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Stromal cells and stem cells in clinical bone regeneration

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Abstract

Stem-cell-mediated bone repair has been used in clinical trials for the regeneration of large craniomaxillofacial defects, to slow the process of bone degeneration in patients with osteonecrosis of the femoral head and for prophylactic treatment of distal tibial fractures. Successful regenerative outcomes in these investigations have provided a solid foundation for wider use of stromal cells in skeletal repair therapy. However, employing stromal cells to facilitate or enhance bone repair is far from being adopted into clinical practice. Scientific, technical, practical and regulatory obstacles prevent the widespread therapeutic use of stromal cells. Ironically, one of the major challenges lies in the limited understanding of the mechanisms via which transplanted cells mediate regeneration. Animal models have been used to provide insight, but these models largely fail to reproduce the nuances of human diseases and bone defects. Consequently, the development of targeted approaches to optimize cell-mediated outcomes is difficult. In this Review, we highlight the successes and challenges reported in several clinical trials that involved the use of bone-marrow-derived mesenchymal or adipose-tissue-derived stromal cells. We identify several obstacles blocking the mainstream use of stromal cells to enhance skeletal repair and highlight technological innovations or areas in which novel techniques might be particularly fruitful in continuing to advance the field of skeletal regenerative medicine.

Introduction

Bone has an innate propensity to regenerate following traumatic injury. Upon fracture, resident stromal, stem and progenitor cells work in tandem with pro-inflammatory and anti-inflammatory macrophages^{1,2} and circulating blood cells³ to orchestrate a complex signalling cascade that leads to scarless healing.⁴ In spite of this tremendous capability, a number of clinical indications remain that require therapeutic intervention to facilitate bone repair and regeneration. Autologous bone grafting, in which bone from another part of the

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Competing interests

J.M.G. is co-founder, co-owner and Chief Scientific Officer of LaCell, a biotechnology company focusing on the clinical translation of stromal-cell and stem-cell science. The other authors declare no competing interests.

Author contributions

W.L.G., B.P.H. and J.M.G. researched data for the article and wrote the article. W.L.G., B.A.B., E.M., T.F. and J.M.G. made substantial contributions to discussions of the content. All authors reviewed and/or edited the manuscript before submission.

body is transplanted to the defect site, remains the gold standard; however, this approach is associated with numerous drawbacks, including donor-site morbidity, the availability of limited grafting material and compromised bone quality in patients with osteoporosis.⁵ Bone-tissue engineering (BTE) has been developed as a potential alternative to overcome the critical shortcomings associated with autografts and allografts. In general, BTE involves the use of various combinations of cells, growth factors and/or cytokines, and bioactive carriers (scaffolds and/or hydrogels). Even though it has been ~30 years since the first efforts in this area,⁶ few BTE techniques have translated into clinical practice and none of them has become the standard of care in regenerative medicine.

This Review focuses specifically on the successes and challenges of using stromal or stem cells in the clinical translation of BTE techniques. Some controversy remains over the specification of adipose-tissue-derived and bone-marrow-derived progenitors as stem cells. Although the authors consider that each of the two descriptions has merits, these cells will be referred to in the remainder of this Review as stromal cells. Currently, the role of transplanted stromal cells in mediating regeneration remains poorly understood, particularly in the clinical trials that have been conducted. The original premise of many early *in vitro* and preclinical studies was that transplanted cells would undergo differentiation and morphogenesis to form the regenerated tissue; however, this paradigm has been challenged by experimental findings documenting that very few regenerative cells actually survive following transplantation.⁷ In spite of the clear benefits associated with cell delivery, the poor mechanistic understanding of stem-cell-mediated regeneration is an obstacle to optimizing regenerative approaches. Animal models have the potential to provide some insight; however, many of the available models do not effectively recapitulate the clinical situation, which is either due to the size of the defects or the timing of cell delivery relative to when the defect was created. In addition to the lack of mechanistic insight, logistical, regulatory and technical challenges continue to limit the clinical application of stromal and stem cells for skeletal regeneration. In this Review, we briefly discuss the history of stromal cells, their use in clinical trials, the challenges facing their widespread implementation and current approaches to bone regeneration that are based on stromal and stem cells. This Review also highlights novel technologies and future studies that are needed to establish stromal-cell-mediated and stem-cell-mediated BTE as a standard component of clinical care.

Stromal cells

Historical and developmental relationships

Pioneering reports in the 1960s by Alexander Friedenstein and colleagues at the University of Moscow laid the foundations for the modern era of multipotent-stromal-cell and mesenchymal-stem-cell (MSC) research.^{8–10} Friedenstein's team was the first to demonstrate that bone marrow contains fibroblast-like stromal cells, termed mechanocytes, which are capable of osteogenic differentiation and are necessary for the creation of the haematopoietic microenvironment or niche.⁸ Additionally, the researchers demonstrated that similar cell populations are present in the thymus, liver and other organs. These findings were advanced in the 1980s by the demonstration that primary cultures of bone-marrow-derived stromal cells are adipogenic and chondrogenic.^{11,12} During the same period,

techniques for the long-term culture of primary bone marrow cells were developed.^{13,14} Adherent stromal-cell populations, which are required to support proliferation and differentiation of haematopoietic progenitors and stem cells, were also shown to be capable of mediating adipogenesis in the presence of glucocorticoid-containing horse serum.¹⁵ In pursuit of these findings, haematologists across the globe developed clonal cell lines from adherent stromal cells in long-term cultures, which were isolated from bone marrow, spleen, liver and other tissues capable of supporting B-cell lymphopoiesis.^{16–18} When co-cultured in the presence of stromal cell clones, haematopoietic stem cells and progenitor cells (which routinely died in culture) were able to proliferate and differentiate as a result of the release of as yet unidentified growth factors. In many cases, stromal-cell clones went on to be used as critical reagents for the isolation and characterization of haematopoietic cytokines, such as IL-7 and IL-11.^{19–21}

The stromal-cell field was advanced in the early 1990s by the adoption of the term ‘mesenchymal stem cells’ to classify the adherent bone marrow cells that are characterized by their ability to differentiate along the adipocyte, chondrocyte, osteoblast, skeletal myocyte and tenocyte pathways.^{22,23} Several research groups were among the first to generate monoclonal antibodies against the human bone-marrow-derived mesenchymal-stromal and stem-cell (MSC) surface antigens STRO-1, CD73 (which targets the Src homology [SH] 3 and SH4 domains) and CD105 (which targets the SH2 domain).^{24,25} Some researchers have argued that MSCs do not meet the scientific standards required to define them as stem cells, as no reports have documented their ability to be serially passaged through multiple recipients whilst retaining tissue-generating functionality. As a compromise, in 2006, the International Society for Cellular Therapy issued a consensus statement defining MSCs as ‘multipotent stromal cells’ on the basis of the following criteria: firstly, capability of plastic adherence and self-renewal in culture; secondly, staining positive for CD73, CD90 and CD105, and staining negative for CD11B or CD14, CD19 or CD79 α , CD34, CD45 and HLA-DR; and thirdly, differentiating along the adipocyte, chondrocyte and osteoblast pathways *in vitro*.²⁶ At present, these criteria have been used to define MSCs in the majority of the published literature; however, questions have been raised as to whether or not this *in vitro* evidence is sufficient for the characterization of MSCs as stem cells. The growing number of MSC filings at the FDA have employed an ever-widening array of surface antigenic biomarkers.²⁷ The inconsistency of these characterizations has been complicated by the fact that an increasing percentage of MSC products are derived from tissues other than bone.²⁷ The FDA consortium has suggested that additional bioactivity assays, which involve proteomic analysis of membrane proteins, and adipocyte differentiation be used to identify and define MSCs that are isolated from these various tissues.^{27–31} Some researchers have long advocated that the *in vivo* criteria should constitute the ‘gold standard’ for MSC definition.³² These researchers and others have documented the robust ability of bone marrow MSCs to form mineralized bone that contains a haematopoietic marrow after insertion on a hydroxyapatite or related scaffold and implantation subcutaneously in rats.³² Although the pharmaceutical and biotechnology industries continue to pursue *in vitro* surrogate assays, the *in vivo* assay of bone differentiation remains the definitive standard for many academic laboratories.³³

Dependence on tissue of origin

Multiple independent studies have isolated cells with MSC-like characteristics from amniotic, placental and umbilical-cord tissues, as well as adult adipose, dental, dermal and skeletal muscle tissues.^{34–43} Tissue-derived MSCs have been isolated and/or identified, in part, on the basis of their adherence properties to tissue culture surfaces and by flow cytometric sorting on the basis of the surface antigens they express.^{24,43–45} Perivascular cells isolated from multiple tissues on the basis of their expression of CD146, chondroitin sulphate proteoglycan 4 (commonly known as NG2) and platelet-derived growth factor receptor β display clonal multipotency and express mesenchymal markers, which suggests that stromal cells and pericytes are functionally equivalent.⁴⁶ A limited number of studies have directly compared stromal cells that were isolated from distinct tissues. Although failing to demonstrate substantial differences in immunophenotype or morphology, an initial comparison of cells that were isolated from adipose tissue, bone marrow and umbilical cord found a greater frequency of colony-forming units in adipose-tissue-derived cells than in bone-marrow-derived and umbilical-cord-derived cells and greater proliferative and inferior adipogenic capacities in umbilical-cord-derived cells than in adipose-tissue-derived and bone-marrow-derived cells.⁴² A later analysis determined that although adipose-tissue-derived stromal cells (ASCs) shared multiple features in common with their bone-marrow counterparts, ASCs display subtle differences in their immunophenotypic profile.⁴⁷ These differences have been encapsulated in a joint International Society for Cellular Therapy and International Federation for Adipose Therapeutics and Science consensus statement.⁴⁸ ASCs have demonstrated osteogenic potential *in vitro* and *in vivo* both in preclinical models and in clinical trials.^{34,49–55} *In vitro* studies have demonstrated that the extent of ASC osteogenesis can be enhanced by manipulating the concentrations of ascorbate and dexamethasone in cell-culture media.⁵⁶ Nevertheless, some studies have highlighted concerns that the osteogenic capacity of ASCs is significantly lower than that of MSCs isolated from bone.^{57,58} In spite of this concern, the ease of accessibility and relative abundance of ASCs in comparison to that of MSCs confer practical advantages and have contributed to the continued interest in the clinical use of these cells in bone regeneration.^{52,54,59} Whether or not the tissue of origin regulates the epigenetic memory of MSCs and their subsequent differentiation potential remains to be determined.⁶⁰

Skeletal-muscle-derived stem cells (MDSCs) are distinct from the resident satellite cells, which are stimulated upon muscle damage to repair the tissue. MDSCs, isolated from muscle tissues on the basis of expression of CD34 and apoptosis regulator Bcl-2, demonstrate robust osteogenic capacity and thus have potential for use in skeletal repair.⁶¹ Similarly to MSCs and ASCs, the MDSC population exhibits multipotency, can regenerate various tissues *in vivo* and can secrete a number of trophic factors that are capable of stimulating endogenous repair.^{62,63} However, as a therapy for bone defects, MDSCs lag behind ASCs and MSCs and they are not currently being investigated in clinical trials.

Skeletal regeneration

The field of skeletal regeneration continues to face multiple challenges that would potentially benefit from approaches that involve cell-based therapeutics. Of these

challenges, the most common is acute trauma, which accounts for the majority of orthopaedic surgical procedures in the USA and internationally. The body responds to trauma by initiating a cascade of inflammatory and regenerative events. Sequentially, these actions include local and systemic release of proinflammatory cytokines, homing of immune cells to the site of injury, soft-tissue inflammation and oedema, mobilization of osteogenic progenitor cells, local release of bone morphogenetic proteins, callus formation, bone remodelling and eventual bone replacement. A rationale exists for introducing exogenous MSCs during one or more of these events. Immediately following the acute injury (hours to days), MSCs can dampen or modulate local and systemic inflammatory responses by producing immunosuppressive factors, such as transforming growth factor β , prostaglandin E2 and indoleamine 2,3-dioxygenase 1 (commonly known as IDO). Additionally, the release of stromal cell-derived factor 1 (also known as SDF-1) and other cytokines from MSCs can alter the types of immune cells that are recruited to the site of injury. During intermediate periods (from days to weeks) following the injury, MSCs can contribute to the repair process by differentiating into chondrocytes and osteoblasts, thereby augmenting the recruitment of local endogenous osteoprogenitor cells. Although whether exogenous MSCs have a substantial benefit when introduced late (from weeks to months) following acute trauma remains to be determined, at least one instance in which late delivery of MSCs can be beneficial is delayed union or non-union of bone.

Non-union can occur in up to 15% of cases of complex trauma as a result of mechanical factors, as seen in comminuted fractures with multiple bone fragments; infection, as seen with bacterial contamination of the injury site or a patient's underlying viral diseases (for example, hepatitis and HIV); smoking and other tobacco-related or drug-related toxins; and endocrine disorders, such as type 2 diabetes mellitus, obesity, osteopenia and osteoporosis. Finally, exogenous MSCs have the potential to benefit treatment of bone-related tumours, such as Ewing sarcoma, osteosarcoma and metastatic bone disease. The introduction of a healthy exogenous MSC population might enable bone metabolism to recover more rapidly following chemotherapy, radiation and/or surgical ablation of the tumour by improving the local microenvironment. Additionally, MSCs that are genetically modified to deliver specific proteins, radioisotopes or microRNAs can be used as antitumour vectors owing to their ability to home to sites of active primary or meta-static cancers.^{64–71} Nevertheless, as MSCs can promote the proliferation of breast, prostate and other tumours *in vitro* and *in vivo*,^{72,73} preclinical safety studies need to be performed before using native or genetically modified MSCs in the context of bone tumours. The safety concerns regarding the use of ASCs or MSCs, which include tumour formation in bone regeneration applications, are significantly lower than those regarding the use of pluripotent stem cells (embryonic stem cells or induced pluripotent stem cells). To date, MSCs and ASCs have been used in a small number of studies for bone regeneration in humans (Table 1). In the majority of studies, autologous cells (with or without prior expansion *in vitro*) were either directly injected into the defect site or injected with the aid of biomaterial carriers. Owing to the immunoprivileged characteristics of ASCs and MSCs, a number of clinical trials are also currently being performed with genetic cells.

Clinical trials

Craniofacial bone regeneration

Ectopic vascularized bone formation—The treatment of large craniofacial defects presents unique challenges owing to the complex 3D geometry of bone. The current gold standard for the treatment of large bone defects is autologous bone transplantation, which can be vascularized or nonvascularized. In nonvascularized bone, the lack of vasculature can lead to graft resorption with resultant loss of the geometric structure of bone. To transplant vascularized, autologous bone, the surgeon has to painstakingly dissect out suitable portions of the patient's own iliac crest, fibula or ribs, shape them into an approximate anatomical shape and then use microsurgical techniques to restore the blood supply. This immediate supply of oxygen and nutrients is critical for graft survival and long-term integration. Consequently, one clinical approach to utilize BTE grafts incorporates an *in vivo* cultivation period. The grafts are implanted into large, highly vascularized muscle tissues (for example, latissimus dorsi⁷⁴ or rectus abdominis⁷⁵) for several months to facilitate vascular ingrowth and the development of a vascular pedicle suitable for microsurgical anastomosis. To achieve this vascular growth, a preshaped titanium mesh is used to enclose mineralized matrix (for example, autograft cancellous bone chips or xenograft bone blocks), osteoinductive recombinant human (rh) growth factors (such as rhBMP-2 or rhBMP-7) and cells (Figure 1). Both bone-marrow aspirates for mandibular reconstruction⁷⁴ and ASCs expanded using good manufacturing practice (GMP) standards to treat maxillary defects⁷⁵ have been successfully used to regenerate craniofacial bone with this methodology. The resulting bone had sufficient structural integrity to support dental implants 4 months following surgical reconstruction of the defect with the BTE graft.⁷⁵

In situ bone formation—In spite of the considerable successes of the two-step process described above, ectopic bone formation requires additional surgeries, which increases the risk of comorbidities and involves a substantial investment in time to facilitate new mineral deposition in the graft. Therefore, several groups have taken a single-step approach to form new, structurally sound bone matrix in orthotopic sites. Treatment of a 10 cm anterior mandibular defect (left after tumour excision) using additive manufacturing recreated the exact geometry of a patient's mandible from radiographic images.⁷⁶ A titanium mesh was then prefabricated with the customized patient geometry and filled with β -tricalcium phosphate (β -TCP) granules that were soaked in rhBMP-2 for 48 h before implantation of ASC cultures (expanded under GMP conditions). By 10 months post-surgery, sufficient new bone had developed to support dental implants. A similar *in situ* approach was used to treat calvarial defects in a child with multiple fractures.⁷⁷ The surgical team applied milled bone from the iliac crest, ASCs isolated from fat tissue harvested from the gluteal region and autologous fibrin glue, which was used to hold the cells and milled bone grafts in place. The patient displayed bone regeneration within 3 months of surgery. Although the patient numbers were limited and the studies were not randomized with double-blinded controls, these case reports indicate that MSCs can be used successfully to repair defects in nonweight-bearing craniofacial bones.^{76,77} In both cases, a titanium mesh was used to provide the regenerated bone with the appropriate anatomical geometry, whereas the granules, cancellous bone chips or bone blocks were osteoconductive and osteoinductive.

Advanced biomaterial scaffolds capable of integrating these structural elements and biological signals can also be used to guide new bone growth (discussed elsewhere^{78–81}).

Distal tibial non-union

In addition to the treatment of craniofacial defects, which requires highly invasive procedures, the clinical relevance of stromal cells in minimally invasive procedures has also been assessed. Specifically, non-unions and delayed fracture healing, in which a deficiency of fracture repair exists, are ideal situations in which to harness the regenerative potential of stromal and/or stem cells. In a randomized controlled clinical study, which included 24 patients who were considered to be at low risk of non-unions of the tibia, the prophylactic effects of MSCs in expediting fracture healing were assessed.⁸² Autologous MSCs ($\sim 10^8$) isolated from the iliac crest and peripheral blood were injected into the fracture site together with platelet-rich plasma (containing 1.1×10^9 platelets) and allograft demineralized bone matrix. This treatment resulted in a significant reduction in the time to union, from 3.0 months to 1.5 months in the intervention group of patients who received the biological composite compared with the control group of patients who did not receive this treatment. Additionally, subcutaneous grafting of a portion of the injectable composites into immunodeficient mice resulted in bone formation; however, as the authors were unable to assess the origin of the newly formed bone *in vivo*, the precise role of MSCs in promoting the improved response remains unclear.

Osteonecrosis of the femoral head

Another potential minimally invasive application of stromal cells is the treatment of osteonecrosis of the femoral head (ONFH).^{83–86} Nontraumatic ONFH is a debilitating skeletal disorder that can lead to collapse of the femoral head and the need for total hip replacement. In 2004, a double-blind nonrandomized study was conducted to assess the effect of delivering autologous bone marrow mononuclear cells (following core decompression of the lesion) to patients with stage I or stage II ONFH.⁸⁴ Cells were injected directly into the defect site via a trephine without any scaffold or hydrogel to enhance retention at the site. Within 3 months, a statistically significant reduction in the lesion-volume:femoral-head-volume ratio was observed in the cell-grafting group compared with the untreated control group. Further decreases in this volume ratio occurred by 24 months, which indicated the possibility of a slight progression in healing over time. At 60 months of follow-up, the number of patients who progressed to fracture in the cell-grafting group was markedly decreased compared with those in the control group;⁸³ however, the use of core decompression might have introduced additional trauma to the region. A less-invasive approach, in which iliac-crest-derived bone marrow mononuclear cells (BMMCs) are delivered through the medial circumflex femoral artery (using fluoroscopy to locate the injection site) was subsequently developed.⁸⁵ The 62 patients in this study were all treated with autologous BMMCs, which enabled the safety, but not the efficacy, of the treatment to be assessed. One limiting factor in these approaches is the low concentration of MSCs in bone-marrow aspirates from the iliac crest. This limitation was overcome and the safety and efficacy of the approach was demonstrated in a randomized trial of 100 patients by use of culture-expanded autologous BMMCs that were isolated from the subtrochanteric region (aspirated through the decompression tunnel) together with iliac-crest-derived BMMCs.⁸⁶

Limitations, challenges and opportunities

Animal models

The available clinical data strongly support an enhanced regenerative effect of stromal cell delivery to the site of bone defects. However, advancing these approaches in order to bring them into the realm of standard clinical care requires insight into the underlying mechanisms of cell-mediated effects. Animal models provide the best proxy to investigate cellular mechanisms. Small rodent models are, traditionally, the first choice to assess *in vivo* responses. Cranial defects in mice have been used to demonstrate bone regeneration following the delivery of ASCs.^{87–89} By use of fluorescent *in situ* hybridization staining of female (donor) chromosomes in male recipient mice,⁸⁹ up to 99% of new bone was seen to be formed by the transplanted cells. An important consideration, however, is that the mouse calvarium is <500 µm thick and, therefore, oxygen gradients throughout the graft that might lead to the generation of hypoxic regions in the core are not a complication. Consequently, cell survival in this model is not heavily dependent on revascularization, which does not mimic the reality of the clinical situation. The model of femoral defects in rats shows greater reliance on sufficient revascularization to facilitate bone healing than that in mice.⁹⁰ Using this model, transplanted cells were confirmed to enhance regeneration even though they are not incorporated into the new bone.⁷ Such reports provide indirect evidence that the primary mechanism through which transplanted stem cells mediate tissue repair might involve the secretion of paracrine factors that stimulate the recruitment and activation of endogenous stem cells.^{91,92}

The critical dependence on trophic factors to facilitate regeneration rather than to direct differentiation, tissue morphogenesis and integration of transplanted cells is further supported by studies in which blocking VEGF signalling impaired stem-cell-mediated bone repair.^{93–95} Hypoxia, acting through the hypoxia-inducible factor 1 (HIF-1) transcription factor, reduced the expression of vascular endothelial growth factor receptor 1 (VEGFR-1; commonly known as FLT-1) on bone marrow MSCs.⁹⁴ Similarly, *in vitro* studies using murine muscle-derived stem cells showed that overexpression of soluble FLT-1, a VEGF antagonist, promoted chondrogenesis in pellet cultures.⁹⁵ Overexpression of soluble FLT-1 improved articular cartilage repair *in vivo*, whereas overexpression of VEGF-A₁₆₅ (the main biological isoform of VEGF-A) led to arthritic changes in the joint, which were consistent with hypertrophic cartilage formation.⁹⁵ Furthermore, gain-of-function and loss-of-function expression studies in the mouse embryo have shown that VEGF, released by the limb bud mesenchyme, is required for the development of the skeletal vasculature.⁹³

Long-bone defects in larger animal models such as dogs and sheep might be better approximations of the clinical scenario than similar defects in mouse. Treatment of 3 cm full-thickness, segmental defects in 6–7-year-old sheep by use of polycaprolactone–tricalcium phosphate (PCL–TCP) scaffolds with nonautologous, culture-expanded MSCs or rhBMP-7 has been reported.⁹⁶ In this study, rhBMP-7 elicited the greatest new bone formation, whereas sheep that received MSCs showed similar healing to the group that received PCL–TCP only. Large animal models might also be used to assess bone healing in skeletally immature animals. For example, a porcine model was used to evaluate the effect

of BMP-7 delivery on bone healing in an immature pig.⁹⁷ The porcine model has also been used to assess the effect of direct or indirect ASC delivery to noncritical-sized bone defects in the mandible.⁹⁸ None of these models precisely mimics the challenges involved in regeneration of large craniofacial defects, non-unions or ONFH in humans. At this time, no single species has been identified as the 'gold standard' preclinical animal model for human skeletal regeneration. Consequently, future studies to investigate the mechanisms of stromal-cell-mediated bone repair will continue to rely on a variety of small and large animal models.

Cell survival and vascular integration

Widespread adoption of BTE grafts as the clinical standard of care for the treatment of massive bone defects requires the development of simple and effective techniques to rapidly vascularize grafts and enhance the survival of transplanted stromal cells. To address this need, multiple groups have investigated methods of prevascularization to harness the proangiogenic potential of endothelial cells, which are co-cultured with pro-osteogenic stromal cells. Endothelial cells have the ability to self-assemble into primitive capillary-like networks in response to proangiogenic growth factors in permissive hydrogel environments. Ideally, these nascent networks can anastomose with blood vessels, which infiltrate the defect site from its periphery. Once perfused, the resulting blood flow stimulates maturation and subsequent pruning of the vasculature. In purely vascular tissue-engineering systems, these nascent networks have been shown to remain viable for up to 1 year after intervention.⁹⁹ Several groups have employed this technique using endothelial cells and either MSCs or ASCs to engineer vascularized bone grafts in preclinical models.^{100–104} The main limitation of this technique is the extensive *in vitro* manipulation and precultivation required to facilitate blood vessel development and mineralization. Angiogenesis and osteogenesis are tightly coupled processes during bone development and healing. However, the development of vascularized osteogenic grafts has resulted in the requirement for separate cultivation conditions for cells, followed by a reintegration phase. The extensive manipulation and use of multiple culture conditions and growth factors might continue to substantially limit the clinical utility of this approach. Cell pre-aggregation methods, which enhance cell survival following transplantation^{105–107} and harness the potential of stromal cells to self-assemble into complex tissues,^{4,108} might facilitate translation of this approach into clinical practice. These techniques could be combined with the development of biomaterials capable of time-released, serial delivery of angiogenic factors followed by delivery of osteogenic factors; biomaterials with these attributes have already been described.^{109–113}

Rapid vascularization is essential for graft viability. In the absence of an immediate vascular supply to BTE grafts in the defect site (as was the case in the clinical trials conducted to date), transplanted cells are immediately exposed to severe hypoxia and ischaemia. To minimize the negative effect of hypoxia, several groups have investigated the potential of localized oxygen delivery using perfluorocarbons or peroxide-based scaffolds. Perfluorocarbons are capable of delivering oxygen for 2–3 h before becoming depleted.¹¹⁴ Revascularization occurs over a period of 1–2 weeks in clinically sized grafts and bone formation takes months. In spite of this obvious mismatch, delivery of oxygen for just a few

hours after transplantation resulted in statistically significantly enhanced bone formation within 6 weeks in a murine model.¹¹⁵ In contrast to perfluorocarbons, oxygen-generating peroxide scaffolds are able to continuously supply oxygen for weeks to months. The main drawback of peroxides is that they produce reactive oxygen species (ROS), which are detrimental to cell viability. Thus, methods to improve biomaterial design for localized oxygen delivery remain a promising opportunity for future research to enhance the outcomes of cell-based therapies.

An alternative approach for delivering oxygen along with the scaffold is to prime cells to be resistant to low-oxygen conditions. MSCs and ASCs are known to be highly resilient in hypoxic conditions and, under these conditions, they upregulate expression of the HIF-1 α pathway. The transcription factor HIF-1 α controls both the production of angiogenic growth factors and cytokines and the ability of vascular cells to respond to these proteins. Additionally, HIF-1 α activates the expression of multiple proangiogenic cytokines, including SDF-1, placenta growth factor and angiopoietins,^{116,117} which are required for physiological vascularization and which cannot be effectively stimulated by VEGF signalling through VEGFR-2 alone. HIF-1 α modulates metabolic responses to hypoxia in order to maintain homeostatic levels of cellular energy, pH and the redox state. These responses include cellular adaptations regulated by downstream HIF-1 α signalling that limit the production of ROS during periods of hypoxia; upregulation of levels of glucose transporters and glycolytic enzymes; and modified protein expression that facilitates pH homeostasis. Interestingly, the expression of HIF-1 α in osteoblasts has been identified as a mechanism for coupling angiogenesis and osteogenesis in native bone.^{118–120} By culturing cells in low-oxygen conditions or chemically inducing upregulation of levels of HIF-1 α before transplantation, cell survival and homing of the cells following transplantation (mediated by upregulation of levels of CXCR4) can be augmented, which thereby enhances the regenerative properties of the cells, as has been shown in cardiovascular applications.^{121,122}

Cell dosing and optimal concentrations

Cell numbers for transplantation are often determined empirically through intuition on the basis of prior experience or from *in vitro* data. In clinical applications for which the size and geometry of the graft needs to be customized, it is impossible to ascertain optimal cell numbers *a priori* without knowledge of the mechanisms of cell-mediated bone regeneration. However, mechanistic data derived from preclinical models can be used to develop computational approaches that model bone repair as a function of initial bone condition.¹²³ Computational models can be used predictively to rigorously define the number of cells and mode of delivery required for any specific application.

In vitro expansion

In cell culture, the term hypoxia is used to describe oxygen levels lower than the 20% found in the atmosphere in which cells are cultured *ex vivo*. However, average oxygen levels within tissues in the body can be as low as 5%. Consequently, culturing cells *ex vivo* exposes them to hyperoxic conditions, which might lead to elevated levels of intracellular ROS production. Another drawback of extended *ex vivo* culture is the potential for

development of genetic and epigenetic mutations as a consequence of rapid cell division. Thus, methods that employ culture-expanded allogenic or autologous cell sources require robust assays to monitor and validate biological changes that might negatively influence the safety and subsequent efficacy of cell-based therapies.

Genetic modification of stromal and stem cells

Stromal and stem cells can be transduced to overexpress growth factors (in particular, BMPs¹²⁴) and transcription factors (such as sonic hedgehog¹²⁵) in order to enhance the efficacy of promoting new bone formation. This approach has been shown to be efficacious in small-animal models and its potential has been reviewed elsewhere.^{126,127} However, genetically modified stromal cells and stem cells remain a highly experimental model, which has proven difficult to advance through the regulatory process as a result of substantial safety concerns.

Approved cell-based products

The most established commercial cell product available for orthopaedic disabilities is Carticel[®] (Genzyme, USA), which uses culture-expanded, autologous chondrocytes for the treatment of degenerated articular cartilage. The use of MSCs or ASCs rather than chondrocytes can be advantageous owing to the potential to obtain larger quantities of cells and because it is associated with less donor-site morbidity. Although studies assessing the role of cell adhesion in improving chondrogenic outcomes with MSCs have been reported,¹²⁸ no comparable commercially available cell products for bone regeneration exist. Osteocel[®] (NuVasive, USA), which retains viable MSCs within bone allografts, is now available for spine fusion applications. Trinity[®] Evolution[™] (Orthofix, Netherlands Antilles) is a similar product for spine and other orthopaedic conditions. Lately, culture-expanded allograft MSCs have been used in a clinical trial to treat meniscectomies.¹²⁹ Such clinical successes might herald the routine use of allograft cell-based products for bone regeneration.

To achieve widespread usage, cell-based products must be readily available at all levels of the healthcare system. This advance will require the development, validation and standardization of guidelines and protocols for the shipment and storage of cell therapeutics. Fortunately, the decades of experience obtained, and the advances made, by blood and transfusion centres serve as a foundation for the nascent MSC field. Nevertheless, multiple questions remain to be addressed. At present, no national or international infrastructure of facilities to manufacture and ship cells for skeletal regeneration to points of care exists. Few hospitals or universities are equipped with certified current GMP laboratories that are suitable for the isolation, expansion, characterization and processing of MSCs. Although private current GMP contract research organizations have emerged to fill this gap, their numbers and locations are limited. Furthermore, cells are being produced, stored and shipped under multiple environmental conditions, ranging from room temperature to -196° C (by use of liquid nitrogen); however, not all hospitals or outpatient surgical centres have the capability of storing cell products reliably at low temperatures. Owing to the fact that regulatory authorities and clinical research societies have not yet agreed on definitions for the quality assurance and control of MSC products, the distribution of skeletal regenerative cells across national borders is difficult. Such issues related to international standardization

remain an obstacle to the continued growth of the cell-therapy field. Another difficulty relates to obtaining regulatory approval from the FDA for combination products, such as cells combined with growth factors and other bioactive biomaterials (for example, osteoinductive scaffolds). Without appropriate precedence, the safety standards of these approaches remain undefined, a challenge compounded when one considers the huge costs associated with animal testing.

Conclusions

Stem-cell-mediated bone regeneration provides a number of potential therapeutic advantages to the use of autograft tissues. Currently, there is a large amount of preclinical and clinical data that support the delivery of stromal and stem cells to defect sites to enhance bone repair and regeneration. The successful clinical applications of MSCs and ASCs firmly establish proof-of-concept of the clinical feasibility of complex, multistage surgical processes, as well as the indications requiring minimally invasive approaches. The number of ongoing clinical trials with both MSCs and ASCs (Table 1) bodes well for the future of cell-based therapies. Therefore, what are the obstacles limiting the more extensive use of stromal cells and stem cells in the clinic and what steps need to be taken in order to bring ASC-mediated and MSC-mediated bone repair up to the standard of care currently reserved for autografts? In the near term, more prevalent therapeutic use of stromal and stem cells for skeletal regeneration does not require any major changes in methodology. The techniques for cell delivery involving hydrogel encapsulation in combination with a mineralized component have been well established over the past 30 years. The critical restriction at this stage is the development of internationally recognized, standardized regulatory guidelines that define the minimum safety criteria and, consequently, robust methods for cell expansion, storage and shipping that minimize or eliminate potentially harmful changes in the cells' genetic make-up whilst retaining their potency. The growing number of cell products on the market will facilitate movements in this direction.

However, in the longer term (that is, beyond the next 10 years), elevating stromal-cell-mediated and stem-cell-mediated bone regeneration to the standard of clinical care requires technological advances that maximize cell retention, viability, homing (for systemic delivery), vascular network formation, osteogenic differentiation capacity and tissue assembly properties. These advances require a fundamental understanding of the functioning of cells following transplantation. Put simply, are transplanted cells actively undergoing tissue morphogenesis and integration or are they merely mediators, emitting signals to recruit and stimulate their endogenous counterparts into action? The answer might be context-dependent and simple techniques, such as cell aggregation (reviewed elsewhere^{130,131}) might profoundly influence the fate of cells after transplantation. Similarly, continued research into cell–biomaterial interactions (particularly *in vivo* interactions in immunocompetent animals) that spawn novel techniques for oxygen delivery^{132,133} or growth-factor tethering, retention and presentation¹¹¹ might profoundly enhance regenerative outcomes. Most critically, systematic studies that deepen our level of understanding to an extent that we can model predicted regenerative outcomes on the basis of specific input parameters will ultimately facilitate the creation of customized therapies

that are founded on rational design and usher in a new standard of care in the field of regenerative medicine.

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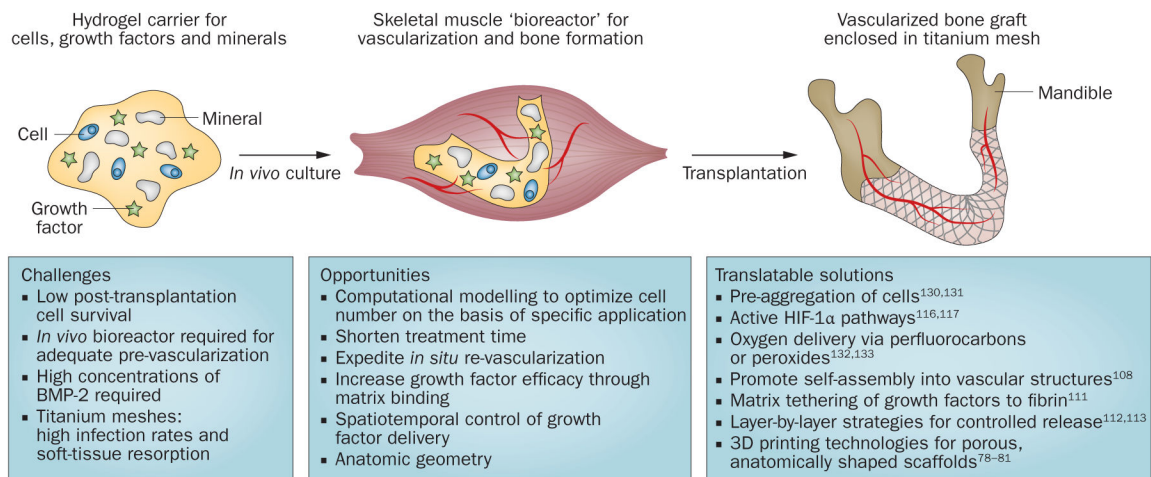
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Key points

- Stromal cells and/or stem cells can be isolated from different tissues on the basis of plastic adherence and surface-antigen profiles, thereby providing opportunities for bone regeneration
- The regenerative potential of therapies that are based on adipose-tissue-derived and bone-marrow-derived mesenchymal stromal or stem cells is being tested clinically for the treatment of craniofacial bone defects, tibial non-unions and osteonecrosis of the femoral head
- Although most approaches in this area use autologous cells, allogeneic sources that include commercially available allograft cell-based products are being investigated
- Widespread use of cell-based products requires the development and standardization of guidelines and protocols for the shipment and storage of cell therapeutics
- Despite strong clinical data, which indicates enhanced regenerative outcomes following stromal-cell or stem-cell transplantation, further insight is needed into the mechanisms of action of these strategies
- Opportunities exist to develop technologies that improve cell survival, morphogenesis and functionality to advance cell therapy as standard care for the treatment of bone defects

Review criteria

The articles selected for this Review were identified by searching PubMed, ISI Web of Knowledge and ClinicalTrials.gov. Search terms used included, but were not limited to: “stromal cells”, “stem cells”, “MSCs”, “ASCs”, “muscle-derived stem cells”, “pericytes”, “bone tissue engineering”, “oxygen”, “hypoxia”, “VEGF”, “BMP”, “HIF”, “angiogenesis”, “osteogenesis”, “craniofacial bone”, “non-union”, “osteonecrosis”, “spine” and “gene delivery”. Often, the names of renowned researchers within the field were combined with key words in Boolean searches. Articles spanning the late 1960s to the mid 1990s were used to write the brief history of the field. Known clinical trial reports were researched and articles from those reference lists were used to find other clinical trial reports. Major advances that continue to affect current paradigms were cited to describe the development of the field over time without considerations of publication date. A major focus of the article concerns developing trends and novel technologies. Hence, many of the studies cited were published within the past 5 years. In citing other review articles on specific topics, the most recent reviews were used. All cited articles were published in the English language.

**Figure 1.**

Generalized clinical approach for stem-cell-based regeneration of large craniofacial bone defects. Adipose-tissue-derived stromal and/or stem cells are mixed with growth factors (such as BMP-2) and combined with mineral blocks in a preshaped titanium mesh, cultured *in vivo* and transplanted to repair the bone defect (the mandible is shown as an example). Abbreviations: BMP-2, bone morphogenetic protein 2; HIF-1 α , hypoxia-inducible factor 1 α .

Table 1

Clinical trials in which stromal cells were used for skeletal regeneration

Indication	Cell source	Cell processing and delivery	Clinical trial
MSC			
Non-union of bone	Autologous	Direct injection	NCT00512434, ¹³⁴ NCT01206179, ¹³⁵ NCT01429012, ¹³⁶ NCT01788059 ¹³⁷
		Implantation with carrier	NCT00250302, ¹³⁸ NCT01435434, ¹³⁹ NCT02177565, ¹⁴⁰ NCT01626625, ¹⁴¹ NCT01842477, ¹⁴² NCT01725698, ¹⁴³ NCT01958502 ¹⁴⁴
ONFH	Autologous	Direct injection	NCT02065167, ¹⁴⁵ NCT01700920, ¹⁴⁶ NCT01544712 ¹⁴⁷
		Implantation with carrier	NCT01605383 ¹⁴⁸
Other (spine fusion, osteoarthritis)	Autologous	Direct injection	NCT01210950 ¹⁴⁹
		Implantation with carrier	NCT01552707, ¹⁵⁰ NCT01389661 ¹⁵¹
	Allogeneic	Direct injection	NCT01603836, ¹⁵² NCT02172885, ¹⁵³ NCT00186914 ¹⁵⁴
		Implantation with carrier	NCT00001391, ¹⁵⁵ NCT01207193, ¹⁵⁶ NCT00221130 ¹⁵⁷
ASC			
Non-union of bone	Autologous	Implantation with carrier	NCT01532076 ¹⁵⁸
	Allogeneic	Direct injection	NCT02140528 ¹⁵⁹
ONFH	Autologous	Direct injection	NCT01643655 ¹⁶⁰
Other (spine fusion, osteoarthritis)	Autologous	Direct injection	NCT01501461, ¹⁶¹ NCT01739504, ¹⁶² NCT01585857, ¹⁶³ NCT01885819, ¹⁶⁴ NCT02241408, ¹⁶⁵ NCT01885832, ¹⁶⁶ NCT02142842, ¹⁶⁷ NCT01947348 ¹⁶⁸
		Implantation with carrier	NCT01633892, ¹⁶⁹ NCT01645722, ¹⁷⁰ NCT01218945 ¹⁷¹

Abbreviations: ASC, adipose-tissue-derived stromal cell; MSC, bone-marrow-derived stromal cell; ONFH, osteonecrosis of the femoral head.