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TOPIC HIGHLIGHT

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Adipose mesenchymal stem cells in the field of bone tissue engineering

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Abstract

Bone tissue engineering represents one of the most challenging emergent fields for scientists and clinicians. Current failures of autografts and allografts in many pathological conditions have prompted researchers to find new biomaterials able to promote bone repair or regeneration with specific characteristics of biocompatibility, biodegradability and osteoinductivity. Recent advancements for tissue regeneration in bone defects have occurred by following the diamond concept and combining the use of growth factors and mesenchymal stem cells (MSCs). In particular, a more abundant and easily accessible source of MSCs was recently discovered in adipose tissue. These adipose stem cells (ASCs) can be obtained in large quantities with little donor site morbidity or patient discomfort, in contrast to the invasive and painful isolation of bone marrow MSCs. The osteogenic potential of ASCs on scaffolds has been examined in cell cultures and animal models, with only a few cases reporting the use of ASCs for successful reconstruction or accelerated healing of defects of the skull and jaw in patients. Although these reports extend our limited knowledge concerning the use of ASCs for osseous tissue repair and regeneration, the lack of standardization in applied techniques makes the comparison between studies difficult. Additional clinical trials are needed to assess ASC therapy and address potential ethical and safety concerns, which must be resolved to permit application in regenerative medicine.

Key words: Adipose-derived stem cells; Bone tissue engineering; Osteogenic differentiation; Scaffold; Regenerative medicine

Core tip: The complex and dynamic process of bone tissue engineering is a challenging field in regenerative medicine. Current research is focused on the optimization and facilitation of bone regeneration by combining growth factors and mesenchymal stem cells with the many types of materials that have been studied as scaffolds. This review presents an overview of ideal scaffold properties and discusses the application of adipose-derived stem cells in bone tissue engineering and translational medicine.

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INTRODUCTION

Recent progress in the field of bone tissue engineering has led to new and exciting research concerning regenerative medicine. This interdisciplinary field is focused

on the development of biological substitutes that restore, maintain or improve tissue function by applying the principles of engineering and the life sciences[1]. The primary target of clinical therapeutic strategies is the regeneration of bone for skeletal reconstruction of large bone defects created by trauma, infection, tumor resection and skeletal abnormalities, or cases in which the regenerative process is compromised, including avascular necrosis, atrophic non-union and osteoporosis. Strategies that stimulate bone healing to reduce or treat complications are becoming more important, due to the increase in life expectancy and ageing of the world population.

Autologous grafts represent the "ideal graft bone substitutes" and are currently the gold standard therapeutic strategy as they combine all essential components to induce bone growth and regeneration, including osteogenic cells, osteoinductive growth factors and bone-supporting matrix. Autografts are non-immunogenic and histocompatible, as they are the patient's own tissue. Although they reduce the likelihood of immunoreaction and transmission of infection[2], autografts are limited and commonly result in donor site morbidity as a result of the additional surgical harvesting procedures, and are accompanied by the risk of infection, hematoma and chronic pain, which can all lead to implant failure[3-7]. An alternative approach involves the use of allogenic bone grafts obtained from human cadavers or living donors, which bypasses the complications associated with harvesting and quantity of graft materials. However, allogenic grafts are limited by tissue matching, disease transmission, batch variability and an inability to survive and integrate following implantation[8-10].

The limited success of auto- and allografts in some clinical situations has stimulated the investigation of a wide variety of biomaterials to be used as scaffolds, and the development of promising clinical therapies[11]. Advantages to utilizing sophisticated bone scaffolds include the elimination of the risk for disease transmission, fewer surgical procedures, and reduced risk of infection or immunogenicity. Moreover, there is an abundant availability of synthetic or natural biomaterials that can be employed, including collagen, hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), calcium phosphate cements and glass ceramics. The concept of bone substitution involves the replacement of bone structure to allow the migration, proliferation and differentiation of bone cells and to promote vascularisation, thus utilizing the body's natural biological response to tissue damage in conjunction with engineering principles. Current models of *in vitro* bone formation are based on the idea that the same factors known to play a role during embryonic development can be used to induce cellular differentiation and function in the process of regeneration[12]. In order to engineer an environment supporting bone formation, combinations of biochemical and biophysical signals need to be presented to the cells in a three-dimensional setting in a way that allows interactions between the surrounding cells and the extracellular matrix. The complexity of signaling, with temporal and spatial gradients of molecular and physical factors affecting bone morphogenesis, presents significant challenges to engineering fully viable, functional bone. This "diamond concept" has allowed the scientific community to consider more complex interactions between scaffolds, cells and growth factors in order to induce tissue regeneration in bone defects[13]. This article presents a concise review regarding the main properties of scaffolds, the most recent progress in bone tissue engineering using human adipose-derived stem cells and current models used for bone regeneration.

PROPERTIES OF ENGINEERED BONE SCAFFOLDS

An ideal scaffold must address multiple physical and biological requirements in order to optimize bone regeneration. One of the most important stages of bone tissue engineering is the design and processing of a porous, biodegradable three-dimensional (3D) structure. This scaffold provides a structural and logistical template for developing tissue, which can markedly affect cell behavior. The properties of scaffolds that are important for bone formation include the size, distribution and shape of the pores, the surface roughness, the presence of cell attachment sites and the biomechanics of both the material and the scaffold structures[14-17]. The most suitable scaffolds for bone formation are those made of osteoconductive materials, such as bone proteins and HA, with mechanical properties similar to those of load-bearing native bone that stimulate osteogenesis and have large and interconnected pores to facilitate cell infiltration and matrix deposition, and rough inner surfaces to promote cell attachment. Additionally, scaffolds should be anisotropic structures that can be fashioned into anatomically correct shapes that also have the capacity for vascularization. Scaffolds should also incorporate and control the delivery of bioactive molecules, such as growth factors or drugs that regulate cellular function, accelerating healing and preventing pathology[18,19]. Furthermore, as scaffolds will be replaced over time by new formed bone, they should be comprised of resorbable materials, or materials that degrade in an enzymatic or hydrolytic way, such as polymers, or can be dissolved by cells such as osteoclasts[20,21].

The majority of studies are currently focused on the development of 3D structures that mimic the anatomical and biochemical organization of cells and native matrix in order to achieve suitable mechanical properties for bone tissue[22]. Numerous materials have been shown to support *in vitro* bone formation by human cells, including bioceramics like HA, β-TCP, bio-glasses and biodegradable polymers[23,24], and natural or synthetic collagen, fibrin, chitosan or polyesters[25,26]. Scaffolds containing composites of these materials provide an optimized and convenient alternative as they combine the advantages of both bioactive ceramics and biodegradable polymers[27-31].

OSTEOINDUCTIVE BIOMOLECULES

One of the most challenging tasks for the development of bone graft substitutes is to produce scaffolds with osteoinductive properties, which can involve the application of biologically active molecules. Growth factors that naturally occur within a healthy bone matrix or are expressed during fracture healing can be used to direct the development of structures, vascularization and differentiation of bone cells[19]. Growth factors, such as cytokines, are endogenous proteins that act on a wide variety of cells and direct their actions by binding to and activating cell-surface receptors. As developmental bone formation is an orchestrated cellular process tightly controlled by actions of growth factors, their use in engineered scaffolds is an obvious strategy when the bone integrity is compromised and bone tissue needs to be repaired[32,33]. This strategy aims to enhance the local presence of bone-depositing osteoblasts, either by attracting the cells to the repair site or by inducing the proliferation of local undifferentiated precursor cells, followed by the transformation of precursor cells into an osteoblastic phenotype[34].

The introduction of specific biomolecules has been shown in animal models to enhance the union of non-union type (a fracture that does not heal by itself after several months) bone fractures[32]. Many growth factors that have been used in bone repair with some degree of success include mitogens such as platelet-derived growth factors, metabolic regulators such as insulin-like growth factors, angiogenic proteins such as basic fibroblast growth factors, and morphogens such as bone morphogenetic proteins (BMPs)[35-39]. BMPs, which are members of the transforming growth factor beta (TGF-β) superfamily, have been the most extensively studied, as they are potent osteoinductive factors that induce the mitogenesis and differentiation of mesenchymal stem cells and other osteoprogenitors[35,11]. They are a very promising candidate for the treatment of bone diseases and defects, as a number of experimental and clinical trials demonstrate their safety and efficacy[40-42]. However, the clinical application of BMPs is currently limited to the use of BMP-2 for open tibial fractures and spinal fusion, and BMP-7 (OP-1) for non-unions with limited indication for spinal fusion[43,44], which were approved by the U.S. Food and Drug Administration in 2004. The clinical and scientific utility of bone tissue engineering largely depends on the ability to create scaffolds with specific characteristics that predictably direct cells to differentiate into the right phenotypes in a spatially and temporally defined pattern guided by molecular and physical factors.

HUMAN ADIPOSE-DERIVED MSCS

The combination of engineered scaffolds with recent developments in the emerging field of stem cell science may allow the use of stem cells to repair tissue damage and, eventually, to replace organs. MSCs are non-hematopoietic cells of mesodermal derivation that are present in a number of postnatal organs and connective tissues. The stroma of bone marrow contains bone marrow mesenchymal stem cells (BMSCs) capable of differentiating into osteogenic, chondrogenic, adipogenic and endothelial lineages[45-48], and thus is the most well studied source of MSCs for bone regeneration. Bone marrow transplantation is also being used clinically in combination with osteoconductive materials to augment bone healing[9].

In the last few years, MSCs have been isolated from other tissue sources including trabecular bone[49], synovium[50], umbilical cord[51], periodontal ligament[52] and other dental tissues[53], skeletal muscle, cord blood and skin[54-56]. Although the stem cell populations derived from these sources are valuable, common problems include limited amounts of available tissues and low numbers of harvested cells, which necessitate at least some degree of *ex vivo* expansion or further manipulation before preclinical or clinical use. In contrast, a promising population of MSCs has been identified within adipose tissue, termed adipose-derived stem/stromal cells (ASCs) by the regenerative medicine community during the Second Annual International Fat Applied Technology Society Meeting in 2004. Human adipose tissue is ubiquitous and can easily be obtained in large quantities with little donor site morbidity or patient discomfort[45], in contrast to the invasive and painful procedure for isolating BMSCs. Moreover, stem cell yields are greater from adipose tissue than from other stem cells reservoirs, a significant factor for use in regenerative medicine. As many 1×10^7 ASCs can routinely be isolated from 300 mL of lipoaspirate, with greater than 95% purity. ASCs comprise 2% of nucleated cells in processed lipoaspirate, with a yield of 5000 fibroblast colony-forming units (CFU-F) per gram of adipose tissue, compared with estimates of about 100-1000 CFU-F per milliliter of bone marrow[57,58]. In general, cell isolation protocols include density gradient centrifugation of the collagenase-digested tissue (lipoaspirate or minced adipose tissue)[57-61], followed by the seeding of the pelleted stromal vascular fraction (SVF) on monolayer culture plastics. The adherent cell population can then be expanded and used in a variety of assays.

Although the study of human ASCs (hASCs) is emerging, the standardization of isolation and culture procedures could improve quality control and facilitate comparisons between different studies. There are discrepancies in the results of studies from different laboratories due to differences in the methods and quality of hASC isolation, which can affect the composition of the initial cell culture, as well as in the procedures used to culture the cells. Cell culture basal medium, generally containing 10% fetal bovine serum, is often supplemented with epidermal growth factor, fibroblast growth factor-2 and/or TGF-β[58,62,63]. In addition, some protocols may recommend differing initial cell seeding densities, though evidence suggests that low seeding densities and subconfluent passaging are recommended[64,65]. Other variables that may affect the composition of the initial isolated cell culture cannot be standardized, such as donor age, gen-

der, body mass index, ethnicity and medical history[66]. It is therefore important to standardize hASC isolation and culturing methods to maximize the reliability and reproducibility of results from different laboratories.

COMPOSITION AND CHARACTERIZATION OF CULTURED hASCs

The SVF that is obtained from processed adipose tissue contains a highly heterogeneous cell population, including non-adherent cell populations. A complete characterization of SVF cell populations was done by Yoshimura *et al*[64] in which they identified endothelial cells, pericytes, blood-derived cells, fibroblasts, vascular smooth muscle cells and preadipocytes, in addition to the potential hASCs. Although the adherence of hASCs allows for their selection from the SVF during subsequent tissue culture passages, other cell types, such as fibroblasts, can also adhere to the culture plastic. Thus, other cell types, or subpopulations, may compromise the proliferation and/or differentiation potential of hASCs.

To reduce the heterogeneity of cultured ASCs, a washing procedure in the beginning of the cell culture can be used, as various cell types adhere to the plastic at different time points[66]. Additionally, flow cytometric sorting or immunomagnetic separation with specific cell surface markers can be used to isolate and purify specific subpopulations of hASCs. However, there is considerable heterogeneity in commonly analyzed hASC surface markers, which can be modified by the culturing procedure. The cell phenotype can also be influenced by differences in the cell purification procedure and by the number of passages[66-70]. Mitchell *et al*[59] identified hematopoietic lineage cells from the SVF using flow cytometry based on their expression of CD1, CD14, CD45 and other markers, which were lost with progressive passages. The loss of these markers indicates that they do not represent the adherent population. Moreover, SVF cells exhibit low levels of classic stromal cell markers (CD13, CD29, CD44, CD73, CD90, CD105, CD166) in the earliest stages of isolation, and assume a more homogeneous profile with consistently high levels of stromal markers after four to five passages, a temporal expression pattern that resembles what has been reported in human BMSCs[54]. Work from Rada *et al*[71] demonstrated the complexity of hASC populations by showing that they are composed of several subpopulations that express different levels of hASC markers and exhibit distinctive differentiation potentials. In their study, hASC subpopulations were isolated using immunomagnetic beads specific for CD29, CD44, CD49, CD73, CD90, CD105, p75 and STRO-1, and cultured with specific chondrogenic or osteogenic media in order to evaluate their differentiation potential into these lineages. Among all the hASC subpopulations isolated, STRO-1-containing populations had the highest osteogenic potential, with the highest chondrogenic differentiation potential in populations expressing CD29 and CD105. These data clearly demonstrate that SVF from adipose tissue is comprised of several stem cell subpopulations that exhibit *in vitro* chondrogenic and osteogenic differentiation profiles. Therefore, these subpopulations should be studied in order to select those most suitable for application in bone and cartilage regenerative medicine.

APPLICATION OF hASCs AND SCAFFOLDS FOR BONE TISSUE ENGINEERING

Since the discovery of hASC osteogenic differentiation, substantial progress has been made toward the use of these cells as an optimal source for bone regeneration. Although initial applications involved the direct administration of stem cells into the target fracture site, current paradigms using scaffolds loaded with stem cells are preferred as they provide support for cell colonization, migration, growth and differentiation[72]. Combined with the support of a scaffold, the directed osteogenesis of hASCs confirms that adipose tissue is a promising autologous source of osteoblastic cells for bone regeneration. Utilization of hASCs in scaffolds for bone tissue engineering has been heralded as the alternative strategy of the 21st century to replace or restore the function of traumatized, damaged or lost bone.

In the last ten years, several cell characterization studies have extensively described the differentiation potential and function of hASCs *in vitro*[58,62,67,69]. Many types of materials have been used to confirm these positive hASC characteristics, which have become available for scaffold-assisted bone regeneration in a variety of tissue engineering strategies. The importance of the scaffold in hASC osteogenesis has been demonstrated in a number of studies that recommend the use of different materials, including ceramics[73], titan alloys[74,75], natural and synthetic polymers[76,77], and natural or semi-synthetic grafts[78,79], with variable porosity, roughness, and methods of fabrication for future regenerative applications. A clear trend has emerged toward the use of composite scaffolds due to their superior properties and structures[80-82] derived from the combination of two or more materials[83-87].

The study of hASCs for bone regeneration has largely involved the insertion of biomaterials in rat and nude mouse models[88-92]. Furthermore, a femoral defect in nude rats is available and calvarial defect models have been described for other species, to demonstrate the application and optimization of hASCs in regenerative medicine[93-97]. However, relatively few reports are available concerning the utilization of hASCs for human bone tissue regeneration (Table 1). The first compelling evidence supporting the clinical application of an hASC scaffold to promote fracture healing was reported by Lendeckel *et al*[98] in 2004. In this work, a combination of autologous hASCs obtained from the gluteal region

Table 1 Summary of current representative bone tissue engineering models combined with human adipose-derived stem/stromal cells

Scaffold origin	Type of scaffold	Active molecule	Study type	Differentiation pre-implant	Implant area	Species	Ref.
Synthetic	BCP	-	*In vitro*	Yes	-	-	[73]
Synthetic	Ti6Al4V	-	*In vitro*	Yes	-	-	[74]
Synthetic	Ti6Al4V	-	*In vitro*	Yes	-	-	[75]
Semi-synthetic	CMCA	Sr2+	*In vitro*	Yes	-	-	[76]
Semi-synthetic	MPLA/CNC	-	*In vitro*	-	-	-	[77]
Semi-synthetic	Silk/fibroin	-	*In vitro*	Yes	-	-	[79]
Semi-synthetic	Apatite-coated CH/CS	rhBMP-2	*In vitro*	Yes	-	-	[80]
Synthetic	Bioactive glass	-	*In vitro*	Yes	-	-	[81]
Synthetic	PCL	-	*In vitro*	Yes	-	-	[82]
Synthetic	PLA/β-TCP	-	*In vitro*	Yes	-	-	[83]
Synthetic	PLA/β-TCP	-	*In vitro*	Yes	-	-	[84]
Synthetic	BCP	-	*In vitro/In vivo*	Yes	Femur	Rat	[86]
Semi-synthetic	Collagen/PCL	-	*In vitro*	Yes	-	-	[87]
Synthetic	PEG/PCL	-	*In vitro/In vivo*	-	Subcutaneous	Rat	[88]
Synthetic	HA	-	*In vitro/In vivo*	-	Subcutaneous	Rat	[89]
Synthetic	HA/ β-TCP	-	*In vitro/In vivo*	-	Subcutaneous	Mouse	[90]
Synthetic	PCL/β-TCP	-	*In vivo*	-	Subcutaneous	Rat	[91]
Synthetic	PLA	-	*In vivo*	Yes	Palate	Rat	[92]
Synthetic	HA/β-TCP	-	*In vivo*	-	Femur	Rat	[93]
Synthetic	Apatite-coated PLGA	rhBMP-2	*In vivo*	-	Calvaria	Mouse	[94]
Semi-synthetic	ABB/titanium	-	*In vivo*	-	Calvaria	Rabbit	[95]
Natural	Fibrin matrix	BMP-2	*In vivo*	-	Femur	Rat	[96]
Synthetic	Carbon nanotube	rhBMP-2	*In vitro/In vivo*	Yes	Subcutaneous	Mouse	[97]
Natural	Fibrin glue	-	*In vivo*	-	Calvaria	Human	[98]
Synthetic	β-TCP/titanium	rhBMP-2	*In vivo*	-	Maxilla	Human	[99]
Synthetic	β-TCP	rhBMP-2	*In vivo*	Yes	Mandibula	Human	[100]
Natural	ABB	PRP	*In vivo*	Yes	Maxilla/mandibula	Human	[101]
Synthetic	β-TCP/bioactive glass	rhBMP-2	*In vivo*	Yes	Craniofacial	Human	[103]

BCP: Biphasic calcium phosphate ceramics; Ti6Al4V: Titanium alloy; CMCA: Amidate carboxymethilcellulose; PLA: Poly(L-lactic acid); MPLA/CNC: Maleic anhydride grafted PLA/cellulose nanocrystals; CH/CS: Chitosan/chondroitin sulfate; PCL: Polycaprolactone; β-TCP: β-tricalcium phosphate; PEG: Polyethylene glycol; HA: Hydroxyapatite; PLGA: Poly(L-lactic acid-co-glycolic acid); ABB: Anorganic bovine bone; Sr^{2+}: Strontium ion; rhBMP-2: Recombinant human bone morphogenetic protein; PRP: Platelet-rich plasma.

and bone grafts from the dorsal iliac crest was used for the treatment of a multi-fragment calvarial fracture in a 7-year-old girl. An autologous fibrin glue was applied using a spray adapter to keep the cells in place, and post-operative healing was uneventful after three months. In 2009, Mesimäki *et al*[99] described a novel method to reconstruct a major maxillary defect in an adult patient using autologous hASCs that were produced in clean room facilities free of animal-derived reagents, combined with recombinant human BMP-2 and β-TCP granules. The patient's healing was also clinically uneventful in this case, thus paving the way for extensive clinical trials using ASCs in custom-made implants for the reconstruction of bone defects. Moreover, the use of autologous cells, handled and prepared without animal-derived materials with good manufacturing practices in standard clean rooms, demonstrates that these cells can be considered safe for applications in tissue regeneration, according to the clinical cell therapy safety standards of the European Union.

Defects of the skull and jaws have been successfully reconstructed or their healing has been accelerated by the use of hASCs[98-102], extending our limited knowledge regarding the potential use of hASCs for osseous tissue repair and regeneration. Work published in 2012 by Sándor demonstrates the synergistic effect of hASCs, resorbable scaffolds (β-TCP and bioactive glass) and growth factors (BMP-2), in the treatment of 23 patients with craniofacial osseous defects[103]. He has established the utility of hASCs in combination with biomaterials in 85% of the cases followed after bone reconstruction, though the long-term success of this procedure needs to be verified using a large sample.

CONCLUSION

The emerging application of hASCs on engineered scaffolds for bone tissue regeneration represents the most exciting challenge for the scientific community in future translational medicine. The ability to obtain a large quantity of MSCs from easily accessible adipose tissue, combined with the growing research on new biomaterials incorporating bioactive molecules such as drugs and growth factors, opens the way to new therapeutic applications. Although clinical trials have demonstrated the use of hASCs for the reconstruction of craniofacial defects in humans, there are many aspects that need to be examined and resolved. Further investigations are needed to standardize procedures for harvesting, isolating, cultivating and preparing hASCs for clinical applications. The differences in currently applied techniques make

comparisons across studies difficult. Moreover, the lack of guidelines for the proper utilization of different bone scaffold materials may provoke safety concerns, impeding clinical trials and the translation of scaffold technologies to the clinical environment. Prospective randomized clinical trials are needed to identify clear indications for and to demonstrate clinical outcomes of the hASC therapies. Ethical and safety concerns must be resolved to prevent human testing as the first stage in novel scaffold development.

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