Amniotic Fluid Stem Cells Produce Robust Mineral Deposits on Biodegradable Scaffolds

Alexandra Peister, Ph.D.,^{1,2} Eric R. Deutsch, B.S.,² Yash Kolambkar, M.S.,²
Dietmar W. Hutmacher, Ph.D.,^{2,3} and Robert E. Guldberg, Ph.D.²

Insufficient availability of osteogenic cells limits bone regeneration through cell-based therapies. This study investigated the potential of amniotic fluid-derived stem (AFS) cells to synthesize mineralized extracellular matrix within porous medical-grade poly-ε-caprolactone (mPCL) scaffolds. The AFS cells were initially differentiated in two-dimensional (2D) culture to determine appropriate osteogenic culture conditions and verify physiologic mineral production by the AFS cells. The AFS cells were then cultured on 3D mPCL scaffolds (6-mm diameter × 9-mm height) and analyzed for their ability to differentiate to osteoblastic cells in this environment. The amount and distribution of mineralized matrix production was quantified throughout the mPCL scaffold using nondestructive micro computed tomography (microCT) analysis and confirmed through biochemical assays. Sterile microCT scanning provided longitudinal analysis of long-term cultured mPCL constructs to determine the rate and distribution of mineral matrix within the scaffolds. The AFS cells deposited mineralized matrix throughout the mPCL scaffolds and remained viable after 15 weeks of 3D culture. The effect of pre-differentiation of the AFS cells on the subsequent bone formation *in vivo* was determined in a rat subcutaneous model. Cells that were pre-differentiated for 28 days *in vitro* produced seven times more mineralized matrix when implanted subcutaneously *in vivo*. This study demonstrated the potential of AFS cells to produce 3D mineralized bioengineered constructs *in vitro* and *in vivo* and suggests that AFS cells may be an effective cell source for functional repair of large bone defects.

Introduction

CELL SOURCING is critical for cell-based therapies to regenerate musculoskeletal tissues.^{1,2} Cell-based tissue engineering strategies represent a clinical alternative to bone grafting and the delivery of osteoinductive proteins. Tissue engineering approaches that combine biodegradable scaffolds with stem cells capable of osteogenesis have shown promise as an effective bone graft substitute.³ Purified mesenchymal stem cells (MSCs) derived from bone marrow have been shown to enhance repair of critical-sized defects in preclinical animal studies.^{4,5} However, the low frequency of MSCs in healthy tissues, which is further reduced in aged bone marrow, has limited their widespread use.⁶

In vitro mineralization of cell-seeded scaffolds before implantation can be used to assess the osteogenic potential of stem cells as an indicator of their ability to enhance *in vivo* bone repair.⁷ However, initial attempts to mineralize tissue engineered bone thicker than 1 mm *in vitro* have typically

resulted in a shell of viable cells and mineral surrounding a necrotic core because of poor scaffold design, the absence of nutrient perfusion, or both.⁸ Recent studies have shown that dynamic culture systems dramatically increase osteogenic differentiation at the core of large scaffolds, producing mineralized matrix throughout constructs 9 mm thick.⁹

Human amniotic fluid-derived stem (AFS) cells are a recently characterized stem cell source that expresses a combination of embryonic and adult stem cell markers and displays some but not all characteristics of both.¹⁰ Unlike embryonic stem cells, undifferentiated AFS cells expand extensively and are not tumorigenic.¹⁰ In contrast to adult-derived stem cells, the AFS cell lines expanded for more than 250 population doublings retained long telomeres and a normal chromosomal karyotype.¹⁰ AFS cells are broadly multipotent and have been induced to differentiate into cell types representing each embryonic germ layer, including cells of osteogenic, adipogenic, chondrogenic, myogenic, endothelial, neuronal, and hepatic lineages.^{10–13} AFS cells thus have

¹Morehouse College, Atlanta, Georgia.

²Georgia Institute of Technology, Atlanta, Georgia.

³Queensland University of Technology, Queensland, Australia.

the advantage of being a versatile precursor cell with great expansion capability; however, little is known about the extent of their osteogenic potential.

The goal of this study was to determine the potential of human AFS cells to reproducibly produce mineralized matrix *in vitro* and to study AFS cells as a potential source for scaffold-based bone tissue engineering. After exposure to osteogenic supplements, human AFS cells differentiated into mineral-producing cells, and the amount of calcium deposition increased over 4 weeks of culture: 10 times more than previously reported protocols for AFS cell mineralization.¹⁰ A clonogenic osteogenesis assay further demonstrated that more than 85% of colonies formed were able to successfully mineralize, and Fourier transform infrared (FTIR) analysis confirmed that the mineral was biological in nature.

Demonstration of two-dimensional (2D) osteogenic differentiation is not sufficient to show that cells are capable of robust mineralized extracellular matrix synthesis. Therefore, the ability to produce mineralized matrix throughout a large, 3D biodegradable scaffold *in vitro* was analyzed. The AFS cells produced extensive mineralization throughout 6-mm-diameter×9-mm-thick cylindrical mPCL scaffolds, and the mineral volume continued to increase for the duration of the culture period. Live-dead staining confirmed that the human AFS cells were highly viable after 15 weeks *in vitro*. The AFS cells continued to produce mineral once placed subcutaneously in an immunodeficient rat but only when pre-differentiated *in vitro* for 4 weeks. The robust mineralization potential observed *in vitro* and at an ectopic site *in vivo* suggests that AFS cells are a candidate for cell therapy strategies aimed at restoring function to damaged or degenerated bone.

Materials and Methods

AFS cell isolation and culture

The Institute for Regenerative Medicine at Wake Forest University (Winston-Salem, NC) kindly provided human AFS cells.¹⁰ As previously described, cells were harvested using trypsinization from confluent back-up human amniocentesis cultures and subjected to immunoselection. The c-kit-positive cells were immunoselected with an antibody to CD117 (c-Kit), and clonally derived lines were subsequently expanded.¹⁰ In this study, two cell lines were analyzed: A1 and H1 human AFS cells (Fig. 1 A, B).

The AFS cells were received at passage 14 and further passaged two to three times in alpha minimum essential medium (α -MEM) containing 15% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U of penicillin, 100 μ g of streptomycin (all Invitrogen, Carlsbad, CA) supplemented with 18% Chang B and 2% Chang C (Irvine Scientific, Santa Ana, CA) (modified Chang medium) at 37°C in a 5% carbon dioxide atmosphere.¹⁰ AFS cells were subcultured at a dilution of 1:10 and not permitted to expand beyond 70% confluence in the modified Chang medium on Integrid plates (BD Falcon, San Jose, CA). Cells were frozen in the modified Chang medium supplemented with 5% dimethyl sulfoxide. For experimental use, 1×10^6 AFS cells were quickly thawed to 37°C and placed in ten 150-mm Integrid culture plates with modified Chang medium. After five to six days, the cells were harvested with 0.25% trypsin-ethylenediaminetetraacetic acid (Invitrogen), counted, and used experimentally.

Osteogenic differentiation

AFS cells were cultured at 20,000 cells per cm^2 in six-well plates (Nunc, Rochester, NY) in modified Chang medium ($n=6$) for all conditions. Twenty-four h after plating, the modified Chang medium was changed to the experimental conditions. For control samples, the cells were grown in the control medium consisting of α -MEM supplemented with 17% FBS (Atlanta Biologicals, Lawrenceville, GA) and 2 mM of L-glutamine, 100 U of penicillin, and 100 μ g of streptomycin or in osteogenic medium consisting of control medium supplemented with 1 μ M of dexamethasone, 6 mM of β -glycerol phosphate, 50 μ g/mL of ascorbic acid 2-phosphate, and 50 ng/mL of thyroxine (all Sigma, St. Louis, MO).¹⁴ The medium was changed two times per week. Cells were analyzed 2 and 4 weeks after the initiation of differentiation.

Alizarin red S staining for calcium

The cells were washed with phosphate buffered saline (PBS) (Mg^{2+} and Ca^{2+} free, Invitrogen) and fixed in 10% neutral buffered formalin (Sigma) for 15 min. The cells were washed three times with water and then stained with 2 mL of 0.4 mM alizarin red S (pH 4.2, Sigma) for 20 min with shaking. Calcium forms an alizarin red S–calcium complex in a chelation process, producing a dark red stain. The excess alizarin red S stain was removed using vigorous washing with excess water (4 times for 5 min each, with shaking). Stained monolayers were visualized using phase microscopy using an inverted microscope (Nikon, Melville, NY).

The alizarin red S was quantified spectrophotometrically, as previously described.¹⁵ Briefly, the calcium was extracted with 800 μ L of 10% (v/v) acetic acid with shaking for 30 min. The cell layer and acetic acid were scraped from the dish and transferred to a microcentrifuge tube. The samples ($n=6$) were vortexed and then heated to 85°C for 10 min, 500 μ L of supernatant was then neutralized with 200 μ L of 10% (v/v) ammonium hydroxide, 150 μ L was transferred to a 96-well plate, and the absorbance determined at 405 nm with a spectrophotometer (PowerWave_x 340, Bio-Tek Instruments, Winooski, VT).

FTIR spectroscopy

Osteogenic or control AFS cells were harvested using a cell scraper and placed in microcentrifuge tubes. The cells were fixed in methanol and dried in an oven at 70°C overnight to remove all aqueous components. The samples were prepared and analyzed using FTIR spectroscopy as previously described.¹⁶ Briefly, the mineral was combined with potassium bromide (KBr, Sigma) and compressed between two platens to form a thin film of mineral and KBr. KBr contains an ionic salt bond that the FTIR beam cannot excite; therefore, only the mineralized matrix is examined. Spectra 64 scans were acquired at 4 cm^{-1} , collected on a Nexus 470 FTIR spectrometer (Thermo Nicolet, Madison, WI) under N_2 purge, and displayed from 400 to 2000 cm^{-1} , spanning the range that includes bands characteristic of biologic hydroxyapatite.

Scaffold preparation

Scaffolds were fabricated using fused deposition modeling and produced with a 6-mm dermal biopsy punch from a

100- $\times$ 100- $\times$ 3-mm or 100- $\times$ 100- $\times$ 9-mm sheet made of medical grade poly ϵ -caprolactone (mPCL, Osteopore International, Singapore), yielding scaffolds 3 and 9 mm thick by 6 mm in diameter with a 0°, 60°, 120° strut pattern and porosity of 85%.¹⁷ The cylinders were incubated in 5 M of sodium hydroxide for 2 h at 37°C to increase surface roughness and hydrophilicity.⁹ The scaffolds were then washed extensively three times in excess sterile water and sterilized using 70% ethanol evaporation.

To produce a collagen network throughout the fully interconnected pores of the mPCL cylinders, a collagen gel was produced with type 1 rat tail collagen (Vitrogen, Fremont, CA). Briefly, 100 parts collagen (1.4 mg/mL in 0.05% acetic acid) was combined with nine parts sodium bicarbonate, and 250 μ L of the mixture was placed in a custom mold. The mPCL scaffold was then placed in the mold, and the collagen was allowed to gel for 30 min at room temperature. The collagen was frozen at -80°C for 2 h and lyophilized overnight (Labconco, Kansas City, MO). After lyophilization, the scaffolds were removed from the mold and placed in a 12-well tissue culture dish (Nunc, low cell binding surface). To maintain scaffold orientation during culture, the cylinders were placed in a holder consisting of a sterile 3/4" Teflon disk with four stainless steel pins surrounding the cylinder (Fig. 2A).

Cell seeding onto mPCL/collagen scaffolds

Six million AFS cells were resuspended in 150 μ L of modified Chang medium and slowly administered dropwise onto the top surface of the scaffold. The collagen mesh readily absorbed the medium, with minimal pooling at the bottom. The cell-scaffold constructs were placed in the 37°C incubator for 1 h to promote cell attachment, after which time 4 mL of modified Chang medium was carefully added to each well. Cell-free controls were prepared by adding 150 μ L modified Chang medium instead of cells and then cultured in the same way as the osteogenic samples.

After 3 days in culture, the medium was carefully aspirated, and osteogenic medium or modified Chang medium (control) was added to each well. At this time, the scaffolds were placed on an orbital shaker to increase medium transport through the scaffold and facilitate exchange of nutrients and waste products (Belly Button orbital shaker, 7.5 rpm, minimal pitch, Stovall, Greensboro, NC). The medium was changed every two to three days for 15 weeks.

Micro computed tomography scanning

At 3, 5, 10, and 15 weeks, the scaffolds were removed from the Teflon holders and placed in a sterile polysulfone sample

FIG. 1. Two-dimensional (2D) osteogenic differentiation of amniotic fluid-derived stem (AFS) cells. Cell line A1 (A) and H1 (B) AFS cell morphology of AFS cells, Phase, 100 $\times$ B. Cell line A1 (C) and H1 (D) stained with alizarin red S forms a dark red complex with the calcium indicating areas of mineral deposition. (E) The quantity of mineral produced by the AFS cells increased as time in osteogenic medium increased, as determined according to alizarin red S stain quantification. (F) The number of AFS cells that could differentiate into osteogenic cells was high, with more than 85% of AFS cells capable of osteogenic differentiation. No osteogenic differentiation was seen in colonies grown in control medium, ensuring that there was not spontaneous differentiation of AFS cells to osteoblasts ($n = 6$). * $p < 0.001$ vs. control. Color images available online at www.liebertonline.com/ten.

FIG. 2. Three-dimensional osteogenic differentiation of AFS cells on the medical-grade poly-ε-caprolactone (mPCL) scaffolds. (A) MicroCT image of the 6-mm diameter by 9-mm high mPCL scaffold. A diagram of the Teflon scaffold holder is shown for *in vitro* culture of the AFS cells on the mPCL scaffold. (B) An increase in mineral is apparent as the AFS cells are cultured on the mPCL scaffold for a longer time. A single scaffold is shown, scanned repeatedly over the 15 week culture. (C) MicroCT images of mineralized matrix produced by the AFS cells within the mPCL scaffolds when cultured in static or dynamic medium perfusion for 10 weeks. (D) The microCT images of four representative scaffolds of each AFS cell line at 15 weeks are shown. Little variability is seen in the mineral production from the two cell lines, and it is reproducible ($n = 6-12$).

holder. Mineralization of the scaffolds was quantified using a VivaCT scanner (Scanco Medical, Switzerland) at a 21.5 μm voxel resolution. Samples were evaluated at a threshold of 80, a filter width of 1.2, and filter support of 1, as previously described.⁹ For each scaffold, a measurement of the mineralized matrix volume was determined. The samples were then removed aseptically from the sample holder and returned to the Teflon holder for further culture ($n = 12$, repeated twice).

Sample preparation for biochemical assays

After the volume of mineralized matrix was determined using micro computed tomography (microCT) at 15 weeks, the samples were placed in an aluminum matrix with a 6-mm channel to cut the scaffolds. Each scaffold was cut into four pieces, first in half to create cylinders 4 mm and 5 mm thick

and then longitudinally in half to form two half-cylinders. The 5-mm half-cylinders were placed in acetic acid for calcium extraction, and the 4-mm half-scaffolds were placed in PBS with 0.05% Tween 20 for DNA extraction. Additional samples were cut longitudinally for cell viability analysis.

Calcium assay

At 15 weeks in osteogenic or control medium, a portion of each scaffold was placed in 500 μL of 1-M acetic acid and placed on a vortex overnight at 4°C to extract the calcium from the mineralized matrix. In a 96-well clear polycarbonate plate, 25 μL of cell extract, or known standards, was mixed with 300 μL of calcium reagent (arsenazo III, Diagnostic Chemicals Limited, Charlottetown, Canada) and the absorbance determined at 615-nm with a spectrophotometer ($n = 5-6$, repeated twice).

FIG. 3. Quantification of mineralized matrix according to microCT. (A) AFS cells grown in osteogenic medium produced significantly more mineral than the control AFS cells at all time points. (B) The rate of mineralization by the AFS cells increased as culture time increased. The rate of mineralization for the AFS cells in osteogenic culture medium was significantly higher than that of the AFS cells in control culture medium at all time points, and the rate was significantly higher for the H1 cell line than the A1 cell line at 15 weeks ($n=6-12$). * $P < 0.001$ vs all control groups, # $p < 0.05$ H1 vs A1 AFS cells.

DNA assay

The PicoGreen DNA quantification kit (Invitrogen) was used following the protocol recommended by the manufacturer to determine the relative amount of DNA within scaffolds from the different experimental groups. Lambda DNA standards were produced from 1 μ g to 1 ng. The cell lysates were diluted 1:10 in Tris-EDTA buffer, 100 μ L of the PicoGreen working solution and 100 μ L of each sample were placed in triplicate in black 96-well plates. After a 5-min incubation, the fluorescence was determined at an excitation of 485 nm and an emission of 535 nm (Perkin-Elmer HTX 7000 fluorescent plate reader, Waltham, MA) ($n=5-6$, repeated twice).

Cell viability staining

Cell viability was determined using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells according to the manufacturer's instructions (Molecular Probes, Invitro-

gen). Briefly, scaffolds were washed three times in 3 mL of PBS and then incubated for 45 min in 4 μ M of calcein AM and 4 μ M of ethidium homodimer-1 with rocking. The scaffolds were then washed three times with PBS and analyzed using a confocal microscope (LSM 510 UV, Carl Zeiss, Thornwood, NY). Random fields were picked on the outside surface, the top and bottom surface, and the cut center surface. The top-center, middle-center, and bottom-center regions of each scaffold were imaged. No evidence of spatial variation in cell viability was seen throughout the cut surface, so only the middle-center image is shown.

Effect of in vitro pre-culture on in vivo mineralization

All animal studies were performed within the guidelines of an approved protocol by the Institutional Animal Care and Use Committee at Georgia Institute of Technology (Protocol number A06020). Athymic nude female rats were purchased from Charles River Laboratories (Wilmington, MA) and housed in pairs in sterile housing.

mPCL scaffolds 6 mm in diameter and 3 mm in height were prepared as described above. Two million AFS cells were seeded on each scaffold. The mPCL/AFS cells were then incubated in osteogenic medium for 1, 7, or 28 days. In the day 1 samples, the cells were pre-osteogenic; at day 7, the cells were early osteogenic with no mineralized matrix; and at day 28, the AFS cells were osteogenic and had initiated mineralized matrix formation. As a control, acellular mPCL scaffolds were also examined. The amount of mineralization of each construct was determined pre-implantation according to a sterile microCT scan. The constructs were then implanted subcutaneously on the dorsal side of athymic nude rats. One construct from each group was implanted in each rat, for a total of four constructs per rat. After 28 days, the animals were sacrificed, and the constructs were removed. They were then analyzed using 2D *ex vivo* digital X-rays (Faxitron MX-20 Digital; Faxitron X-ray Corporation, Wheeling, IL) and microCT with the parameters described above to determine the amount and distribution of the mineralized matrix ($n=8$).

Results

2D osteogenic differentiation

AFS cells are small cells with moderate heterogeneity (Fig. 1A, B). Two-dimensional differentiation of the AFS cells formed a dense layer of mineral that was clearly visible (Fig. 1C, D). This mineral stained intensely with alizarin red S, a histological stain that chelates calcium to form a bright red lake and is routinely used for bone mineral identification. Additionally, the chelated alizarin red S/calcium complex can be extracted from the mineral and quantified spectrophotometrically. The amount of alizarin red S increased with culture time during osteogenic differentiation (Fig. 1E).

Clonogenic osteogenic differentiation

The potential of the AFS cells to mineralize was further studied using a colony-forming mineralization assay. The ability to grow as colonies has been related to having a high proliferation and engraftment potential;^{18,19} 80% of the colonies in osteogenic medium mineralized, but no colonies in control medium showed mineral deposition (Fig. 1F). The

FIG. 4. Cell viability of the AFS cells on the mPCL scaffold. Confocal microscopy after 15 weeks of culture on mPCL scaffolds demonstrates the high viability of the AFS cells. Photomicrographs were taken of the exterior surface, the top and bottom surface, and the middle by cutting the scaffold longitudinally. Many viable cells were found on all surfaces examined, as seen by the abundance of green (live) cells and few dead (red) cells. Additionally, the AFS cells can be seen filling the voids between the mPCL struts (100 $\times$, $n = 6$). Color images available online at www.liebertonline.com/ten.

number of colonies in the osteogenic and control media was not significantly different, indicating that there was not a selective proliferation of AFS cells with osteogenic potential but rather that a high percentage of AFS cells were capable of osteogenic differentiation.

3D osteogenic differentiation

AFS cells readily attached to the mPCL–collagen scaffolds (Fig. 2A). The cells produced extracellular matrix that fully occluded the scaffold pores by 5 weeks in control and osteogenic growth media (not shown). The scaffolds were placed in sterile sample holders that allowed the scaffolds to be scanned repeatedly. Therefore, the rate of mineralized matrix production could be determined for each individual scaffold (Fig. 2B, Fig. 3B). Additionally, a 3D image of the mineralized matrix is produced using the microCT, allowing comparison of the spatial distribution of the mineral (Fig. 2, Fig. 3). The perfusion of the medium through the scaffolds produced by the orbital shaker allowed mineral deposition throughout the scaffold. In static culture, the AFS cells were able to mineralize only the exterior of the scaffold (Fig. 2C). The mineralized matrix production by the AFS cells on 3D constructs is highly reproducible, as the four representative microCT images of each AFS cell line shown in Figure 2D illustrate.

The mineral volume produced by the AFS cells in the mPCL scaffold can be quantified and visualized using microCT. As shown quantitatively in Figure 3A, the volume of mineralized matrix increased with time in culture. Additionally, the rate of mineralization was found to increase approximately linearly as a function of culture time (Fig. 3B). No mineralization was detected in the AFS-seeded scaffolds

cultured in control medium or in the unseeded scaffolds in osteogenic medium, so representative microCT images are not shown.

Cell viability

The molecular probe assay for cell viability allows simultaneous detection of live and dead cells by discriminating the cells based on intracellular esterase activity and plasma membrane integrity, two measures of cell viability. The large number of green live cells and small number of red nuclei of dead cells seen throughout the scaffolds, as shown in Figure 4, confirmed the high viability of the AFS cells, even after almost 4 months of culture. The cells appeared to line and fill the space between the mPCL struts. No differences in cell viability or number were apparent between control and osteogenic samples. Additionally, no difference in cell viability was seen between the two AFS cell lines examined.

Biochemical analysis

FTIR analysis confirmed the presence of biologic hydroxyapatite produced by the AFS cells grown in osteogenic medium (Fig. 5A). The spectrum was displayed on a relative absorbance scale. The mineral formed by the AFS cells in osteogenic culture conditions exhibited the amide I and II peaks that correlate to protein. The stretching (st) phosphate peaks near 1100 cm^{-1} , the stretching (st) carbonate peak at 870 cm^{-1} , and the phosphate bending (bd) doublet at 605 cm^{-1} and 560 cm^{-1} were also apparent in the mineral. The observed spectrum of mineralized matrix produced by the AFS cells is thus characteristic of biological hydroxyapatite. To confirm the mineralized matrix quantification using

FIG. 5. Biochemical conformation of osteogenesis. (A) Fourier transform infrared confirmed that the extracellular matrix produced by the AFS cells had the spectra of biological hydroxyapatite, the major component of physiologic bone. (B) Calcium was quantified from the scaffolds after 15 weeks in osteogenic medium. Significantly more calcium was contained in the matrix produced by the AFS cells when cultured in osteogenic medium. $*P < 0.001$ (C) No significant difference was seen the number of cells contained in the scaffolds, as determined according to DNA quantification ($n = 6$).

microCT, calcium content from a portion of the scaffold was quantified using the arsenazo III reagent. The calcium content of the matrix produced by the AFS cells under osteogenic conditions was significantly higher than that of the control AFS cells (Fig. 5B). The DNA content was not significantly different between the scaffold groups; therefore, the difference in mineralized matrix production could not be attributed to a difference in cell number (Fig. 5C).

In vivo osteogenic differentiation

The effect of *in vitro* osteogenic differentiation on *in vivo* mineralization of the AFS cells was examined. The AFS cells were placed on mPCL scaffolds and cultured in osteogenic medium for 1, 7, or 28 days. An AFS cell-free scaffold was used to control for host cell-derived osteogenesis. The scaffolds were analyzed using microCT the day before implantation to determine pre-implantation mineralization. There was significantly more mineral found on the scaffolds pre-cultured for 4 weeks, with negligible mineral on the three other groups (Fig. 6A).

After subcutaneous implantation in the athymic nude rat for 4 weeks, all four groups had significantly more mineral than the pre-implantation mineral quantification. No significant differences were seen between the acellular control and the short pre-culture groups of 1 and 7 days after 4 weeks *in vivo* (Fig. 6A). The scaffolds with 28-day osteogenic pre-culture had significantly more mineral than the other three groups, as well as significantly more mineral than their pre-implantation value. From the microCT analysis, it was determined that the amount of mineral present was approximately seven times as great from pre-implantation to post-implantation. A representative scaffold from the 28-day pre-culture group pre- and post-implantation illustrates the increase in mineral produced by the AFS cells during the 4 weeks *in vivo* (Fig. 6B). Additionally, the dense regions can be seen using high-resolution x-ray solely in the 4-week pre-culture group (Fig. 6C).

Discussion

Critical-sized segmental bone defects require surgical intervention and are one of the most challenging problems faced by orthopedic surgeons.²⁰ Trauma, degenerative diseases, and tumor resection are among the potential causes of large clinical bone defects. The current standard of care for bone repair augmentation is autologous bone grafting, but the small amount of tissue available for transplantation and the lack of structural integrity of the autograft fragments limit this approach. Structurally intact allografts are also commonly used to reconstruct large bone defects because they can withstand functional loads but are associated with a high complication and re-fracture rate due to slow or incomplete revascularization and remodeling.²¹

Engineered bone graft substitutes are therefore attractive as a clinical alternative to allografts or autografts.^{5,22,23} The delivery of biologics such as cells or growth factors within biomaterial scaffolds has shown promise for promoting the regeneration of damaged musculoskeletal tissues. Cell-based therapies may prove particularly effective for treating patients with a limited endogenous supply of stem or progenitor cells due to advanced age, severe multi-tissue injury, or tumor irradiation. Although cell-based therapies have great potential, several critical questions remain to be answered, including which cells to deliver to the site of injury, how to maximize survival of transplanted cells, what the most effective strategy is for programming delivered cell function, and what the consequences are of host physiology (e.g., immunogenicity, sex, or age). The first of these challenges requires identification of a plentiful cell source capable of robust mineralized matrix synthesis. The purpose of this study was to evaluate the osteogenic potential of a

FIG. 6. *In vivo* osteogenesis of AFS cells. **(A)** The volume of mineralized matrix produced by the AFS cells before and 4 weeks after subcutaneous implantation in an immunodeficient rat. The scaffolds and AFS cells cultured *in vitro* for 28 d before to implantation had significantly more mineral volume than the other groups (*). After 4 weeks *in vivo*, the AFS cells pre-cultured for 28 d had increased the mineralized matrix within the mPCL scaffold (#). * $P < 0.001$. **(B)** MicroCT images of the mineralized matrix pre- and post-implantation for a representative scaffold from the 28 d pre-culture group. **(C)** High-resolution x-ray images of the mPCL scaffolds 4 weeks post-implantation. Note the bright white densities in the 28 d pre-culture scaffold ($n = 8$).

fetal-derived cell source collected from amniotic fluid *in vitro* and *in vivo*.

The 2D osteogenic differentiation showed that the AFS cells were capable of robust mineralization. The mineralized matrix produced by the AFS cells increased over the 4 weeks in culture. Multiple osteogenic medium supplementation protocols were evaluated, and the protocol described here produced 10 times as much mineralized matrix as the previously published AFS cell mineralization protocol (data not shown).^{10,11} This matrix had the characteristic chemical composition of biological hydroxyapatite, as determined using FTIR analysis. Additionally, 85% of the AFS cells were capable of osteogenic differentiation. This is higher than that found in human MSCs; approximately 50% of human MSCs are reported to form osteogenic colonies.^{24,25} Future studies that directly compare the osteogenic differentiation of AFS cells and MSCs are therefore warranted.

The Food and Drug Administration has approved the scaffold chosen in this study, medical-grade PCL, which has good mechanical stability and is highly porous, allowing a large surface area for cell attachment, differentiation, and extracellular matrix deposition, for human use. Lyophilized collagen was used to increase cell retention during seeding and produce a biologically active surface to which cells adhere well.^{9,26,27} To further increase mineralization of the AFS cells throughout the scaffold, it was necessary to differentiate the cells in dynamic culture. Three-dimensional culturing of cells on large scaffolds is challenging because of mass transport difficulties. Previous studies have shown the feasibility of using rat MSCs on PCL scaffolds and demonstrated the need for perfusion of the scaffolds to allow mineralization at the construct core.^{9,28} The AFS cells were able to produce mineralized matrix only on the periphery of

the scaffold in static culture; dynamic culture produced with the orbital shaker allowed a large sample size to be analyzed simultaneously while producing robust mineralized matrix throughout the scaffold and maintaining viability of the AFS cells at the scaffold core.

Long-term culture of the AFS cells on the scaffold combined with sequential microCT analysis allowed the determination of the rate of mineral deposition of individual scaffolds throughout the 15 weeks in culture. The AFS cells increased the rate of mineral deposition as the study progressed. Additionally, the AFS cells produced mineralized matrix comparable with that produced in the previous rat MSC studies at 5 weeks, the latest time points studied in those studies.⁹ Two AFS cell lines were analyzed in this study and found to have comparable mineralized matrix deposition in the 3D scaffolds. Although the H1 cell line had significantly more mineral at the 15-week time point, they were indistinguishable up to 10 weeks *in vitro*. It will be interesting to compare additional AFS cell lines to determine the full range of osteogenic differentiation capacity among different donors.

Little is known about the relative immunogenicity of different stem cell sources, but this is an important issue for clinical translation. Conflicting reports of the immunological response to MSCs has been reported, but most studies agree that the cells express MHC class I molecules and that these surface proteins are upregulated upon exposure to interferon gamma, which is typically found at cell implantation sites.^{29,30} MSCs do not appear to express MHC class II molecules or ABO blood group antigens, but it is speculated that they might after differentiation.³¹ Despite this possible immunogenicity, many studies have revealed long-term engraftment of allogeneic MSCs in immunocompetent animals.

AFS cells are much less studied because they have only recently been identified. DeCoppi *et al.* found that human AFS cells would engraft in the twitcher transgenic or normal neonatal mouse brain.¹⁰ Additionally, human AFS cells seeded onto an alginate–collagen scaffold contained mineral 8 weeks post-implantation at an ectopic site.¹⁰ Conversely, human AFS cells were shown not to engraft in the rat model of myocardial infarction.³² More data are needed with the AFS cells to understand these differences and compare their engraftment and immunogenicity with those of other stem cell sources such as MSCs.

To determine whether preconditioning was needed for *in vivo* osteogenic differentiation of the AFS cells, we placed the cell-seeded mPCL scaffolds subcutaneously in an athymic nude rat for 4 weeks. The athymic nude rat is deficient in T cells and is thus useful to evaluate human cell therapies. There was no mineral accumulation on the acellular scaffolds; therefore, the mPCL alone was not sufficient to promote endogenous cell osteogenic differentiation. The naïve AFS cell constructs were also not able to produce mineralized matrix *in vivo*. The 7 day pre-culture treatment of AFS cells was chosen as a time point to examine early differentiation without detectable mineral deposition. However, the 7-day pre-culture group did not produce significantly more mineral than the naïve AFS cells *in vivo*. Only the AFS cells with the longest pre-culture period (28 days), when mineral was detectable using microCT before implantation, were capable of robust mineralization *in vivo*. These AFS cells increased the mineralized matrix within the mPCL scaffold seven times during the 4 weeks *in vivo*.

The AFS cells were thus not able to mineralize at an ectopic *in vivo* implantation site unless they underwent extensive pre-differentiation *in vitro*. It remains to be determined how the cells will differentiate when placed in an orthotopic environment or an immunocompetent animal. Although pre-culture under osteogenic medium conditions was required in the present study for continued mineralization at an ectopic site *in vivo*, AFS cells may not require pre-differentiation when placed in a site of bone injury because they will receive differentiation cues from the fracture hematoma and adjacent bone.

The greater rate of mineralization during the *in vitro* mineralization and the need for pre-differentiation before *in vivo* implantation suggests the need for extensive programming of AFS cells before osteogenic differentiation. The AFS cells have multi-lineage differentiation capabilities similar to those of embryonic stem cells and are of fetal origin. Therefore, it is feasible that these cells need a greater differentiation stimulus than adult-derived stem cells to fully differentiate to osteoblasts. Even so, the increasing rate of mineralization during 3D osteogenic culture suggests that these cells may be useful for enhancing bone regeneration because of their long-term viability and robust mineralized matrix production.

In conclusion, this is the first study to clearly establish the impressive osteogenic potential of AFS cells. Previously, AFS cells have been shown to be able to form mineralized matrix, but the culture conditions were not well optimized.^{10,11} The conditions described here produce 10 times more mineral production than shown in the published protocol. The AFS cells were able to produce mineralized matrix throughout large, 3D mPCL scaffolds, and the rate of mineralization increased throughout the 15-week culture period. Although

naïve AFS cells and those receiving short-term osteogenic stimulation exhibited minimal mineralization after implantation *in vivo*, AFS cells that received longer osteogenic signaling *in vitro* demonstrated extensive mineralization *in vivo*. These results suggest that AFS cells are an exciting potential source of cells for the production of large, mineralized constructs and augmentation of clinical bone repair.

Acknowledgments

This work was supported by Georgia Tech/Emory Center for the Engineering of Living Tissues National Science Foundation grant EEC-9731643. AP was supported by National Institutes of Health Institutional Research and Academic Career Development Awards Post-doctoral training Grant 2K12GM000680-06.

Disclosure Statement

No competing financial interests exist.

References

1. Bianco, P., Robey, P.G. Stem cells in tissue engineering. *Nature* **414**, 118, 2001.
2. Oreffo, R.O., Triffitt, J.T. Future potentials for using osteogenic stem cells and biomaterials in orthopedics. *Bone* **25**, 5S, 1999.
3. Meinel, L., Karageorgiou, V., Fajardo, R., Snyder, B., Shinde-Patil, V., Zichner, L., Kaplan, D., Langer, R., Vunjak-Novakovic, G. Bone tissue engineering using human mesenchymal stem cells: effects of scaffold material and medium flow. *Ann Biomed Eng* **32**, 112, 2004.
4. Schantz, J.T., Teoh, S.H., Lim, T.C., Endres, M., Lam, C.X., Huttmacher, D.W. Repair of calvarial defects with customized tissue-engineered bone grafts I. Evaluation of osteogenesis in a three-dimensional culture system. *Tissue Eng* **9 Suppl 1**, S113, 2003.
5. Bruder, S.P., Fox, B.S. Tissue engineering of bone. *Cell based strategies. Clin Orthop Relat Res* **S68**, 1999.
6. Tokalov, S.V., Gruner, S., Schindler, S., Wolf, G., Baumann, M., Abolmaali, N. Age-related changes in the frequency of mesenchymal stem cells in the bone marrow of rats. *Stem Cells Dev* **16**, 439, 2007.
7. Einhorn, T.A. Clinically applied models of bone regeneration in tissue engineering research. *Clin Orthop Relat Res* **S59**, 1999.
8. Ishaug-Riley, S.L., Crane-Kruger, G.M., Yaszemski, M.J., Mikos, A.G. Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers. *Biomaterials* **19**, 1405, 1998.
9. Porter, B.D., Lin, A.S., Peister, A., Huttmacher, D., Guldberg, R.E. Noninvasive image analysis of 3D construct mineralization in a perfusion bioreactor. *Biomaterials* **28**, 2525, 2007.
10. De Coppi, P., Bartsch, G., Jr., Siddiqui, M.M., Xu, T., Santos, C.C., Perin, L., Mostoslavsky, G., Serre, A.C., Snyder, E.Y., Yoo, J.J., Furth, M.E., Soker, S., Atala, A. Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* **25**, 100, 2007.
11. Delo, D.M., De Coppi, P., Bartsch, G., Jr., Atala, A. Amniotic fluid and placental stem cells. *Methods Enzymol* **419**, 426, 2006.
12. De Coppi, P., Callegari, A., Chiavegato, A., Gasparotto, L., Piccoli, M., Taiani, J., Pozzobon, M., Boldrin, L., Okabe, M.,

- Cozzi, E., Atala, A., Gamba, P., Sartore, S. Amniotic fluid and bone marrow derived mesenchymal stem cells can be converted to smooth muscle cells in the cryo-injured rat bladder and prevent compensatory hypertrophy of surviving smooth muscle cells. *J Urol* **177**, 369, 2007.
13. Kolambkar, Y.M., Peister, A., Soker, S., Atala, A., Guldberg, R.E. Chondrogenic differentiation of amniotic fluid-derived stem cells. *J Mol Histol* **38**, 405, 2007.
 14. Peister, A., Mellad, J.A., Larson, B.L., Hall, B.M., Gibson, L.F., Prockop, D.J. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood* **103**, 1662, 2004.
 15. Gregory, C.A., Gunn, W.G., Peister, A., Prockop, D.J. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem* **329**, 77, 2004.
 16. Byers, B.A., Garcia, A.J. Exogenous Runx2 expression enhances in vitro osteoblastic differentiation and mineralization in primary bone marrow stromal cells. *Tissue Eng* **10**, 1623, 2004.
 17. Zein, I., Hutmacher, D.W., Tan, K.C., Teoh, S.H. Fused deposition modeling of novel scaffold architectures for tissue engineering applications. *Biomaterials* **23**, 1169, 2002.
 18. Digirolamo, C.M., Stokes, D., Colter, D., Phinney, D.G., Class, R., Prockop, D.J. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* **107**, 275, 1999.
 19. Smith, J.R., Pochampally, R., Perry, A., Hsu, S.C., Prockop, D.J. Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma. *Stem Cells* **22**, 823, 2004.
 20. Rommens, P.M., Coosemans, W., Broos, P.L. The difficult healing of segmental fractures of the tibial shaft. *Arch Orthop Trauma Surg* **108**, 238, 1989.
 21. Berrey, B.H., Jr., Lord, C.F., Gebhardt, M.C., Mankin, H.J. Fractures of allografts. Frequency, treatment, and end-results. *J Bone Joint Surg Am* **72**, 825, 1990.
 22. Salgado, A.J., Coutinho, O.P., Reis, R.L. Bone tissue engineering: state of the art and future trends. *Macromol Biosci* **4**, 743, 2004.
 23. Hutmacher, D.W., Schantz, T., Zein, I., Ng, K.W., Teoh, S.H., Tan, K.C. Mechanical properties and cell cultural response of polycaprolactone scaffolds designed and fabricated via fused deposition modeling. *J Biomed Mater Res* **55**, 203, 2001.
 24. Morito, T., Muneta, T., Hara, K., Ju, Y.J., Mochizuki, T., Makino, H., Umezawa, A., Sekiya, I. Synovial fluid-derived mesenchymal stem cells increase after intra-articular ligament injury in humans. *Rheumatology (Oxford)* **47**, 1137, 2008.
 25. Sakaguchi, Y., Sekiya, I., Yagishita, K., Ichinose, S., Shinomiya, K., Muneta, T. Suspended cells from trabecular bone by collagenase digestion become virtually identical to mesenchymal stem cells obtained from marrow aspirates. *Blood* **104**, 2728, 2004.
 26. Lan, C.W., Wang, F.F., Wang, Y.J. Osteogenic enrichment of bone-marrow stromal cells with the use of flow chamber and type I collagen-coated surface. *J Biomed Mater Res A* **66**, 38, 2003.
 27. Lan, C.W., Wang, Y.J. Collagen as an immobilization vehicle for bone marrow stromal cells enriched with osteogenic potential. *Artif Cells Blood Substit Immobil Biotechnol* **31**, 59, 2003.
 28. Cartmell, S.H., Porter, B.D., Garcia, A.J., Guldberg, R.E. Effects of medium perfusion rate on cell-seeded three-dimensional bone constructs in vitro. *Tissue Eng* **9**, 1197, 2003.
 29. Liu, H., Kemeny, D.M., Heng, B.C., Ouyang, H.W., Melendez, A.J., Cao, T. The immunogenicity and immunomodulatory function of osteogenic cells differentiated from mesenchymal stem cells. *J Immunol* **176**, 2864, 2006.
 30. Tse, W.T., Pendleton, J.D., Beyer, W.M., Egalka, M.C., Guinan, E.C. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* **75**, 389, 2003.
 31. Le Blanc, K., Samuelsson, H., Lonnies, L., Sundin, M., Ringden, O. Generation of immunosuppressive mesenchymal stem cells in allogeneic human serum. *Transplantation* **84**, 1055, 2007.
 32. Chiavegato, A., Bollini, S., Pozzobon, M., Callegari, A., Gasparotto, L., Taiani, J., Piccoli, M., Lenzini, E., Gerosa, G., Vendramin, I., Cozzi, E., Angelini, A., Iop, L., Zanon, G.F., Atala, A., De Coppi, P., Sartore, S. Human amniotic fluid-derived stem cells are rejected after transplantation in the myocardium of normal, ischemic, immuno-suppressed or immuno-deficient rat. *J Mol Cell Cardiol* **42**, 746, 2007.

Address correspondence to:

Alexandra Peister, Ph.D.

Morehouse College

Department of Biology

830 Westview Drive SW

Atlanta, GA 30317

E-mail: [apeister@morehouse.edu](mailto:apeaker@morehouse.edu)

Received: September 25, 2008

Accepted: April 3, 2009

Online Publication Date: June 30, 2009