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Osteogenesis of Adipose-Derived Stem Cells

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1. Introduction

Mesenchymal stem cells (MSCs) are a group of multipotent adult-derived stem cells that can be isolated from the organs and tissues, including the bone marrow, ligaments, muscle and adipose tissue [1, 2]. MSCs may undergo self-renewal for several generations while maintaining their capacity to differentiate into multi-lineage tissues such as bone, cartilage, muscle and fat [3]. Bone marrow stem cells (BMSCs), one of the earliest multipotent stem cells to attract researchers' attention, have been studied for years and have gained some achievements. However, the stem cell population in bone marrow is estimated to be approximately 1 per 10^5 cells, and other tissues contain even fewer stem cells. Recently, research interest in the therapeutic potential of adipose derived stem cells (ASCs) has grown rapidly. Compared with BMSCs, ASCs are easier to obtain, have lower donor site morbidity, grow quickly, and are harvested in large numbers from small volumes of adipose tissue [4]. During culture in vitro, ASCs can be expanded for more passages because of their proliferative capacity, and they maintain their function after expansion or cryopreservation like BMSCs. ASCs demonstrate substantial in vitro and in vivo bone formation capacity that is similar to or greater than that of BMSCs [5]. Moreover, ASCs secrete potent growth factors, such as fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF), to stimulate angiogenesis, which is of vital importance for osteogenesis [6]. Bone tissue engineering offers a promising method for the repair of bone deficiencies caused by fractures, bone loss, and tumors. In bone regeneration, the use of ASCs has received attention because of the self-renewal ability and high proliferative capacity of these cells and because of their potential for osteogenic differentiation. Therefore, it is of significance to study the osteogenesis mechanism of ASCs for future clinical applications.

2. The isolation and culture of ASCs

Adipose tissue is composed of adipocytes and a heterogeneous set of cell populations, which, upon isolation, are termed the stromal vascular fraction (SVF), that surround and support the adipocytes [7]. The SVF includes ASCs, cells from the microvasculature such as vascular endothelial cells and their progenitors, vascular smooth muscle cells, cells with hematopoietic progenitor activity and leukocytes. Despite the fact that the SVF is a heterogeneous cell population, the subsequent expansion of ASCs selects for a relatively

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homogeneous cell population that is enriched for cells expressing a stromal immunophenotype, when compared with the heterogeneity of the crude SVF.

The ASCs that were isolated from the inguinal fat pads of mice were harvested as follows. Eight-week-old BALB/c mice were used in the study, in accordance with the International Guiding Principles for Animal Research (1985). All of the surgical procedures were performed under approved anesthetic methods using Nembutal at 35 mg/kg. Inguinal fat pads were harvested from the mice and extensively washed with sterile phosphate-buffered saline (PBS) to remove contaminating debris. Then, the fat pads were incubated with 0.075% type I collagenase in PBS for 60 min at 37°C with agitation. After removing the collagenase by dilution with PBS, the cells that were released from the adipose specimens were filtered through a 100 µm mesh to remove the tissue debris and were collected by centrifugation at 1,200 g for 10 min. This treatment resulted in the separation of harvested fat into three layers: the infranatant (the lowest layer, which is composed of blood, tissue fluid and local anesthetic), the middle portion (primarily fatty tissue), and the supranatant (the upper layer, which is the least dense and consists of lipids). The pellet from the infranatant was resuspended and incubated to remove contaminating red blood cells. Then, the pellet was washed three times with PBS and seeded on plastic tissue culture dishes in growth medium containing α-MEM, 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. The ASCs were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. The cells were passaged three times prior to osteogenic differentiation. After their transfer into specific medium containing dexamethasone (10⁻⁸ mol/L), ascorbic acid (50 mg/L), and β-glycerophosphate (10 mmol/L), the ASCs exhibited an obvious phenotype alteration and became osteogenic. The medium was replaced every 3-4 days for 14 days until the differentiated cells were confluent.

ASCs are adherent cells, which display a fibroblast-like morphology and align with a spindle-like or eddy-like shape. ASCs have proven to be difficult to identify in culture. Some studies have focused on particular cell markers to more easily recognize ASCs. Dominici et al. demonstrated that ASCs must express CD105, CD73 and CD90, and lack expression of the CD45, CD34, CD133, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules [6]. Mitchell et al. found that stromal cell-associated markers (CD13, CD29, CD44, CD63, CD73, CD90, and CD166) were initially low on SVF cells and increased significantly with successive passages [7]. Lin et al. observed the behavior of ASCs in culture, likened them to vascular and endothelial cells, and pinpointed markers CD34+/CD31-/CD104b- /SMA- in this differentiation [8]. The markers that are uniformly reported to be highly expressed are CD13, CD29, CD44, CD73, CD90, CD105, CD166 and MHC-I, while markers of the hematopoietic and angiogenic lineages, such as CD31, CD45 and CD133, have been reported to be lowly expressed or unexpressed on ASCs. MHC-II has also been found to be absent on ASCs. Moderate expression, in which the surface marker expression level is lower than 50%, has been reported for markers CD9, CD34, CD49d, CD106, CD146 and STRO-1.

ASCs have the ability to differentiate into cells of several lineages such as adipocytes, osteoblasts, chondrocytes, myocytes, endothelial cells, hematopoietic cells, hepatocytes and neuronal cells.

3. The mechanisms of osteogenesis – Growth factors and cytokines

The bone regeneration and repair process is not completely understood, and its molecular mechanisms have recently been paid an increasing amount of attention. Traditionally, the

process of bone healing has been defined by four stages: inflammation and clot formation, cellular infiltration and soft-callus formation, hard-callus formation, and remodeling. The mechanisms that drive the ASCs into the osteoblast lineage are still not clear, but research on growth factors and cytokines have provided much information about the effect of signaling molecules on cell proliferation, differentiation, adhesion, migration, and ultimate bone formation. Engineered tissues can be formed more efficiently by delivering genes that encode growth factors into ASCs through the use of electroporation, calcium phosphate precipitation transfection or lipofection of plasmids or viral vectors [9]. It is possible to accelerate the bone healing and regeneration process by gene transfection [10]. Therefore, the incorporation of the appropriate growth factors or cytokines within a progenitor population will allow for their use in bone regeneration.

A host of growth factors and cytokines are involved in the process of bone formation in developmental biology and distraction osteogenesis. BMPs, which are already used in the clinic, seem to be the most promising candidate cytokines in osteogenesis and ectopic bone formation. With the exception of BMP-1, BMPs are members of the TGF- β superfamily that were originally isolated from bovine bone extracts and were found to induce ectopic bone formation subcutaneously in rats [11]. This group of proteins includes sixteen BMPs and comprises nearly one-third of the TGF- β superfamily. BMPs are also involved in mesoderm induction, skeletal patterning and limb development [12]. BMPs transmit their signals via ligand binding to the heteromeric complex of types I and II serine/threonine kinase receptors on the cell surface[13]. The ligand signal is then transduced intracellularly via activation of the SMAD (signaling mothers against decapentaplegic) proteins, and then phosphorylated R-Smads and Smad4 subsequently migrate to the nucleus to effect the expression of the target gene and promote the osteogenic differentiation. BMP signaling also has been known to be transmitted via the MAPK (mitogen-activated protein kinase) pathway. Various subtypes of BMPs are observed to be expressed in obviously relevant tissues; for example, BMP-2 is expressed in the cartilage, periosteum and compact bone tissues. BMP-2, BMP-4, and BMP-7 exhibit good bone-forming activity when combined with collagen, hydroxyapatite (HA) and degradable high molecular polymer (HMP) in different animal bone defects experiments[14]. BMPs control both intramembranous and endochondral ossification through the chemotaxis and mitosis of mesenchymal cells, the induction of a mesenchymal commitment to osteogenic or chondrogenic differentiation, and programmed cell death. BMPs stimulate osteogenic differentiation in multiple cell lines, including fibroblasts, chondrocytes, BMSCs and ASCs. The effect of BMPs has also been noted to be concentration dependent. At low concentrations, BMPs foster chemotaxis and cellular proliferation, while at high concentrations, BMPs induce bone formation [15]. BMPs are more potent at inducing bone formation as heterodimers than as homodimers. In culture, BMP-2/6, BMP-2/7, and BMP-4/7 heterodimers have been shown to promote higher alkaline phosphatase levels than homodimer combinations [16, 17, 18]; these data have also been corroborated in vivo [19]. BMP-2, BMP-4, BMP-6, BMP-7, and BMP-9 are considered to be the most osteoinductive of the BMP proteins [20]. It is believed that BMPs regulate osteoblast differentiation via the increased transcription of core-binding factor-1/Runt-related family 2 (Cbfa1/Runx2), a molecule that is known to be necessary for commitment along an osteoblastic lineage [14]. The BMP and Wnt signaling pathways regulate the osteoblastic differentiation of mesenchymal stem cells (MSCs). The Wnt pathway plays an essential role in bone regeneration, and it has been observed that Notch-1 overexpression inhibits osteoblastogenesis by suppressing Wnt/beta-catenin but not BMP

signaling [21]. Notch enhances the BMP-2-induced osteoblastic differentiation by overexpression of Delta1/Jagged1-activated Notch1 signaling in MC3T3-E1 cells [22]. Hence, there are many singling pathways play role in osteogensis. Here, our group has focused on BMP-2 and Notch.

BMP-2 is a pleiotropic regulator that governs the key steps in the bone induction cascade such as the chemotaxis, mitosis and differentiation of mesenchymal stem cells in the process of bone healing [23, 24]. There have been some reports describing the effectiveness of BMP-2 in the osteogenesis of BMSCs and ASCs, but it is unclear whether BMP-2-enhanced ASCs can heal large bone defects [10, 25]. Our group harvested ASCs from normal SD rats and transfected them with the BMP-2 gene before they were loaded onto alginate gel. The ability for bone regeneration was determined in critical-size rat cranial defects. An 8-mm diameter defect was created in the calvarias of 36 rats, and then these rats were divided into three groups. In the experimental group, the defects were filled with alginate gel combined with BMP-2-transfected ASCs; in the negative control group, the defects were filled with alginate gel mixed with normal ASCs; in the blank controls, the defects were filled with cell-free alginate gel. To identify the molecular events leading to the formation of new bone, we investigated the expression of biochemical markers by using RT-PCR and western blotting over the course of the BMP-2 enhanced ASCs differentiation. In the experimental group, weak osteogenesis was noted in the epidural region of the border of the defect at 8 weeks. After 16 weeks of treatment, the continued formations of new bone throughout the defects were observed. In the negative control group, bone islets formed by interstitial osteogenesis were observed in various connective tissues after 16 weeks. In the blank control group, the alginate gel was absorbed at 4 weeks. The RT-PCR analysis of OPN, OCN, RUNX2 and BMP-2 demonstrated that there was a significant difference in the expression of these genes between the experimental and control groups. Continued high expression of OPN, OCN, RUNX2 and BMP-2 was observed throughout the progression of the experimental group both in vitro and in vivo. In the negative control group, these genes were observed neither in vitro nor in vivo at 8 weeks; only at 16 weeks after surgery, a weak expression of these genes was observed. In the blank control group, these genes were not detected at 8 and 16 weeks post surgery. The western blot results were similar to the RT-PCR results, but the OPN, OCN, RUNX2, and BMP-2 proteins were not observed in the negative and blank control groups. The expression of OPN and OCN inside of the cranial defects made sure that osteogenesis and the maturation of BMP-2-enhanced ASCs occurred. Our research indicated that alginate gel with BMP-2-enhanced ASCs was necessary for critical-size defect repair, and load-bearing alginate with BMP-2-enhanced ASCs can be applied in engineering approaches for further clinical use.

Notch signaling plays a key role in the determination of cell fate and in the progenitor's maintenance in the normal development of many tissues and cell types. An evolutionarily conserved mechanism is to maintain a balance between the differentiation and proliferation of a diverse range of stem/progenitor cells and to enable them to adopt distinct cell fates [26]. Previous investigations have shown that Notch signaling positively regulates the osteoblastogenesis of several types of cells, such as murine bone marrow mesenchymal progenitors [27], ST-2 marrow stromal cells [21], Kusa mesenchymal progenitor cells [28], M3T3-E1 osteoblastic cells [29] and C2C12 myoblasts [30]. Other research has concluded that Notch is a positive regulator of osteogenesis in COS-7 cells [31] and MC3T3-E1 cells [32]. However, the enhancement of osteogenic gene expression was not observed in Tezuka's report.

In mammals, Notch signaling is mediated by the intracellular interactions of type I transmembrane ligands, such as Delta and Serrate, with Notch receptors (Notch-1, Notch-2, Notch-3 and Notch-4). Once it is bound to its ligand, the Notch receptor is cleaved by the metalloprotease TNF- α converting enzyme and the γ -secretase complex, at two sites, to generate the Notch intracellular domain (NICD) [33]. This domain is transported to the nucleus and binds a CCAAT-binding protein (CBF-1), which is also called CSL. CSL acts as a transcriptional repressor in the absence of NICD, which recruits a co-repressor complex and inhibits the transcription of target genes that contain the CCAAT binding sites [34,35]. As a consequence of binding, NICD displaces the repressor complex of CSL and recruits nuclear co-activators, such as mastermind-like 1 (MAML1) and histone acetyltransferases [36], to convert CSL into a transcriptional activator. Notch activation through the CSL-NICD interactions can activate the transcription of various target genes, including Hes (Hairy/Enhancer of Split) [37], Hes-related repressor protein (HERP) [38,39], nuclear factor- κ B (NF- κ B) [40] and PPAR (peroxisome-proliferator-activated receptor) [41].

The Notch system is known to be an evolutionarily conserved mechanism that balances the differentiation and proliferation of stem/progenitor cells [42], with NICD acting to keep the cells in an undifferentiated state during development [43]. N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester (DAPT) is a γ -secretase inhibitor that can block Notch signaling by preventing the cleavage of Notch receptors, which has been widely used to evaluate the biological behaviors and Notch signaling pathway in various cells such as BMSCs, muscle stem cells, neural stem cells, and human tongue carcinoma cells [42]. It will be beneficial to consider the influence on the osteogenesis of ASCs by regulating Notch signaling with DAPT. We investigated, for the first time, the effects of DAPT on the proliferation and osteogenesis of ASCs by using an *in vitro* 1,25-Dihydroxyvitamin D₃(VD₃)-induced osteogenic differentiation system. The results showed that ASCs cultured in DAPT had significantly decreased CFU numbers in comparison with those cultured in control medium during a 2-week culture period. DAPT clearly inhibited the ASCs' proliferation at all doses, which indicated that ASCs responded with decreased growth when the Notch pathway was blocked. The alizarin red results indicate that the addition of DAPT to the VD₃ treatments increased osteogenesis in ASCs. Real-time PCR showed the expression levels of the Notch downstream target genes, Hes-1 and Hey-1, were decreased after DAPT treatment. Immunofluorescence staining revealed that Hey-1 was down-regulated when Notch signaling was inhibited by DAPT. However, Real-time PCR and Western blot analysis showed the up-regulation of Runx2 and OSX after DAPT treatment. Hey-1, which is expressed in the nucleus of ASCs and acts as a transcriptional repressor, was down-regulated when Notch signaling was inhibited by DAPT, whereas the expression of Runx2, an essential transcription factor that is required for osteogenesis, was increased in the nucleus of osteogenic ASCs after DAPT treatment. This finding indicates that the Runx2 dependent osteogenic differentiation of ASCs was enhanced when the interaction between Runx2 and the Notch target gene Hey-1 was suppressed in the presence of DAPT. In accordance with what has previously been reported, Notch repressed osteoblastic differentiation through its target genes and Runx2 [44]. Therefore, our study demonstrated that DAPT reduced the proliferation and enhanced the osteogenesis of ASCs via the regulation of Notch and Runx2 expression. We also found that the adipogenesis of mouse adipose-derived stem cells (mASCs) can be enhanced by the coordinated regulation of Notch and PPAR- γ . DAPT comprehensively inhibited the Notch signaling pathway and consequently influenced Hes-1 expression, which may directly or indirectly reduce DLK-1

/Pref-1, an inhibitor of the adipogenic transcription activator PPAR- γ . The continuous repression of DLK-1/Pref-1 with the activation of PPAR- γ dephosphorylation promotes the adipogenesis of mASCs. All of these findings imply that Notch signaling plays an important role in the fate determination of ASCs.

The FGFs (fibroblast growth factors) are a highly conserved family of twenty-four proteins that transmit their signals via a family of four transmembrane tyrosine kinases. The most abundant ligand of the family, FGF-2 may increase osteoblast proliferation and bone formation both *in vitro* and *in vivo* [45]. Exogenous FGF-2 was able to rescue the decreased bone nodule formation in osteoblast cultures from these transgenic mice.

TGF- β (transforming growth factor- β) enhances the osteogenic differentiation of MSCs by promoting mitosis, calcium phosphate deposition, Col I synthesis and adipogenesis suppression. The expression of IGF-1 mRNA, which is up-regulated by TGF- β s, may also promote the osteogenic differentiation of BMSCs.

IGF-2 (insulin-like growth factor) has been known to stimulate bone collagen synthesis, osteogenesis and chondrogenesis. In a transgenic mouse, where the IGF expression was up-regulated in osteoblasts, bone formation of the distal femur increased as compared with the control group [46]. Histology showed no increase in the number of osteoblasts, which suggests that IGF-1 up-regulated the activity of the existing bone-forming cells. The size and bone-formation rates of the IGF-1 knockout mice were significantly reduced as compared with their wild-type littermates [47].

PDGF (platelet-derived growth factor) has also been demonstrated to be a potent stimulus for osteoblast proliferation, chemotaxis, and collagen activity. PDGF is now being used clinically in periodontics; the application of recombinant human PDGF in a tricalcium phosphate matrix significantly increased periodontal bone formation. [48]

Hormones, including estrogen, glucocorticoid, and parathyroid hormone, are also considered to influence the bone metabolism directly or indirectly. Estrogen up-regulates the transcriptional expression of osteoblast-related genes such as ALP, Cbal, BMP-2 and TGF-1. Physiological concentrations of glucocorticoid can stimulate the osteoblastic differentiation of MSCs. However, an inhibition of osteoblast proliferation and apoptosis and a reduction of active osteoblast-composition, which may lead to osteoporosis, may be observed if large doses of glucocorticoid are applied for a long period of time. In mature bone tissue, parathyroid hormone either decomposes or synthesizes bone by promoting bone growth or filling up lacunas created by osteoclast[49].

Bone formation by the implantation of ASCs must be preceded by the *in vitro* osteogenic differentiation of these cells. The differentiation procedure has the disadvantage of requiring additional culture time and steps, including the use of large amounts of costly growth factors such as bone morphogenetic protein (BMP), and dexamethasone, which may be cytotoxic to cells, prior to implantation to achieve therapeutic efficacy. New methods aimed at reducing the culture period and the amount of required growth factors and enhancing the efficiency of osteogenesis and thus of bone regeneration should be developed. One approach is the delivery of cytokines by incorporating these molecules into scaffolds such as microspheres and liposome. This approach would allow the growth factors to be retained at the site of interest for an extended period of time while maintaining the proteins' biological activity. Moreover, engineered ASCs that are produced by gene transduction by various virus-vectors have evolved to be an attractive option to ameliorate bone repair, especially large bone defects. Transfecting ASCs with genes for BMP-2, BMP-4, BMP-7, Runx-2 or

Osterix is considered to promote bone formation *in vivo* following implantation of the ASCs [10, 24, 25].

4. Ectopic bone formation of ASCs and In situ repair of critical – Size cranial defects

Bone grafting and bone substitutes are required in many orthopedic and dental procedures such as spinal fusion, the revision of hip prostheses, the repair of non-healing fractures, or the reconstruction of large bone defects. Although autografts are the gold standard for the clinical repair of large defects, unsatisfactory results occur in as many as 30% of cases [50], and autografting can be restricted by donor tissue shortage and morbidity [51]. Allografts are limited in usage owing to immunological rejection, possible transmission of infectious diseases and premature resorption. Bone marrow-derived mesenchymal stem cells (BMSCs) are particularly promising as they can heal large segmental defects and can be genetically modified to augment *in vivo* bone formation [52,53]. ASCs and BMSCs are similar with respect to growth, morphology, immunoprivileged properties and the ability to differentiate into chondrocytes, osteoblasts and adipocytes [54]. Furthermore, ASCs are reported to be slightly better than BMSCs with regard to osteogenic and chondrogenic differentiation potential; ASCs are also easy to isolate through liposuction and are available in large quantities, which has prompted the use of ASCs to repair cranial defects in animals and in clinical studies [55].

ASCs have osteogenic differentiation potential. Additionally, the biomaterial and the medium that were used enhanced the osteogenic differentiation of the cells. The ASCs showed an ability to adhere to and proliferate on scaffolds *in vitro*. *In vivo*, ASCs survive in low oxygen environments, which makes them good candidates for cell-based therapies in which the oxygen supply may be limited during the post implantation period when a blood supply is lacking[56]. However, ASCs secrete angiogenic cytokines such as vascular endothelial growth factor and hepatocyte growth factor, and these are considered to contribute to the angiogenic properties of ASCs[57]. It was considered that the transplanted ASCs produce cytokines and chemokines that act as homing signals to attract endogenous stem cells and progenitor cells to the site of injury. Thus, the presence of ASCs may enhance the osteogenic and angiogenic conditions of the construct *in vivo*, and the bone-forming capacity of ASCs in combination with various scaffold materials has been well reported [58]. Inorganic materials such as bioceramics, biodegradable polymer materials and composite materials have been commonly used in combination with ASCs to repair bone defects; for example, hydroxyapatite (HA)/tricalcium phosphate (TCP)[59], PLGA[60], chitooligosaccharide (COS)[61], fibrin/HA[62], and biphasic calcium phosphate nanocomposite (NanoBCP)[63] have all been used for these purposes. Heather L. et al. [64] examined the cell coverage and cell function of ASCs on different biomaterials, such as silicone rubber, fibronectin, dualligand , oxygen plasma plus fibronectin, polyimide and polyurethane. They found that cell attachment was very strong on both polyimide and polyurethane for all of the attachment methods; none of the attachment methods caused any differences in basic cell functions, including proliferation, metabolism, intracellular ATP concentration, and caspase-3 activity. However, ectopic bone formation inside of the porous ceramic blocks revealed that the material properties such as composition, geometry, porosity, size, and microstructure might be important but not sufficient parameters for evaluating appropriate bone formation [65].Moreover, β -TCP granules have been in clinical

use in Europe for over 20 years under the name CEROS 82, and investigations have been published concerning the clinical value of Chronos1 β -TCP in the bone environment [66].

Cytokines can induce healing in satisfactory biologic environments and are reported to improve the ability of ASCs to form bone when supplied in osteoinductive medium or coated onto biomaterials [62]; however, contradictory reports have been published with regards to this finding [67]. Engineered ASCs combined with gene modification have evolved to be an attractive option to ameliorate bone repair, especially large bone defects [68]. The co-delivery of BMP-2 and Runx-2 was a useful tool to enhance the osteogenesis of ASCs both in vitro and in vivo [69]. BMP-2/VEGF-[70], Runx2- or Osterix-transfected ASCs promoted bone formation in vivo following implantation [71].

Some researchers believed that ASCs should be induced into osteogenic lineages before they are seeded into scaffolds as more new bone tissues were observed when osteogenetic differentiation ASCs were seeded on PLGA in a rat critical-sized calvarial defect model [72,63]; this approach also had the advantage of avoiding the use of cytotoxic dexamethasone and had an additional culture period when it was used in a clinical application. Additionally, the physiological differences between individuals might influence the osteogenic and proliferative capacity of the expanded cells, as well as the microenvironment in the recipient site. The number and concentration of osteogenic cells in a scaffold are important for successful bone formation in vivo [73, 74].

Ectopic and in situ repair of cranial bone defects with ASCs and various scaffolds have been observed in mouse, rabbit and canine models [75, 76, 77]. Ectopic bone formation, inside of a muscle free flap, with autoASCs has been performed to reconstruct a large bony defect [56]. Ectopic bone formation was found when BMP-2- (BMP-2-ASC) or BMP-2/ Runx2- (BMP-2/ Runx2-ASC) transfected ASCs were seeded on PLGA biodegradable scaffolds and then implanted into the dorsal subcutaneous spaces of the mice [69]. ASCs are considered to be a suitable resource for cranial defects. The preferential expression of the HMWFGF-2 form is associated with a more osteogenic differentiated state of calvarial osteoblast. Murine ASCs undergoing osteogenesis recapitulate the in vivo osteogenic differentiation expression pattern of FGF ligands and receptors of calvarial mesenchymal cells during their own osteogenic differentiation[78]. Chin-Yu Lin et al. [70] confirmed the potential of the FLP/Frt-mediated baculoviral vector recombination for sustained BMP-2 /VEGF expression in ASCs, and implantation of the engineered ASCs not only accelerated the weight-bearing segmental bone defect healing but also ameliorated the bone metabolism, bone volume, bone density, angiogenesis and mechanical properties so as to repair the massive bone defects. Additionally, 84% to 99% of the in situ new bone was derived from implanted cells when hASCs were transplanted onto PLGA to repair critical-size rat cranial defects successfully [72]. ASCs have been used clinically in a microvascular flap composed of autoASCs, and β -TCP and BMP-2 have been successfully used in a large bone defects reconstruction surgery [79]. Additionally, ASCs cultured in platelet- rich plasma have been successfully used to regenerate bone in rats' periodontal tissue defects [80].

Our group has performed research on the formation of ectopic and in situ new bone by osteogenic ASCs combined with biphasic calcium phosphate nanocomposite (NanoBCP), with high strength and porous structures. The NanoBCP constructs containing osteogenic ASCs were transplanted into nude mice subcutaneously for 8 weeks to acquire the physiological behavior of induced ASCs during ectopic differentiation in vivo. Critical-size rat cranial defects were used as the model to determine the efficiency of engineered constructs in the generation of new bone in situ. Histological analysis of the retrieved

specimens from nude mice in the experimental group showed obvious ectopic bone formation, and there was positive expression of osteopontin (OPN) and osteocalcin (OCN) at the RNA and protein levels. There was complete repair of the cranial defects in the experimental group, but only partial repair in the negative controls. Combining osteogenic ASCs with NanoBCP can lead to the formation of ectopic new bone. Furthermore, the approach can also stimulate bone regeneration and repair for large bone defects. Based on our results, we thought that load-bearing NanoBCP with ASCs could be applied in engineering approaches for further clinical usage. Patients' own ASCs would be an ideal cell source for bone tissue engineering, and autologous non-immunogenic bone tissues could be easily regenerated with this approach for the repair of large size bone defects.

Our goal is to develop a less invasive and more effective method for clinical use in bone regeneration. However, most of the animal models that are chosen for studies clearly belong to low-order phylogenetic species with a characteristically high potential for osteogenesis, and extending the experimental results relative to rate and amount of bone regeneration from animal models to humans is difficult. Additionally, the size of the defects that are likely to be treated in human subjects is usually much greater than those that were evaluated in this study. Consequently, a further investigation of large animal vertical augmentation models will be necessary before a similar protocol could be applied to bone reconstruction in the clinic.

5. New research in the field

The use of ASCs as an autologous and self-replenishing source for a variety of differentiated cell phenotypes provides much promise for reconstructive surgery. Therefore, research of ASCs and osteogenesis has been the focus of attention in recent years, both in basic research and clinical application.

The animal species for ASC cell sourcing have been expanded. ASCs can be recovered from wild Scandinavian brown bears and then grown in standard cell culture medium in monolayer cultures; ASCs from yearlings spontaneously formed bone-like nodules surrounded by cartilaginous deposits, which suggested the differentiation into osteogenic and chondrogenic lineages[81]. This is the first report of ASCs spontaneously forming extracellular matrix that is characteristic of bone and cartilage in the absence of specific inducers, and this ability appears to be lost gradually with age. Therefore, hibernating brown bears are considered as a model to study the osteogenesis mechanisms and disuse osteoporosis. ASCs were reported to be isolated based on a gradient solution and enzymatic digestion, and then several cell components were harvested. Rada T. et al. developed a method based on the use of immunomagnetic beads coated with specific antibodies, which could be used to study niches in ASC populations [82]. ASCs are further found to express stem cell markers (Oct4, Nanog, CD90 and CD105) and lineage-specific markers following induction; the expression of ALP, phosphoprotein (SPP1), Runx2 and OCN mRNA were positive in osteogenic lineages, and peroxisome proliferator activated receptor (PPAR γ 2) mRNA was positive in adipogenic lineages[83]. These cells are similar to but distinct from other adult stem cells. The expression of chemokine receptors such as CCR1/4/7 and CXCR6/4 in hASCs was higher than in BMSCs [84]. These receptors and their ligands and adhesion molecules play an important role in the tissue specific homing of leukocytes and have also been implicated in the trafficking of hematopoietic precursors into and through the tissues. Thus, ASCs may show a better migration and homing capacity, and they may be

a better candidate for bone regeneration. Meanwhile, a protocol for labeling ASCs with a readily available PET tracer, FDG, has been developed [85]. ASCs can be safely labeled with FDG concentrations up to 25 Bq/cell, without compromising their biological function. The initial biodistribution of the implanted FDG-labeled stem cells can be monitored using microPET imaging; this may provide a favorable method for long-term *in vivo* tracking for clinical usage.

Some new research on the osteoblast differentiation of ASCs and related factors should be noted. BMP-2 governs the key steps in the bone induction cascade such as chemotaxis, mitosis, and the differentiation of mesenchymal stem cells, which is applied in the clinical routine [86]. However, BMP-2 has a significant disadvantage; when it is used alone, it may induce a surplus of callus formation, and bone may develop in muscles (heterotopic ossification) [87]. However, Claudia K et al. just reported that the combination of ASCs and BMP-2 in a fibrin matrix significantly reduces callus formation when compared with BMP-2 alone [88]. Lin et al. reported that, compared with ASCs transiently expressing BMP-2, ASCs persistently expressing BMP-2 not only accelerated the healing of a weight-bearing segmental bone defect but also ameliorated the bone metabolism, bone volume, bone density, angiogenesis and mechanical properties [70]. BMP-6 also has been demonstrated to induce the osteogenic and chondrogenic differentiation of MSCs for tissue engineering and regenerative applications [89, 90]. hASCs were considered to express all components of the BMP/BMP receptor signaling pathway and respond to BMP-4 inducing up-regulated expression of its specific target genes Id1-Id4 [91]. BMP-4 effects on hASCs are dose-dependent. High doses significantly increased apoptosis and drastically reduced cell proliferation, whereas low doses of BMP-4 (0.01-0.1ng/mL) significantly increased culture cell content, cycling cells and reduced the number of apoptotic cells. Treatment of hASCs with low doses of BMP-4 did not modify the expression of Nanog or Oct4 or void their osteogenic or osteoblastic differentiation capacities. Natalina Q et al. demonstrated that FGF-2 treatment sustains the proliferative and osteogenic potential state of mASCs, while inhibiting their terminal osteogenic differentiation by antagonizing the retinoic-acid mediated up-regulation of BMPR-IB [96]. In their follow-up study, they further found that FGF ligand genes, such as FGF-2, FGF-4, FGF-8, and FGF-18, displayed a differential and dynamic profile during mouse ASC (mASC) osteogenesis[56]. Fgf-2 transcript was down-regulated, while Fgf-18 transcript level was strongly up-regulated. Also recent research has proven that the transfer of Runx2 or Osterix genes can enhance the *in vitro* and *in vivo* osteogenic differentiation of ASCs [69, 71].

The culture conditions appeared to affect the osteogenic differentiation capacity *in vitro*, with more robust osteogenic differentiation seen in ASCs cultured in medium supplemented with human serum derivatives in or in SF conditions compared with FBS supplemented media [93, 94]. 17 beta-estradiol E(2) may stimulate the osteogenic differentiation of ASCs and therefore, can be used as an inducing agent to improve the efficiency of these cells in *in vitro* and *in vivo* studies [95]. Jing et al. demonstrated that VD3 induced the osteogenic differentiation of ASCs [96]. Song et al. suggested that vitamin D3 treatment, throughout the culture period with BMP-2, added in the later period is an effective and economical way of inducing the osteogenic differentiation of ASCs [87]. Gender differences were found to affect the osteogenic capacity of ASCs, with male ASCs differentiating more rapidly and more effectively than female ASCs *in vitro* [97]; the adipogenic potential was unchanged irrespective of age, while the osteogenic potential appears to decrease with increasing age [98]. These differences are likely due to the different

steroid functions in males and females with hormone levels varying at different phases of life, which must be taken into account when designing clinical treatments for patients.

Osteoblastic differentiation of ASCs is still mainly used in the laboratory experiments or animal trials, and there are few studies about its clinical application. To date, two clinical case studies have been reported where the capacity of ASCs in bone tissue repair has been investigated [99, 56]. In the first case, the patient was a 7-year-old girl, who had sustained severe head injury after a fall that resulted in a closed multifragment calvarial fracture. The calvarial defect was treated with autologous ASCs that were isolated and applied in a single operative procedure in combination with milled autologous bone from the iliac crest. ASCs were supported in place with an autologous fibrin glue, and mechanical fixation was achieved with two large, resorbable macroporous sheets that acted as a soft tissue barrier. The new bone formation and near complete calvarial continuity was observed 3 months after the reconstruction. The harvesting of bone tissue or a composite microvascular flap is frequently followed by morbidity and a donor site defect despite. Furthermore, a large amount of autologous blood is needed for plasmapheresis, which may, in some cases, be difficult to obtain. It is known that ASCs can secrete angiogenic factors that promote neovascularization and vessel-like structure formation [100]. In the second case, K. Mesimäki et al. reported the reconstruction of maxillary defect of a 65-year-old male patient, who underwent a hemimaxillectomy due to a large keratocyst, with a microvascular flap using auto-ASCs, beta-tricalcium phosphate and bone morphogenetic protein-2 [56]. It was the first clinical case in which ectopic bone was produced using autologous ASCs in microvascular reconstruction surgery. The successful outcome of this clinical case paves the way for extensive clinical trials using ASCs in custom-made implants for the reconstruction craniofacial bone defects.

Because ASCs have bright prospects in clinical stem cell therapy, improved methods to assess safety, efficiency, reproducibility and quality of the vitro expanded or osteoblast differentiated stem cells are urgently called for. These methods must be not only safe in vitro, but also in vivo and in the clinical. The cell source, culturing components such as FBS and osteoinductive supplements, and the cell expansion time may have considerable effects on the cells at the gene level and may affect the quality and safety of the cell products. Furthermore, producing cells that are genetically stable and nontoxic is a step towards ensuring that the cells do not transform and lead to a genetically aberrated progeny or virus infection when transplanted into the recipient, especially tissues engineered ASCs with gene transfected by virus vectors. Few studies have been carried out on the mechanism of immunohistochemical characteristic by ASCs. Therefore, assessing the immunogenic properties of the cells in vitro and in vivo is important to assure that anaphylactic reactions in the recipient are avoided. Reports have shown that the immunosuppressive capacity of the ASCs may, in some cases, favor the growth of tumor cells, but contradictory results exist [101, 102, 103]. These controversial results indicate that further studies are necessary to fully elucidate the true effect of ASCs on tumor formation. Hence, further pre-clinical safety and efficacy studies are required to assess and verify the safe outcome of the clinical procedure using in vitro expanded or osteoblastic differentiated stem cells.

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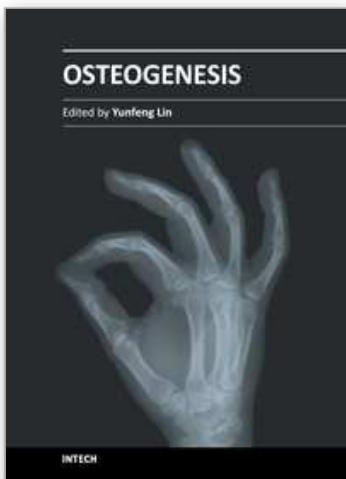
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