developmental and regenerative biology will lead to better exploit and stimulate the normal regenerative process. We still do not fully comprehend the mechanisms of endogenous stem cell activation during normal bone repair and how they are affected in disease conditions. Consequently, new strategies for bone tissue engineering will benefit from advances in the basic cell biology of bone repair.

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