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Osteogenic Differentiation of Human Amniotic Fluid–derived Stem Cells Induced by Bone Morphogenetic Protein-7 and Enhanced by Nanofibrous Scaffolds

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Abstract

Amniotic fluid–derived stem cells (AFSCs) are becoming an important source of cells for regenerative medicine given their apparent advantages of accessibility, renewal capacity and multipotentiality. In the intermediate stage between the embryonic stem cells (ESCs) and adult stem cells, AFSCs may have a distinct mechanism to choose their fate. Unfortunately, until now, little is known about how bone morphogenetic proteins (BMPs) control the osteoblastic differentiation of AFSCs, especially on 3D scaffolds. Our research shows that human AFSCs (hAFSCs) can be induced for osteoblastic differentiation by rhBMP-7, and hAFSCs respond to rhBMP-7 more strongly than human mesenchymal stem cells (hMSCs). As synthetic ECM, scaffolds play a central role in tissue engineering. The hAFSCs, on the nanofibrous scaffolds (NF scaffolds) with morphology similar to that of natural collagen fibers, showed significantly enhanced alkaline phosphatase (ALP) activity, calcium content, von Kossa staining and the expression of osteogenic genes than those on the traditional scaffolds, i.e. solid walled scaffolds. The data on the bone formation *in vivo* presented further evidence that biomimetic NF scaffolds provided hAFSCs a more favorable synthetic ECM, and thus, facilitated the osteogenic differentiation of hAFSCs. The relative strong responsiveness to rhBMP-7 makes hAFSCs promising in bone regeneration. The synthetic NF scaffolds, which mimic the morphology of natural collagen fibers, enhanced the osteoblastic differentiation of hAFSCs *in vitro* and bone formation *in vivo*.

Keywords

Human amniotic fluid–derived stem cell; bone morphogenetic protein; extracellular matrix; nanofiber; tissue engineering scaffold; bone

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1. Introduction

Amniotic fluid-derived stem cells (AFSCs) are becoming an important source of cells for cellular therapy. They have the ability to differentiate into cells of all three embryonic germ layers and have a high proliferation rate [1,2]. Considering the intermediate status of AFSCs, between embryonic stem cells (ESCs) and adult stem cell types, we are interested in the unique ways to control their cell fate. Little is known about the cues that regulate the AFSCs ability to differentiate to osteoblasts. This is especially true concerning their interactions with growth factors and scaffolds, which are the essential elements in tissue engineering.

Among the growth factors related to bone, bone morphogenetic proteins (BMPs) are probably the most important growth factors in bone formation and healing [3]. These growth factors have been extensively studied during recent decades and are widely used in bone tissue engineering. Among the BMP members, BMP-2, 4, 6, 7 and 9 are found to have distinct osteogenic activity. rhBMP-2 and rhBMP-7, are most studied *in vitro* and *in vivo* [4]. A number of clinical studies have assessed the efficacy of rhBMPs in the healing of critical-sized bone defects and the acceleration of bone fracture healing in humans [5,6]. While the latter studies showed certain promising results, substantially high doses of BMPs were required to induce adequate bone formation compared with animal models, and large variations in response among individual patients were observed [7]. The level of responsiveness of human mesenchymal stem cells (hMSCs) to rhBMPs was reported to be lower than that of rodent cells [8,9]. It is crucially important to evaluate the responsiveness of hAFSCs to rhBMPs, which can partially predict their potential in bone tissue engineering. Although previous studies have shown that hAFSCs can be induced to differentiate along osteoblastic lineage by dexamethasone (DEX), we are more interested in the role of BMPs in regulating the osteogenic differentiation of hAFSCs because it is BMPs not DEX that regulates the bone formation *in vivo*.

Extracellular matrix (ECM) and growth factors are part of the natural signaling environments to regulate stem cell fate [10]. Many extracellular proteins have a fibrous structure with diameters on the nanometer or submicrometer scales [11,12]. Scaffolds play a pivotal role in tissue engineering by defining the three-dimensional templates and synthetic ECM environments for tissue regeneration [12]. Whereas traditional tissue engineering scaffolds were based on macroporous hydrolytically degradable materials, current approaches emphasize the control over cell behaviors and tissue formation by nano-scale topography that closely mimics the natural ECM [13,14]. We have developed synthetic nanofibrous (NF) scaffolds with morphology similar to that of natural collagen fibers under the hypothesis that synthetic NF scaffolds would mimic the morphological function of collagen fibers and create a more favorable microenvironment for cells than solid walled scaffolds (SW scaffolds) [15–17]. Previously, we reported that the nanofibrous architecture generated by a phase separation technique improved the features of protein adsorption including serum fibronectin and vitronectin, which may mediate cell interactions with scaffolds. This explained why cells attached on NF scaffolds at a higher level than that on SW scaffolds [18]. Further investigation found that the differentiation of MC3T3-E1 pre-osteoblastic cells and neonatal mouse osteoblastic cells was also enhanced on NF scaffolds [19,20].

Although the previous studies have shown that AFSCs can be induced to differentiate to osteoblasts *in vitro* and *in vivo* [1], there are still vital questions need to be answered before AFSCs could be applied clinically for bone regeneration. One primary need is to select a suitable scaffold, which plays the central role in tissue engineering [12,21,22]. Our previous studies have demonstrated that NF scaffolds advantageously mimicked the natural ECM to enhance osteoblastic differentiation of ESCs and pre-osteoblasts [20,23,24]. It is intriguing to examine osteogenic differentiation of AFSCs on the NF scaffolds both *in vitro* and *in vivo*.

2. Materials and Methods

2.1 Preparation of nanofibrous and solid-walled PLLA scaffolds

The NF and SW PLLA scaffolds were prepared using a phase separation process as previously described [17,18,20]. The scaffolds used in the current study have a disc-like shape with a height of 1.5 mm, diameter of 5.2 mm and pore size of 250–420 μm . The average fiber diameter of the NF scaffolds was between 100 and 200 nm. No fibrous architecture was found in SW scaffolds. The numbers of scaffolds used were indicated in respective studies.

2.2 Cell culture and seeding

hAFSCs were provided by the Wake Forest Institute for Regenerative Medicine (Winston-Salem, NC). The isolation method has been described previously [1,25]. Briefly, back-up human amniocentesis cultures were harvested by trypsinization, and subjected to c-kit immunoselection. hAFSCs were subcultured routinely at a dilution of 1:6 and not permitted to expand beyond 70% of confluence. The hAFSCs (H1 cell line) were received at passage 14, and further passaged 2–3 times in α MEM supplemented with 15% fetal bovine serum (ES Cell-qualified FBS), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA), 18% Chang B and 2% Chang C (both from Irvine Scientific, Santa Ana, CA). It should be noted that the hAFSCs have been found to be pluripotent, even at high passage numbers [1].

A commercially available hMSCs line (derived from human bone marrow withdrawn from the posterior iliac crest of the pelvic bone of normal volunteers) was obtained from LONZA (Walkersville, MD) and routinely cultured at 37°C, 5% CO₂ in mesenchymal stem cell growth media (LONZA, Walkersville, MD). Cells from passages 3 through 6 were used.

For the osteogenic differentiation studies on 2D petri dishes, hAFSCs or hMSCs were seeded at a density of 3,000 cells/cm² and were cultured in low-glucose DMEM medium with 10% FBS (Invitrogen, Carlsbad, CA), Pen/Strep and osteogenic supplements (50ng/mL rhBMP-7 from Stryker Biotech (Hopkinton, MA) or 100 nM dexamethasone, 10 mM β -glycerophosphate, 50 $\mu\text{g}/\text{mL}$ ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO)).

For the differentiation studies on 3D scaffolds, the NF and SW scaffolds were soaked in ethanol for 30 min and then exchanged with phosphate-buffered saline (PBS) for three times (30 min each). The scaffolds were then washed twice with low-glucose DMEM medium containing 10% FBS (2 h for each rinse). Two million cells were seeded on each scaffold and transferred 24 h later into 6-well tissue culture plates containing 3 mL of low-glucose DMEM medium per well supplemented with 10% FBS (complete medium), 50 nM rhBMP-7, 50 $\mu\text{g}/\text{mL}$ ascorbic acid, and 10 mM β -glycerophosphate for up to 4 weeks on an orbital shaker in an incubator at 37 °C with 5% CO₂.

2.3 RNA extraction and real time PCR

The total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) from hAFSCs after osteogenic induction for 1, 2 and 4 weeks. RNA concentration was determined using the optical density of the sample measured at 260 nm. 1 μg RNA was initially reverse transcribed for 1st strand cDNA synthesis (TaqMan® Reverse Transcription Reagents, Applied Biosystems, Foster City, CA). Real-time PCR quantitation of the mRNA of osteoblastic markers was performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) by using Power SYBR® Green (Applied Biosystems, Foster City, CA). The primer sequences of the osteogenic genes, including runt related transcription factor 2 (RUNX2), osterix (OSX), bone sialoprotein (BSP), osteopontin (OPN) and osteocalcin (OCN) are listed in Table 1. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH),

was used as an endogenous reference gene to normalize the calculation through the Comparative Ct (Cycle of threshold) value method.

2.4 ALP and calcium content

ALP (alkaline phosphatase) was extracted and detected with an EnzoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec, San Jose, CA) according to the manufacturer's protocol. Briefly, cells on NF scaffolds or SW scaffolds were homogenized in 500 μ L lysis buffer provided in the kit. Lysate was centrifuged for 15 min at 10,000g at 4°C. Supernatant was collected for ALP assay using p-nitrophenyl phosphate (p-NPP) as a phosphatase substrate and alkaline phosphatase provided in the kit as a standard. The amount of ALP in the cells was measured at 405 nm and normalized against total DNA content [23]. The cell-scaffold constructs were also examined for calcium deposition by use of a total calcium LiquiColor® kit (Stanbio laboratory, TX). The constructs for mineralization quantification were washed three times for 5 min each in double-distilled water and then homogenized with a Tissue-Tearor in 1 ml of double-distilled water. The suspension was incubated in 500 μ L 0.5N hydrochloric acid overnight to extract calcium. Then calcium reagent working solution was added to each sample according to the manufacturer's instruction. The absorbance was measured at 550 nm and the calcium content was calculated using the standard provided in the kit [20].

2.5 Von Kossa and Alizerin Red S staining

The cell-scaffold constructs 4 weeks after osteogenic induction were fixed in neutral buffered 10% formalin and then embedded in paraffin. Five-micrometer sections were cut and stained with von Kossa for light microscopic observation as previously described [26]. Mineralization on petri dishes by hMSCs and hAFSCs was visualized using Alizerin Red S staining assay after 2 weeks of osteogenic induction. Briefly, the cells were fixed in cold 70% ethanol for 1 hour after rinsed with cold PBS. Following a rinse with distilled water, the cells were stained in 40 mM Alizerin Red S (pH4.2) for 10 minutes at room temperature.

2.6 Subcutaneous implantation

hAFSCs-scaffold constructs were induced by 50 ng/mL rhBMP-7 for 1 week (medium changed twice) before implantation. For implantation, male outbred athymic nude (nu/nu) mice with an age range of 6–8 weeks (Charles River Laboratories) were used in the study. Surgery was performed under general inhalation anesthesia with isoflurane. The back of the animals was shaved, washed and disinfected with povidoneiodine. One midsagittal incision was made on the dorsa and two subcutaneous pockets were created using blunt dissection. One scaffold was implanted subcutaneously into each pocket. Each animal received 2 constructs. Three samples were implanted for each group (n=3). The scaffolds were placed alternately at different sites for each mouse. After placement of scaffolds, the incisions were closed with staples. The mice were sacrificed 6 weeks later and the cell-scaffold constructs were harvested because this time point was found to be suitable to examine the differences in mineralization in our previous studies. The animal procedures were performed according to the guidelines approved by the University of Michigan Committee of Use and Care of Laboratory Animals. Radiographic analysis was performed on the cell-scaffold constructs using a micro radiography system (Faxitron X-ray Corporation, Wheeling, IL) at conditions of 35 kV and 60 s. The constructs were then fixed in neutral buffered 10% formalin and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin (H & E) or von Kossa for light microscopic observation as previously described [26]. The percentage of mineralized area of every specimen was measured using ImageJ software (NIH, Bethesda, MD).

2.7 Statistical analysis

For all experiments, values were reported as mean $\pm$ SD based on triplicate cell cultures. The experiments were performed twice to ensure reproducibility. To test the significance of observed differences between the study groups, an unpaired Student's t-test was applied. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1 Mineralization by rhBMP-7 induced hAFSCs

Both hMSCs and hAFSCs produced mineralized matrix after induced by 100 ng/mL DEX for 2 weeks (Fig. 1). There was no obvious difference between these two kinds of stem cells in response to DEX. As showed by Alizarin Red S staining and total calcium content, the hAFSCs also responded strongly to the 50 ng/mL rhBMP-7 in terms of mineralization. However, the hMSCs did not respond to rhBMP-7 as strongly as hAFSCs did in terms of mineralization (Fig. 1).

3.2 ALP activity and mineralization of hAFSC-scaffold constructs in vitro

ALP activity of hAFSCs on NF and SW scaffolds was detected after rhBMP-7 induction for 1 and 2 weeks. The ALP activity of hAFSCs on two scaffolds had increased after 1 week of induction. The rhBMP-7-induced ALP activity of hAFSCs was significantly higher on the NF scaffolds than on SW scaffolds at 1 week. However, the ALP activity decreased on both scaffolds at 2 weeks and there was no statistical difference between the two scaffolds (Fig. 2A). Mineralization of NF scaffolds was evident, which was confirmed by von Kossa staining and the determination of calcium contents in the scaffolds. Calcium content assay revealed greater amount of calcium produced in the NF scaffolds compared to that in the SW scaffolds at 2 and 4 weeks (Fig. 2B). Histology with von Kossa staining revealed that there was heavy mineral deposition throughout the NF scaffolds after 4 weeks of culture (Fig. 2C), while there was little observable mineralization in the SW scaffolds (Fig. 2D).

3.3 Gene expression of osteoblastic differentiation markers

Expression of genes associated with the osteoblastic differentiation was examined using real time PCR to investigate the effect of the synthetic NF matrix on gene expression at 1, 2 and 4 weeks. Expression of RUNX2 and OSX, two major transcription factors of the osteogenic differentiation, was examined in cells on both types of scaffolds. Repeated experiments showed that the expression levels of these factors were consistently higher in the cells grown on NF scaffolds than in the cells grown on SW scaffolds (Fig. 3). Both RUNX2 and OSX increased with the induction time. Importantly, these factors were elevated to significant higher levels in cells on NF scaffolds than on SW scaffolds at 1 week. Consistent with the results of ALP activity and mineralization, the expression of OPN, BSP and OCN was much higher on NF scaffolds than on SW scaffolds.

3.5 NF scaffolds facilitating in vivo bone formation by rhBMP-7-induced hAFSCs

In order to study the bone forming ability of hAFSCs on NF and SW scaffolds, the hAFSC-scaffold constructs were induced in rhBMP-7-containing medium for 7 days and were subcutaneously implanted into nude mice. Six weeks after subcutaneous implantation, a radiographic examination showed that radiopacity in NF scaffolds was significantly higher than that in SW scaffolds (Fig. 4A). Significant ectopic bone formation was found on both scaffolds as noted by von Kossa staining (Fig. 4C, D). The areas of mineralization on NF scaffolds were significantly greater than those on SW scaffolds as measured by ImageJ software (Fig. 4B). The *in vivo* data showed that NF scaffolds provided hAFSCs with a more

favorable microenvironment for their osteogenic differentiation and bone formation than SW scaffolds did.

4. Discussion

Amniotic fluid is known to contain a heterogeneous population of cell types, cells derived from which can give rise to diverse differentiated cells including those of adipose, muscle, bone and neuronal lineages [1]. Therefore, the characteristics including the potentiality of the cells isolated from amniotic fluid, are largely dependent on the methods used for the isolation. That's probably the main reason why there are some discrepancies among different research groups [27,28]. hAFSCs used in our study were isolated by selection for expression of the membrane stem cell factor receptor c-Kit, a common marker for multipotential stem cells. Moreover, the AFS cell line that we used was established clonally, which means the cells are homogeneous. From both surface markers and the potential ability, the AFS cell lines are different both from pluripotent ES cells and from multipotential adult stem cells, and may represent a new class of stem cells whose properties of plasticity exist somewhere between embryonic and adult stem cell types [1,2].

While Dex has been widely used to induce osteoblast differentiation of mesenchymal progenitor cells *in vitro* [29], as one of the most used glucocorticoids, Dex suppresses bone-forming cells *in vivo* causing bone loss and increasing the risk of fractures [30,31]. Accordingly, studies on the role of BMPs to induce osteogenic differentiation of hAFSCs are necessary for their tissue engineering application.

One of the challenges in clinical applications of BMPs, is high dosage BMPs required for the weak response of human MSCs to the BMPs, which will increase both the costs and unwanted side effects [32]. Therefore, stem cells with strong responsiveness to BMPs are needed for bone tissue engineering. We found that hAFSCs could be induced by rhBMP-7 to differentiate along the osteogenic lineage. It is encouraging that stronger mineralization by hAFSCs than that by hMSCs was induced by rhBMP-7 at the same concentration in this study. BMP-7 induces osteogenesis through the BMP-Smad pathway. This pathway can be regulated at intracellular space, membrane site and by extracellular mediators. Molecules involved in the BMP-Smad pathway can also have crosstalks with other signaling pathways [33,34]. Further studies are required to understand the underlying mechanisms. Based on the strong response to rhBMP-7, hAFSCs are likely promising stem cells for bone regeneration.

Biomaterials play central roles as designable biophysical and biochemical milieus that direct cellular behavior and function in regenerative medicine and tissue engineering [22,35]. To engineer materials mimicking the NF ECM, several technologies have been developed with varying degrees of success. Electrospinning is one of the methods used to fabricate NF materials [36,37]. Significant challenges exist in using this technique to fabricate complex 3D scaffold shapes or to generate designed internal pore structures. We have developed a phase separation technique to generate NF scaffolds with interconnected spherical macro pores [12]. Consistent with the results discovered in mouse osteoblasts and ESCs [20,24], the mineralization of hAFSCs was enhanced substantially on the NF scaffolds compared to SW scaffolds. The *in vivo* results (Fig. 4) provided more convincing evidence that NF scaffolds are more favorable for the osteogenic differentiation of hAFSCs. The present study confirmed that the biomimetic microenvironment provided by the NF scaffolds is an important element besides the morphogenetic factors for the differentiation of stem cells.

The roles of BMPs and ECM during differentiation of MC3T3-E1 preosteoblastic cells have been reported previously [38]. While MC3T3-E1 cells constitutively produce BMP-2, BMP-4, and BMP-7, very little BMP activity is observed in the absence of ECM synthesis. However,

the addition of BMPs to cells that are actively synthesizing a matrix dramatically stimulates OCN and BSP gene expression. ERK/MAP kinase pathway is required for the expression of both ECM- and BMP-dependent genes, suggesting that these two pathways cooperatively regulate osteoblast activity. It is well established that matrix responsiveness during osteogenesis is caused by the aggregation of the $\alpha 2\beta 1$ [38] and $\alpha v\beta 3$ [39] integrins that subsequently activate intracellular signaling cascades including FAK [40] and ERK/MAPK [38] pathways, resulting in the phosphorylation of the osteoblast-specific transcription factor RUMX2/Cbfa-1 [38]. Integrins ($\alpha 2$, $\beta 1$, αv and $\beta 3$) were highly expressed in neonatal mouse osteoblastic cells seeded on NF scaffolds. Phospho-Paxillin and phospho-FAK were also found at elevated levels in these cell lysates. It is interesting that when the formation of collagen fibrils were inhibited by 3,4-DHP, cells seeded on NF scaffolds sustained the expression of $\alpha 2$ integrin, while suppression of $\alpha 2$ integrin was evident in cells seeded on SW scaffolds [20]. These results provide us with clues to understand why the synthetic nano-fibers facilitate BMP-induced osteogenic differentiation of hAFSCs, while the exact underlying mechanism needs further investigation.

5. Conclusions

hAFSCs can be induced to generate mineralized tissue by rhBMP-7. Compared to hMSCs, the stronger responsiveness to rhBMP-7 makes hAFSCs a promising cell source for bone regeneration. The synthetic NF scaffolds, which exhibit structural features similar to natural collagen fibers, provided hAFSCs with a more favorable microenvironment, and thus, enhanced the osteoblastic differentiation of hAFSCs *in vitro* and bone formation *in vivo*.

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Figure 1.

hAFSCs and hMSCs were induced by 100ng/mL DEX or 50ng/mL rhBMP-7 for 2 weeks. The mineralization was visualized by Alizarin Red S staining at the end of culture. The two types of stem cells showed similar degrees of mineralization in response to DEX. While hMSCs showed weak mineralization induced by rhBMP7, hAFSCs showed strong mineralization under the same conditions (A). The calcium content produced by hAFSCs induced by rhBMP-7 was significantly higher than by hMSCs (B).

Figure 2. ALP activity of hAFSCs on NF and SW scaffolds was detected after 1 and 2 weeks of rhBMP-7 induction. The rhBMP7-induced ALP activity of hAFSCs was significantly higher on the NF scaffolds than on the SW scaffolds at 1 week. However, no difference between two scaffolds was detected at 2 weeks (A). Mineralization of NF scaffolds was much stronger than on the

SW scaffolds, which was confirmed by von Kossa staining and determination of calcium contents in the scaffolds (B-D). The size bars in C and D are 500 μm .

Figure 3.

Expression of genes associated with the osteoblastic differentiation was examined using real time PCR to investigate the effect of the synthetic nanofibrous matrix on gene expression at 1, 2 and 4 weeks. Expression levels of RUNX2 (A) and OSX (B) were significantly higher in cells on NF scaffolds than on SW scaffolds at 1 week. While the levels of expression of OPN (C), BSP (D) and OCN (E) were increased by BMP7 on both scaffolds, the levels of expression were much higher on NF scaffolds than on SW scaffolds. (* $p < 0.05$)

Figure 4. hAFSC-scaffold constructs after induced by rhBMP7 *in vitro* for 7 days, were subcutaneously implanted into nude mice. Six weeks after subcutaneous implantation, radiographic examination showed radiopacity of NF scaffolds was significantly higher than SW scaffolds (A). The newly formed bone was mineralized as noted by von Kossa staining (C & D). Significant ectopic bone formation was found on both scaffolds. The areas of mineralization on NF scaffolds were significantly higher than those on SW scaffolds, measured by ImageJ software (B). The bars in C and D are 500 µm. (* $p < 0.05$)

Table 1

Primers for real time PCR.

Definition	Sequence (Forward/Reverse)	Ta(°C)	Product (bp)	Gene ID
GAPDH	5' CCCACTCCTCCACCTTTGA 3'	60	105	NM_002046
	5' CCACCCTGTTGCTGTAGCC 3'			
RUNX2	5' AGTGGACGAGGCAAGAGTTTC 3'	60	117	NM_004348
	5' CCTTCTGGGTTCCCGAGGT 3'			
OSX	5' TCCTCCTGCGACTGCCCTAA 3'	60	128	NM_152860
	5' TGCGAAGCCTTGCCATACA 3'			
OPN	5' TTTACAACAAATACCCAGATGC 3'	60	110	NM_000582
	5' ATGGCTTTCGTTGGACTTACT 3'			
BSP	5' GACAGTTCAGAAGAGGAGGAG 3'	60	124	NM_004967
	5' AGCCCAGTGTGTAGCAGA 3'			
OCN	5' AGGGCAGCGAGGTAGTGAA 3'	60	151	NM_199173
	5' TCCTGAAAGCCGATGTGGT 3'			