Histol Histopathol (2013) 28: 557-564
DOI: 10.14670/HH-28.557

**http://www.hh.um.es**

**Histology and Histopathology**
*Cellular and Molecular Biology*

## *Review*

# Adipose stem cells and skeletal repair

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**Summary.** Although adipose tissue has been considered a useless tissue, recent investigations have shown that it provides an abundant source of adult stem cells. Adipose stem cells (ASCs) can undergo rapid osteogenenic differentiation, which represents a promising option for bone tissue engineering and treating large bone defects. While bone marrow-derived stem cells have been more extensively studied for bone tissue engineering, a limitation exists in the harvested amount of bone marrow. As adipose tissue can provide a much greater number of adult stem cells without causing morbidity, it offers a good option as a cell source for bone tissue engineering. In this review, we discuss the definition of ASCs, the induction of osteogenic differentiation from ASCs, scaffolding materials for adipose bone tissue engineering, and *in vivo* models for future clinical applications.

**Key words:** Osteogenic differentiation, Tissue engineering

## Introduction

Fat has been considered a useless tissue. So, lipoaspirate obtained from liposuction has been discarded as surgical waste. However, fatty tissue contains numerous preadipocytes, adipocytes, fibroblasts, vascular smooth muscle cells, pericytes, endothelial cells, macrophages, and lymphocytes (Schaffler and Buchler, 2007). A vast population of cells exists within the stromal vascular layer of fatty tissue, which has adult stem cell characteristics. These multipotent adipose stem cells (ASCs) have a shape, differentiation capacity, and phenotype comparable to those of mesenchymal stem cells (MSCs) isolated from bone marrow. So attention has been also paid to ASCs as a therapeutic modality for autoimmune and inflammatory disorders (Gonzalez et al., 2009). Despite promising preliminary ASC results for inflammatory disease, bone defects or skeletal diseases have not been the subject of a large clinical trial (Levi and Longaker, 2011)

ASCs undergo osteogenesis rapidly, which provides a promising option for bone tissue engineering (Cowan et al., 2004). While the gold standard for treating bone defects is autologous bone grafting, the amount of available bone is limited and fraught with morbidity during harvesting. Allogenic bone grafts or bone substitutes such as demineralized bone matrix are other options. However, their high expense and risks, such as viral disease transmission, limit their applications. In addition, bone morphogenic protein-2 (BMP-2), absorbed on a collagen sponge, is used for nonunions and spinal fusion. However, despite promising results, collagen sponges are not rigid. BMP-2 release is limited in duration and fails to provide long-term effect (Levi and Longaker, 2011). These limitations of current methods indicate the need to combine cells and scaffolds to mimic live material, i.e., autologous bone.

Although MSCs harvested from bone marrow have been studied more extensively for bone tissue engineering (Hsu et al., 2008), they require aspiration from the iliac crest, which only yields 10-40 ml of marrow. The procedure is painful and yields low numbers of cells (Pittenger et al., 1999). In contrast, ASCs are easy to harvest and are abundant. The ultimate goal for adipose bone tissue engineering is to harvest adipose tissue from the proper anatomic location, proliferate ASCs with improved osteogenic potential, treat the cells with appropriate small molecules or cytokines, and implant these cells on a scaffold into a skeletal defect in patients. In this review, we discuss the

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definition of ASCs, the induction of osteogenic differentiation from ASCs, scaffolding materials for adipose bone tissue engineering, and *in vivo* models for future clinical applications.

### Definitions and characterization of ASCs

Various terms have been used to describe the multipotent cells derived from white adipose tissue that adhere to plastic. The nomenclature includes adipose tissue-derived mesenchymal stem cells, adipose-derived adult stem cells, processed lipoaspirate cells, and human adipose-derived adherent stromal cells (Levi and Longaker, 2011). An agreement was reached at the Second Annual International Fat Applied Technology Society meeting to call these cells ASCs (Mitchell et al., 2006). ASCs are differentiated from the stromal vascular fraction, minimally processed cells that have not been exposed to a plastic surface (Bianco et al., 2008).

Similar phenotypes and receptor profiles are found between human MSCs and ASCs (Gronthos and Simmons, 1995). However, a definitive surface antigen profile that completely defines ASCs and allows prospective isolation does not exist. In spite of these inconsistencies, ASCs are commonly defined as cells that express the surface receptor molecules CD44 (hyaluronate) CD90, CD29 (integrin ß1), CD105 (endoglin), and CD49 (integrin α4) but do not express the hematopoietic markers CD45, CD34, and CD117 (cKit) (Yoshimura et al., 2006). Despite these definitions, a significant amount of transition occurs in ASC markers with passage, making it difficult to define a pure ASC population (Yoshimura et al., 2006).

### *In vitro* osteogenic differentiation of ASCs

The distribution of adipose tissue between men and women is significantly different. Adipose tissue from various locations also has different blood supplies, cytokine signaling, gene expression profiles, and, consequently, differences in osteogenic capacity (Peptan et al., 2006). The process of cell freezing and long-term storage inevitably alters cellular processes and characteristics (Valeri and Pivacek, 1996). *In vitro* proliferation and osteogenic and adipogenic differentiation decreases significantly after the freeze-thaw process (Oishi et al., 2008; Thirumala et al., 2010). Therefore, although cryopreserved cells can undergo osteogenic differentiation, a fresh harvest of human ASCs allows for more robust osteogenic reconstruction (Levi and Longaker, 2011).

ASCs are treated with osteogenic differentiation medium containing Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 $\mu$g/ml ascorbic acid, and 10 mM ß-glycerophosphate to induce osteogenic differentiation. ASCs proliferate and demonstrate upregulation of osteoblast-specific genes and mineralization, a sign of commitment to an osteogenic lineage.

### Growth and transcription factors involved in osteogenic differentiation of ASCs

Osteogenic growth factors bind to specific transmembrane receptors to initiate signaling cascades (Mayer et al., 2005). Of these, the bone morphogenetic protein (BMP) pathways, in particular, play an important role during osteogenic differentiation. BMPs are potent inducers of osteogenic differentiation (Kawai et al., 2003) and belong to the transforming growth factor (TGF)-ß superfamily. BMP initiates a signaling cascade through BMP receptor types I and II. These activated receptors subsequently phosphorylate the transcription factors Smad 1, Smad 5, and Smad 8 (Heldin et al., 1997). The phosphorylated Smads form a heterodimeric complex with Smad 4 and stimulate target genes such as Msx2, Dlx5, and Id proteins, which regulate proliferation of osteoprogenitors during early osteogenesis (Peng et al., 2004). Various BMPs induce differentiation of multipotent MSCs into both chondrogenic and osteoblast precursor cells (Cheng et al., 2003). BMPs also inhibit differentiation of adipocytes from bone marrow stromal precursors (Yoon et al., 2006). BMP-2 and BMP-7 (a.k.a. osteogenic protein-1) are currently used clinically to stimulate new bone formation (Mei et al., 2002). BMP-2 also acts synergistically with vitamin D(3) to promote osteogenic differentiation of ASCs (Song et al., 2011). On the other hand, TGF-ß generally opposes stimulated osteogenic differentiation by BMP-2 (Lee et al., 2003).

Insulin-like growth factor (IGF)-1 and IGF-2 are growth factors abundantly present in skeletal tissue (Andrew et al., 1993). IGF-1 systemically and locally regulates osteoblastic function (Gazzerro and Canalis, 2006). IGF-1 is a modest mitogenic factor on osteoblastic lineage cells and stimulates osteoblastic function and bone formation (Zhao et al., 2000). IGF-1 stabilizes beta-catenin by inducing phosphatidylinositol-3 kinase (pI3K) and activating Akt, which phosphorylates and degrades glycogen synthase kinase-3ß, an enzyme that phosphorylates beta-catenin before being degraded by ubiquitination (Canalis, 2009). IGF-2 enhances alkaline phosphatase (ALP) activity and the expression of later osteogenic markers and augments BMP-9-induced ectopic bone formation (Chen et al., 2010). Integrin α-5 (fibronectin receptor, alpha polypeptide) is a key IGF-2 mediator that promotes osteogenic differentiation of human MSCs (Hamidouche et al., 2010a,b).

Fibroblast growth factor (FGF)-2 is a growth factor that also has strong anabolic actions in bone *in vivo* and in osteoblasts *in vitro* (Naganawa et al., 2006; Sabbieti et al., 2008). FGF-18 positively regulates osteogenic differentiation in murine MSCs, triggered by FGFR1/FGFR2-mediated extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and PI3K signaling (Hamidouche et al., 2010a,b).

Runt-related transcription factor 2 (Runx2), also known as core binding factor alpha1 or acute myeloid

leukemia 3, is the most important transcription factor regulating osteoblastic commitment and osteogenic differentiation of MSCs (Teplyuk et al., 2009). Runx-2 is considered to be the master gene for osteogenic differentiation (Lian et al., 2006). Runx2-deficient mice completely lack osteoblasts and the ability to form bone, indicating an essential role for this factor in osteoblastogenesis (Hjelmeland et al., 2005). Runx2 promotes departure from the cell cycle and decreases proliferation but increases osteogenic differentiation during MSC differentiation (Lian et al., 2006). Activation of Runx2 in ASCs induces expression of the osteoblast-related markers type I collagen, ALP, and osteocalcin during the early stages of osteoblastic differentiation (Matsumoto et al., 2008). Runx2 expression and activity are closely regulated by other transcription factors as well as protein-DNA or protein-protein interactions (Jonason et al., 2009). Runx2 is regulated by phosphorylation in the ERK/MAPK pathway. Multiple signaling pathways converge on Runx2 to regulate osteoblastic differentiation, including binding with activator protein-1 and activating transcription factor-4, which control osteocalcin and osterix expression (Baek et al., 2009). Transfection of Runx2 in fibroblasts stimulates expression of osteoblast-specific genes such as osteocalcin, whereas inhibition of Runx2 during development in mice leads to the absence of osteoblasts and a severe phenotype of cleidocranial dysplasia, which is associated with perinatal death (Karsenty, 2001).

Osterix is a zinc finger transcription factor that is also important for osteoblastic differentiation and mineralization and acts downstream of Runx2 (Zhang, 2010). Osterix-deficient mice lack the ability to form osteoblasts (Nishio et al., 2006). BMP-2 promotes Runx2 and osterix expression in murine osteoprogenitor and osteoblastic cells (Long et al., 1995). Transfection of an osterix-expressing plasmid induces ASCs to differentiate into functionals osteoblasts without affecting their proliferation potential (Wu et al., 2007). Osterix-null mice have normal cartilage and normal Runx-2 expression, whereas Runx-2 null mice demonstrate delayed chondrocyte maturation and decreased osterix expression (Nakashima et al., 2002). More recent osterix knockdown studies demonstrated significantly impaired osteogenic differentiation of fetal bone cells (Chan et al., 2009).

Growing interest has been paid to adipogenesis, as an inverse relationship exists between adipocytes and osteoblasts in bone marrow (Gimble et al., 2006). Peroxisome proliferator-activated receptor gamma (PPAR γ), a transcription factor, plays a key role in adipogenic differentiation (Akune et al., 2004). A co-activator protein, transcriptional coactivator with PDZ binding motif (TAZ), functions as a link between Runx2 and PPAR γ. TAZ activates Runx2 and osteogenesis while suppressing PPAR γ and adipogenesis (Gimble et al., 2006). PPAR γ downregulation may be targeted to enhance osteogenic capability of ASCs.

## Gene transfer strategies to induce osteogenic differentiation from ASCs

Osteogenic genes can be transferred to cells with the aid of vectors to facilitate osteogenic differentiation and tissue regeneration from ASCs, which fall into two broad categories of viral and non-viral gene transfer.

Viral vectors have been extensively used to genetically modify cells (Koch et al., 2006). Viral vectors reach transfection efficiencies of 80-90%, which is difficult to achieve with non-viral vectors (Saraf and Mikos, 2006). Viral vectors, including retrovirus, lentivirus, adenovirus and adeno-associated viruses, have been used to enhance osteogenic differentiation of MSCs (Nicole et al., 2008). Adenovirus-mediated Runx2 overexpression enhances osteoblastic differentiation and mineralization in ASCs as evidenced by suppression of lipoprotein lipase and PPAR-γ expression at the mRNA level and reduced lipid droplet formation (Zhang et al., 2006).

Plasmid DNA has been largely employed as a non-viral gene transfer vector by crossing the cell membrane using physical methods, such as electroporation or chemical methods, such as lipofection (Lattanzi et al., 2005). Electroporation is the application of electric pulses to disturb cell membranes, creating temporary pores to pass DNA through cells (De et al., 2010). BMP-4 transferred by *in vivo* electroporation induces *in situ* ectopic bone formation in skeletal muscle (Kishimoto et al., 2002). Combined gene transfer of BMP-2 and BMP-7 into rat skeletal muscle using *in vivo* electroporation induces ectopic bone formation (Kawai et al., 2006). Electroporation-mediated transfer of the Runx2 or osterix genes enhances osteogenic differentiation of ASCs by increasing expression of BSP, type I collagen, osteocalcin. Runx2- or osterix-transfected ASC/poly (lactic-co-glycolic acid) (PLGA) scaffold hybrids promote bone formation after *in vivo* implantation in nude mice (Lee et al., 2011: Fig. 1). Cationized liposomes have also been studied extensively as a non-viral gene carrier for efficient gene delivery. Cationized liposomes are composed of various kinds of lipids such as quaternary ammonium detergents, the cationized derivatives of cholesterol and diacylglycerol, and the lipid derivatives of polyamines (Nishikawa and Huang, 2001). Complexation with cationized liposomes converts the charge of plasmid DNA to positive, which permits an interaction with the negative charge on the cell membrane, thus enhancing gene transfection into cells (Jo and Tabata, 2008).

## Bone tissue engineering from ASCs

Scaffolds prepared from biomaterials are very important when applying tissue engineering-based therapies (Tabata, 2009). Scaffolds confer biochemical, mechanical, and organizational motives for cells to attach, grow, differentiate, and form new tissue (Griffith, 2002). The physicochemial nature of the biomaterial

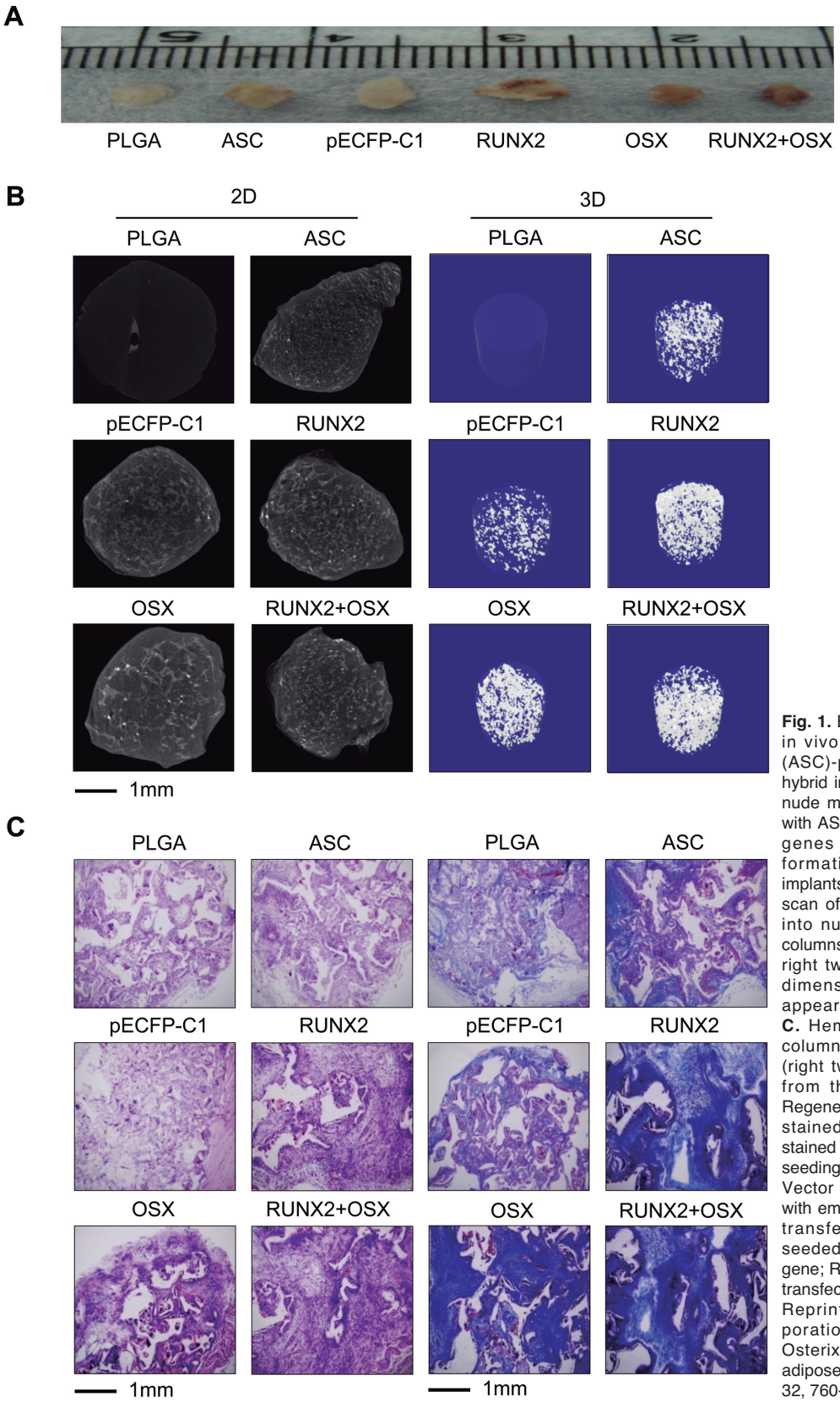


**Fig. 1.** Bone formation and mineralization after in vivo implantation of adipose stem cell (ASC)-poly (lactic-co-glycolic acid) (PLGA) hybrid into the dorsal subcutaneous spaces of nude mice for 6 weeks. The scaffold seeded with ASCs transfected with Runx-2 and osterix genes or both showed increased bone formation. **A.** Gross view of harvested implants. **B.** Microcomputed tomography (CT) scan of the ASC-scaffold complex implanted into nude mice for 6 weeks. The left two columns are two-dimensional images, and the right two columns are reconstructed three-dimensional images. Newly formed bone appears as white dots on the CT images. **C.** Hematoxylin and eosin (HE) (left two columns) and Masson's trichrome staining (right two columns) of histological sections from the implanted ASCs-PLGA hybrid. Regenerated bone is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink. Scale bars: 1 mm. PLGA: no seeding of cells; ASC: seeded with ASCs only; Vector only: seeded with ASCs transfected with empty vector; RUNX2: seeded with ASCs transfected with Runx2 gene; OSTERIX: seeded with ASCs transfected with osterix gene; RUNX-2 + OSTERIX: seeded with ASCs transfected with both Runx-2 and osterix gene; Reprinted with permission from "Electroporation-mediated transfer of Runx2 and Osterix genes to enhance osteogenesis of adipose stem cells". (Lee et al., Biomaterials. 32, 760-768, 2011; with permission).

influences cell behavior and differentiation (Dawson et al., 2008). Scaffolds deliver and retain cells and biochemical factors, and facilitate diffusion of vital cell nutrients (Dawson et al., 2008). Effective scaffolds have mechanical properties similar to the tissue they are replacing, are able to withstand inconsistent mechanical loads, and the final byproducts of degradable scaffolds are nontoxic with low antigenicity (Slaughter et al., 2009). Biomaterial matrices are also used to deliver genetic material or peptides, which can influence differentiation of the delivered stem cells. Scaffolds offer a highly adjustable vehicle for inductive factors such as BMP-2. In a laboratory setting, several investigators have used BMP-2-delivery methods to increase the osteogenic capability of ASCs (Peterson et al., 2005). BMP-2 also enhances osteogenic differentiation of surrounding cells. Tissue engineering is a promising therapeutic approach to treat the loss or malfunction of a tissue or organ. It involves differentiation of MSCs in temporary three-dimensional biomaterial scaffolds to form a new organ or tissue (Leor et al., 2005). A number of fabrication and surface modification methods have been employed to construct scaffolds with a variety of architectures, which can augment cellular behavior, create appropriate environments for tissue growth, and allow manipulation through incorporation of biomolecules (Ximena and Fotios, 2009).

Natural polymers used in bone tissue engineering comprise collagen, fibrin, alginate, silk, and hyaluronic acid (Chung and Park, 2007). Most natural polymers are biocompatible, degradable, and easily solubilized in physiological fluids. However, natural polymers have weak mechanical strength, which is necessary to provide sufficient structural support and protection for the seeded osteoblasts. Other disadvantages include immunogenecity, difficulty in processing, and a potential risk of transmitting animal-originated pathogens (Donzelli et al., 2007).

Synthetic materials have good chemical and mechanical properties with less immunogenicity compared to those of natural materials. PLGA, poly(caprolactone) (PCL), poly (anhydride), poly (phosphazene), poly (propylene fumarate), poly (ethylene glycol) (PEG), and block copolymers of polylactide (PLA) and PEG have been used as bone tissue engineering scaffolds (Lee and Shin, 2007). The combination of PLGA coated with a collagen gel also presents a good scaffold for osteogenesis and bone formation (Ochi et al., 2003). BMP-2 delivery by three-dimensional highly porous hydroxyapatite/collagen-poly(L-lactic acid) (HAC-PLA) scaffolds shows potential for osteogenesis and bone formation (Hu et al. 2003). A rhBMP2-PLLA porous polylactic acid scaffold is also applicable to tissue engineering (Chang et al., 2007). Osteogenic differentiation and bone formation of ASCs are comparable to those of bone marrow MSCs using a beta-tricalcium phosphate (ß-TCP) scaffold in nude mice (Han et al., 2008). Poly (L-lactide-co-caprolactone) quickly and preferentially binds ASCs, which rapidly proliferate and eventually differentiate into the osteogenic phenotype in a goat spinal fusion study (Vergroesen et al., 2011). PLA/ß-TCP composite scaffolds significantly promote ASC proliferation and total ALP activity compared to those in other scaffold types (Haimi et al., 2009). Electrospun composite scaffolds, consisting of PLA and ß-TCP at loading levels of 10 and 20 wt%, caused a dramatic increase in the amount of cell-mediated mineralization from seeded ASCs (McCullen et al., 2009). The bone biomimetic microenvironment, which is a nanocomposite scaffold composed of biphasic calcium phosphates (BCP) coated with a nanocomposite layer of PCL and hydroxyapatite nanoparticles (BCP/PCL-nHA), induces excellent osteogenic differentiation of ASCs (Lu et al., 2012). ASCs encapsulated in a collagen I gel with a PLGA-beta-TCP scaffold enhance skeletal repair in rabbits (Hao et al., 2010).The ASCs in coral scaffolds also induce successful repair of cranial defects in canine models (Liu et al., 2009).

### *In vivo* models for bone regeneration from ASCs

Consistent *in vivo* data is necessary to demonstrate the osteogenic capacity of ASCs and to show that *in vitro* findings can be applied clinically. Long bone skeletal defect models should mimic the ultimate clinical conditions which will be benefited by bone tissue engineering. Either femoral or tibial defects may be used as a load-bearing bone (Niemeyer et al., 2010). The ideal model should closely mimic the clinical condition of the osseous defect. Alternatively, a calvarial bone defect model provides an easier and less expensive defect model, making this a preliminary *in vivo* model that can be used before more extensive studies. Defects in long bone defect models should be created in a load-bearing location, and stable fixation must be provided. The femur usually tolerates a larger defect than that of the tibia. Peterson et al. demonstrated the use of ASCs, which were genetically modified to overexpress BMP-2, to heal a femoral critical-sized defect (Peterson et al., 2005). Human ASCs promote fracture healing and improve biomechanical function in rat femur nonunion (Hsu et al., 2008). A 4-mm mouse parietal bone model provides a reliably reproducible model for assessing calvarial defects. A critical-sized defect (4 mm) is one that will undergo <5% healing when followed for 8 weeks (Levi and Longaker, 2011).

Nude athymic animals should be used when testing human cells to decrease the inflammatory and immune responses. Increases in intereukin-1 and 6, as well as tumor necrosis factor alpha, are observed during bone healing (Gerstenfeld et al., 2003). Nude animals often demonstrate a decreased inflammatory response; they only lack a T cell response. However they still have an inflammatory response with regards to B cells and natural killer cells and possess osteogenic precursor cells

from the periosteum (Gerstenfeld et al., 2003).

## Conclusions

1. Adult stem cells isolated from the stromal vascular fraction of adipose tissue are called adipose stem cells (ASCs).
2. A definitive surface antigen profile that completely defines ASCs and allows prospective isolation does not exist and there is a significant amount of transition in ASC markers with passage, making it difficult to define a pure ASC population.
3. ASCs proliferate and upregulate osteoblast-specific genes and mineralization in standard osteogenic medium.
4. Growth factors or gene transfer of osteogenic genes to ASCs may be used to enhance osteogenic differentiation of ASCs.
5. Osteogenic growth factors for ASCs include bone morphogenetic proteins, insulin-like growth factors, and fibroblast growth factors. Key osteogenic transcription factors are Runx-2 and osterix.
6. Bone tissue engineering scaffolds for ASCs include natural and synthetic materials. PLGA or PCL polymer-coated scaffolds with ß-TCP are the mostly widely investigated biomaterials
7. Long bone skeletal defects in a load-bearing bone (femoral or tibial defects) are ideal models to study *in vivo* bone regeneration with ASCs, whereas the calvarial bone defect model provides an easier and less expensive defect model.

*Acknowledgements.* This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MEST) (2012M3A9B4028566).

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Accepted December 19, 2012