Cell sourcing for bone tissue engineering: Amniotic fluid stem cells have a delayed, robust differentiation compared to mesenchymal stem cells

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Abstract Cell based therapies for bone regeneration are an exciting emerging technology, but the availability of osteogenic cells is limited and an ideal cell source has not been identified. Amniotic fluid-derived stem cells (AFS) and bone-marrow derived mesenchymal stem cells (MSCs) were compared to determine their osteogenic differentiation capacity in both 2D and 3D environments. In 2D culture, the AFS cells produced more mineralized matrix but delayed peaks in osteogenic markers. Cells were also cultured on 3D scaffolds constructed of poly-ε-caprolactone for 15 weeks. MSCs differentiated more quickly than AFS cells on 3D scaffolds, but mineralized matrix production slowed considerably after 5 weeks. In contrast, the rate of AFS cell mineralization continued to increase out to 15 weeks, at which time AFS constructs contained 5-fold more mineralized matrix than MSC constructs. Therefore, cell source should be taken into consideration when used for cell therapy, as the MSCs would be a good choice for immediate matrix production, but the AFS cells would continue robust mineralization for an extended period of time. This study demonstrates that stem cell source can dramatically influence the magnitude and rate of osteogenic differentiation \textit{in vitro}.

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Introduction

Little is known about the optimal cell source for tissue engineering of cell-based therapies for musculoskeletal tissues. Bone graft substitutes composed of a biodegradable scaffold containing stem cells capable of osteogenic differentiation have shown promise as an alternative to bone grafting.
or delivery of osteoinductive proteins. This cellular augmentation is especially important in clinical cases where endogenous cellular supply is diminished, such as in older patients, smokers, or after chemotherapy or radiation therapy. Mesenchymal stem cells (MSCs) are believed to be the source of osteoblastic cells during normal bone growth and remodeling and may be isolated from the bone marrow, among other tissues. Mesenchymal stem cells are a subpopulation of bone marrow-derived cells characterized by their ability to differentiate to the mesenchymal lineage tissues of bone, fat and cartilage, as well as muscle (Owen and Friedenstein, 1988; Owen et al., 1987; Bruder et al., 1998; Tsutsumi et al., 2001; Sekiya et al., 2002; Johnstone et al., 1998; Wakitani et al., 1995). Additionally, MSCs can be isolated from the intended recipient, thus reducing the probability of rejection (Elipoulo et al., 2005). Their relative ease of isolation and differentiation capability makes MSCs potentially useful for many applications, including bone defects (Caplan, 2005). Osteogenic differentiation of MSCs has been well characterized. With the addition of selected supplements to the basal growth media, often referred to as osteoinductive factors including dexamethasone, B-glycerophosphate and ascorbic acid, MSCs will mineralize the surface of a two-dimensional culture (Owen et al., 1987; Peister et al., 2004; Marie and Fromigue, 2006). Furthermore, MSCs have been used in 3D constructs to mineralize bone graft substitutes (Hutmacher et al., 2001; Cartmell et al., 2004; Porter et al., 2007).

Human amniotic fluid-derived stem (AFS) cells are isolated from amniotic fluid after routine amniocentesis (Choi et al., 2011; De Coppi et al., 2007a; Delo et al., 2006; Cananzi et al., 2009; In ‘t Anker et al., 2003; Prusa et al., 2003; Tsai et al., 2004; Roubelakis et al., 2007). The AFS cells express both embryonic and adult stem cell markers and are broadly multipotent; they can be induced to differentiate into cells representing all three embryonic lineages, such as cells of the osteogenic, adipogenic, chondrogenic, myogenic, endothelial, neuronal, and hepatic lineages (Delo et al., 2006; De Coppi et al., 2007b; Kolambkar et al., 2007; Ditadi et al., 2009; Zheng et al., 2009; Cipriani et al., 2007; Carraro et al., 2008; Hauser et al., 2010). Unlike embryonic stem cells, AFS cells are not tumorigenic and can expand extensively without the use of feeder layers or expensive defined media (De Coppi et al., 2007b). The AFS cell lines have been shown to expand over 250 population doublings and retain telomere length and have a normal chromosomal karyotype (De Coppi et al., 2007). Therefore, AFS cells have great potential for cellular tissue engineering. Previously, AFS cells have been shown to be capable of producing robust mineralized matrix in two-dimensional culture and throughout a three-dimensional medical grade poly-ε-caprolactone (PCL) scaffold and nanofibrous scaffolds (Kolambkar et al., 2007; Peister et al., 2009; Sun et al., 2010; Peister et al., 2008). This study compares the ability of the adult and fetal stem cell sources to produce mineralized matrix in 2D and 3D culture.

Tissue engineering strategies using an extracellular matrix combined with stem cells capable of osteogenic differentiation may therefore be used to develop bone graft substitutes. The aim of this study was to compare two cell sources for potential application in the tissue engineering of bone graft substitutes. Comparison of human fetal-derived AFS cells and MSCs for the production of bone graft substitutes will help elucidate the most appropriate cell source for particular applications and may suggest appropriate delivery strategies. By culturing cells on 3D scaffolds, it was determined that the AFS cells had greater osteogenic potential overall, but they were observed to take longer to differentiate than the MSCs.

Results

2D differentiation

The morphology of the AFS cells was heterogeneous, but overall there appeared to be two general morphologies with one very small and compact and the other more spindle shaped (Supplemental Figs. 1A and B). The MSCs were also heterogeneous, but to a lesser extent than the AFS cells. The MSCs were spindle shaped and visibly larger than the AFS cells (Supplemental Fig. 1C). The AFS cells and MSCs both differentiated into mineral producing cells when grown on tissue culture plastic. The mineralized matrix was visualized by the alizarin red S staining of calcium that covers the cells (Supplemental Figs. 1D–F).

Biochemical assay of 2D mineralized matrix

Although the staining appeared to be qualitatively similar between the cell types (Supplemental Figs. 1D–F), when the alizarin red was extracted and quantified, the AFS cells demonstrated significantly more mineral deposition/staining at 2 and 4 weeks (Fig. 1A). The increased mineralized matrix was confirmed by calcium extraction and quantification by Arsenazo III reagent (Fig. 1B). The AFS cell mineralized matrix contained significantly more Calcium than the matrix produced by the MSCs.

Gene expression

The expression levels of mRNA for Runx2, Alkaline Phosphatase and Osteocalcin were examined during osteogenic differentiation of the AFS cells and MSCs. Runx2 is a transcription factor involved in regulating osteogenic and chondrogenic differentiation and maintenance. Alkaline Phosphatase (ALP) is a phosphatase associated with mineralized matrix production. Osteocalcin is a vitamin D and K dependent protein produced by osteoblasts and is a common marker of bone formation.

In all three genes analyzed, the MSCs had a peak in gene expression between days 3 and 5 of differentiation (Figs. 1C–E). The AFS cells did not show this initial peak in gene expression but rather gradually increased expression over the 14 day culture. The ALP gene expression is consistent with previously reported findings that the MSCs have a higher ALP level at 1 week, at 2 weeks the levels are similar, and at 3 weeks the AFS cells have a higher ALP level (Kolambkar et al., 2010). The temporal differences in the gene expression support the hypothesis that the AFS cells are in a more primitive state and require additional time to differentiate into osteoblasts.
The AFS cells and MSCs were seeded onto PCL scaffolds with lyophilized collagen. The lyophilized collagen filled the scaffold pores to enhance cell retention and the composite scaffolds were placed in non-cell binding plates to encourage cell attachment. The seeding efficiencies of the cell types were not significantly different (Fig. 4C). Both the AFS cells and MSCs had visually occluded the pores by 5 weeks.

The mineralized matrix production by the cells was quantified by microCT analysis. The 3D renderings of the mineralized matrix are shown in Fig. 2. The AFS cells had produced significantly more mineral than the MSCs (p < 0.001). Both the AFS cells and MSCs produced significantly more calcium than their control counterpart (n = 6, p < 0.01). Real-time PCR quantification of Runx2 (C), Alkaline Phosphatase (D) and Osteocalcin (E) illustrate the temporal fluctuation of mRNA typical of osteoblasts. The MSCs had an early increase in transcript levels, which then decreased. The AFS cells required a longer induction time before the mRNA levels were increased (n = 3, P < 0.05).

**Figure 1** (A) To compare the amount of alizarin red staining of the mineralized matrix produced by the cells, the dye was extracted and the absorbance determined spectrophotometrically to determine the temporal changes in mineralized matrix. At 2 and 4 weeks the AFS cells had produced significantly more mineralized matrix than the MSCs. (B) Calcium deposition by the AFS cells and MSCs was confirmed and quantified using the Arsenazo III reagent at 4 weeks. The AFS cells deposited significantly more calcium than the MSCs (p < 0.001). Both the AFS cells and MSCs deposited significantly more calcium than their control counterpart (n = 6, p < 0.01). Real-time PCR quantification of Runx2 (C), Alkaline Phosphatase (D) and Osteocalcin (E) illustrate the temporal fluctuation of mRNA typical of osteoblasts. The MSCs had an early increase in transcript levels, which then decreased. The AFS cells required a longer induction time before the mRNA levels were increased (n = 3, P < 0.05).

**Osteogenic differentiation in 3D PCL scaffolds**

The AFS cells and MSCs were seeded onto PCL scaffolds with lyophilized collagen. The lyophilized collagen filled the scaffold pores to enhance cell retention and the composite scaffolds were placed in non-cell binding plates to encourage cell attachment. The seeding efficiencies of the cell types were not significantly different (Fig. 4C). Both the AFS cells and MSCs had visually occluded the pores by 5 weeks.

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**Quantification of 3D mineralized matrix**

The quantity of mineralized matrix was determined by the microCT scan, and the averages are shown in Fig. 3A. The MSCs produced significantly more mineral at the 3 and 5 week time points, but their mineralization capacity was limited to the first few weeks in culture. Alternatively, the AFS cells did not produce substantial mineralized matrix until 5 weeks in culture, with mineralized matrix production increasing throughout the 15 weeks in culture. At 3 and 5 weeks, the MSCs had produced significantly more mineralized matrix than the AFS cells (p < 0.001), but by 15 weeks the AFS cells had produced 3 times more mineralized matrix than the MSCs (p < 0.001). At all time points, cells grown in osteogenic media produced more mineralized matrix than those grown in control media (p < 0.001).

Two AFS cell lines were available at the time of this study and their differentiation potential was compared. Little variability was seen in the osteogenic differentiation of the A1 and H1 AFS cell lines. The mineralized matrix volume did not differ until 15 weeks in culture at which time the H1 AFS cells had produced more mineralized matrix than the A1 AFS cells. Both AFS cell lines produced approximately 5 times the mineralized matrix than the MSCs at 15 weeks. The AFS cell control media constructs also had more mineralized matrix than the MSC control constructs at 15 weeks (p < 0.05), which

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may indicate greater spontaneous differentiation of the AFS cells in the absence of osteogenic cues.

**Rate of 3D mineralization**

The scaffolds were scanned sterile and returned to culture. Therefore, the rate of mineralized matrix production was determined by quantifying the new mineral volume since the previous scan and dividing by the number of weeks between scans. The average rate of mineralized matrix production (Fig. 3B) by the MSCs was greater at 3 weeks than the AFS cells (p < 0.001). By 5 weeks, the rate of mineralization of the cell types was not significantly different. At 10 and 15 weeks, the AFS cells were producing mineralized matrix at a greater rate than the MSCs (p < 0.001). Additionally, at 10 and 15 weeks the MSCs were producing mineralized matrix with a rate that is indistinguishable from the control cells.

**Calcium content and cell number**

The AFS cells produced significantly more mineralized matrix, as analyzed by calcium concentration, than the MSCs (Fig. 4A). Additionally, both the MSCs and AFS cells produced significantly more mineralized matrix when placed in osteogenic media than in control media (n = 6, p < 0.001). This increased mineralization was not due to an increased cell number, but to an increased matrix deposition as the AFS cells and MSCs attached to the scaffold in comparable numbers (Fig. 4C). The seeding efficiency was comparable for both cell types on the PCL scaffold. After 15 weeks in culture, both the AFS cell and MSC seeded scaffolds had similar cell number whether cultured in control or osteogenic media, as determined by DNA content (Fig. 4D). Therefore, human AFS cells demonstrated an increased long-term osteogenic differentiation and mineralized matrix production capacity compared to human MSCs.

**Cell viability**

After 15 weeks in culture, cell viability was determined by a live–dead assay that relied on the intracellular esterase activity and plasma membrane integrity, two measures of cell viability, to discriminate between live and dead cells. Photomicrographs of the exterior surfaces (top, bottom and exterior circumference) are shown (Fig. 4B). Additionally, the cells at the interior of the scaffolds were examined by cutting the scaffold longitudinally. It can be observed that even after 15 weeks in culture, the cell viability is very high in all the scaffolds.

**Histology**

Histological examination of the scaffolds after 15 weeks, using resin embedding and microtome sectioning to 5 μm followed by von Kossa staining revealed no mineralisation for the control (MSCs in CCM) PCL scaffolds (Figs. 5A–D).
MSCs at 3 weeks (p<0.001). At 3 and 5 weeks, significantly more mineral was produced by the MSCs than the AFS cells (p<0.001). At 10 and 15 weeks, both AFS cell lines produced significantly more mineral than the MSCs (p<0.001). At 15 weeks, the H1 AFS line also produced significantly more mineral than the A1 cell line. N=6 for controls, n=12 for osteogenic. (B) The rate of mineral deposition was calculated for each scaffold and then compiled with its cohort. The rate of osteogenic mineral deposition was significantly greater than the A1 cell line. N=6 for controls, n=12 for osteogenic.

Only pink stained fibrous tissue can be observed. Whereas the onset of bone formation was observed for osteoinduced MSC and AFS cells which were seeded into 3D PCL scaffolds. Mineral nodules containing calcium, stain black with the von Kossa staining by virtue of silver ions (positive charge) binding with the mineralised tissue (negative portion of the calcium salt) forming a silver salt which is black in color. Clear black mineral deposition can be observed for osteoinduced MSCs (Figs. 5E–H) and AFS cells (I–P), within a fibrous network penetrating the scaffold pores. Residual PCL scaffold was evident within all transplants as evidenced by voids in the tissue from longitudinal and transverse sectioning of the scaffold struts. It can be noted that the pattern of mineral deposition was different between each cell type and seems to favor a more central scaffold locality for MSC cells whereas the AFS cells seem to deposit throughout the entire scaffold both centrally and at the periphery.

Discussion

This study demonstrates the mineralization potential of a fetal and adult stem cell population. The fetal AFS cells demonstrated an increased duration of mineralized matrix production, but the MSCs were capable of robust differentiation at the earlier time points. After 15 weeks in 3D culture, the AFS cells had produced approximately 20 mm³ of mineralized matrix, whereas the MSCs had produced approximately 4 mm³ by 5 weeks, which did not increase thereafter. MicroCT analysis showed that the mineralization for MSC scaffold constructs was located mainly within the central core of the scaffold as opposed to the AFS cells which mineralized the entire scaffold including the outer edges.

In 2D culture, the AFS cells differentiate readily and using a standard time course it is not evident that there is a lag in differentiation by these cells. Quantitative PCR comparing osteogenic gene expression between the two cell sources in 2D revealed a lag in the AFS cells when compared to the MSCs. This supports the longer induction time necessary for the AFS cells to differentiate to osteoblasts. The sensitivity of the staining of the extracellular matrix in 2D culture was not capable of determining these early differences in the cell differentiation because by 14 days the gene expression by the AFS cells matched or exceeded the expression in the MSCs.

The 15 week in vitro culture protocol used in this study provided a very challenging culture system to examine long term potential of the cell sources. When grown in a large 6 mm×9 mm PCL scaffold, the AFS cells had great potential but the cells required an extensive induction time prior to their osteogenic differentiation. Current studies often culture cells within 3D scaffolds for less than 6 weeks but this time period would not have shown the potential of the AFS cells. Early time points suggest that the MSCs have a greater potential, but the AFS cells have a greater overall mineralized matrix production capacity when examined after an extensive culture period. This supports the hypothesis that the AFS cells have a longer induction period than the MSCs, which may be due to their fetal origins.

The mineral deposition distribution found by microCT was supported by histological examination which demonstrated that the pattern of black stained mineral deposition was different between each cell type and seems to favor a more central scaffold locality for MSC cells whereas the AFS cells seem to deposit throughout the entire scaffold both centrally and at the periphery. This could be due to the MSCs requiring local mineral presence in order to nucleate more mineral production which leads to a tendency to concentrate their cells actively capable of mineralisation within the same area (in the scaffold core in this case) whereas AFS cells might be more capable of spontaneous mineralisation without requiring

**Figure 3** (A) The volume of mineralized matrix produced by the osteogenic and control cells within the PCL scaffold was determined by microCT. At all time points there was significantly more mineral in the osteogenic scaffolds than the control scaffolds (p<0.001). At 3 and 5 weeks, significantly more mineral was produced by the MSCs than the AFS cells (p<0.001). At 10 and 15 weeks, both AFS cell lines produced significantly more mineral than the MSCs (p<0.001). At 15 weeks, the H1 AFS line also produced significantly more mineral than the A1 cell line. N=6 for controls, n=12 for osteogenic.

(B) The rate of mineral deposition was calculated for each scaffold and then compiled with its cohort. The rate of mineral deposition was significantly higher in the osteogenic MSCs at 3 weeks (p<0.001) when compared to osteogenic AFS cells, but at 5 weeks the rate of mineralization between the three osteogenic groups was not significantly different. At 10 and 15 weeks, the rate of mineralization of the osteogenic MSC cell groups was significantly greater than the osteogenic MSCs. At 15 weeks, the H1 mineralization rate was greater than the A1 AFS cell mineralization. At 10 and 15 weeks, the rate of mineralization of the MSCs was not significantly different from the control groups. N=6 for controls, n=12 for osteogenic.

(p<0.001). N=6 for controls, n=12 for osteogenic.
any prerequisite mineral existing in close proximity. Alternatively, the mineral nucleation sites (such as those present on collagen or denatured collagen) (Taubenberger et al., 2010) might have been distributed more centrally in the case of the MSC scaffold constructs compared to the AFS scaffold constructs. It certainly highlights the differences in mineralisation capacities of these different cells from a temporal, spatial and rate perspective.

Serial microCT scans of scaffolds were performed which allowed the determination of the change in matrix within each scaffold over the 15 weeks in culture. The rate of mineralized matrix production was higher for the MSCs at the beginning of the culture but the cells did not produce detectible mineralized matrix after the 5 week scan. The AFS cells had a low rate of mineralization for the first 3 weeks, but the rate increased thereafter and continued to increase over the 15 weeks of culture. This may be due to the more primitive state of the fetal AFS cells needing additional time or cell density before they will differentiate to osteoblasts.

Although the rate of MSC mineralized matrix production decreased after 5 weeks, the cells maintained high viability throughout the culture. At 15 weeks, the cells were found throughout the scaffolds and there was minimal cell death in scaffolds seeded with either cell type without bias for the spatial localization within the scaffold. Additionally, the AFS cells and MSCs had similar cell numbers attach to the scaffold and there was minimal cell death in scaffolds seeded with either cell type without bias for the spatial localization within the scaffold. The long induction time for the AFS cells may make this even more challenging. Co-implantation of both MSCs and AFS cells may prove beneficial as the MSCs could provide immediate mineralized matrix production and the AFS cells will provide long-term support.

Cell-based tissue engineering strategies represent a clinical alternative to bone grafting and the delivery of osteoinductive proteins (Kimelman et al., 2007). However, cell sourcing is a critical issue for cell-based therapies which aim to regenerate musculoskeletal tissues, and this issue needs to be addressed (Caplan, 2005; Barrilleaux et al., 2006). Tissue engineering approaches that combine biodegradable scaffolds with stem cells capable of osteogenesis have shown promise as an effective bone graft substitute (Meinel et al., 2004). Cell-based engineered bone grafts are
an attractive alternative to allografts or autografts, particularly when the endogenous supply of stem cells is depleted through advanced age or concurrent therapy (Hutmacher et al., 2001; Bruder and Fox, 1999; Salgado et al., 2004). Several factors are critical for the choice of transplanted cells, such as: a) availability in sufficient numbers for therapeutic use, b) immune-tolerance, and c) the ability to promote bone formation on a therapeutic timescale.

Despite well known developmental differences in tissue regeneration and scar formation, very little is known about the differences in phenotype and regenerative capacity of stem cells isolated from different human developmental stages. Although there is evidence that allogeneic stem cells promote bone repair, many tissue-engineering studies have been limited by a lack of quantitative outcome measures to allow direct comparisons between different stem cell sources. Purified mesenchymal stem cells (MSCs) derived from bone marrow have been shown to enhance repair of critically sized defects in preclinical animal studies (Bruder and Fox, 1999; Sanchez-Guijo et al., 2009).

Many questions still remain pertaining to the use of stem cells for regenerative medicine. Although we have shown that the AFS cells have an increased osteogenic capacity than the MSCs in vitro, the comparison of this capacity in vivo is of greater clinical significance. Critical factors are still unknown for the optimal delivery strategy, such as: a) should the cells be undifferentiated or pre-differentiated in culture prior to implantation, b) are exogenous factors such as pro-angiogenic growth factors important for revascularization, c) what is the optimal time point for implantation post-trauma, and d) how many cells should be delivered and should they be implanted in one site as a bolus or at multiple implantation sites or specific designated times? There is vast potential for stem cells in regenerative medicine, and determining the optimal cell source will certainly improve patient outcome.

Methods

Amniotic fluid stem cell culture

Human AFS cells were kindly provided by the Institute for Regenerative Medicine at Wake Forest University (De Coppi et al., 2007b). In this study, two cell lines were available and both were analyzed, A1 and H1 human AFS cells. The AFS cells were received at passage 14, and further expanded 2–3 times in α-MEM medium containing 15% FBS, 2 mM L-glutamine, 100 units penicillin, 100 μg streptomycin (all Invitrogen, Carlsbad, CA), supplemented with 18% Chang B and 2% Chang C (Irvine Scientific, Santa Ana, CA) at 37 °C with 5% CO2 atmosphere. This media is referred to in this paper as modified Chang media. AFS cells were sub-cultured at a dilution of 1:10 and not permitted to expand beyond 70% confluence in the modified Chang media on Integrid plates (BD Falcon, San Jose, CA). Cells were frozen in the modified Chang media supplemented with 5% DMSO. For experimental use, 1×106 AFS cells were quickly...
thawed to 37 °C and placed in 10, 150-mm Integrid culture plates with modified Chang media. After 5 days, the cells were harvested with 0.25% trypsin–EDTA (Invitrogen), counted and used experimentally.

**Mesenchymal stem cell culture**

Human MSCs were generously provided by the Texas A&M University Health Science Center through the NIH-funded center for preparation and distribution of adult stem cells (5P40RR017447-08). MSCs from four adult donors were expanded by plating the cells at an initial density of 50 cells/cm² and cultured in complete culture media (CCM) or also referred to as Control Media. CCM consists of alpha-modified minimal essential media (αMEM, Invitrogen) supplemented with 17% FBS (Atlanta Biologicals), 2 mM L-glutamine, 100 units penicillin, 100 μg streptomycin (all Invitrogen). Cells were cultured on tissue culture dishes (Nuncleon A surface, Thermo Fisher, Rochester, NY). After 7–9 days, the cells were harvested with 0.25% trypsin–EDTA (Invitrogen), counted and used experimentally. Cells were not permitted to expand beyond 70% confluence and were frozen in αMEM supplemented with 30% serum and 5% DMSO. For experimental use, all four MSC donor cells were quickly thawed to 37 °C and placed in four 10-mm Nuncleon culture plates with CCM to recover from freezing. The next day the cells were removed from the plates with trypsin–EDTA and counted. Equal quantities of each donor MSCs were combined and placed in Nuncleon culture plates at 50 cells/cm². After 7 days, the cells were harvested with 0.25% trypsin–EDTA, counted and used experimentally.

**Osteogenic differentiation**

AFS cells or MSCs were cultured at 20,000 cells/cm² in 6 well plates (Nunc, Rochester, NY) either modified Chang media (AFS cells) or CCM (MSCs), N=6 for all conditions. After allowing the cells to attach to the culture dish for 24 h, the media was changed to the osteogenic induction conditions, allowing the cells to attach to the culture dish for 24 h, the media was changed to the osteogenic induction conditions, which comprised CCM media supplemented with 1 μM Dexamethasone, 6 mM β-glycerol phosphate, 50 μg/ml Ascorbic acid 2-Phosphate, and 50 ng/ml Thyroxine (all Sigma, St. Louis, MO) (Peister et al., 2004, 2008). Control samples of both MSCs and AFS cells were grown in CCM to exclude spontaneous differentiation. The media was changed 2 times per week. Cells were analyzed 2 and 4 weeks after the start of differentiation.

**Alizarin red S staining for calcium**

The cells were washed with excess PBS (Mg²⁺ and Ca²⁺ free, Invitrogen) and fixed in 10% neutral buffered formalin (Sigma) for 15 min. The cells were washed 3× with water, then stained with 2 ml 0.4 mM Alizarin red S (pH 4.2, Sigma) for 20 min with rocking. 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 and using vigorous washing with excess water (4 times for 5 min each, with rocking). Stained monolayers were visualized by phase microscopy using an inverted microscope (Nikon, Melville, NY).

**Gene expression by real-time RT-PCR**

For each cell type, 50,000 cells/cm² were placed into 6 well dishes and cultured overnight in CCM. After allowing the cells to attach to the culture dish for 24 h, the media was changed to osteogenic media and the media was changed every 3 days throughout the culture. Cells were harvested at days 0, 1, 2, 3, 5, 7, 10 and 14 using a cell scraper (Nunc) and the RNA was isolated using Qiagen RNEasy Plus kit according to the manufacturer’s instructions. cDNA was produced from 1 μg total RNA using Superscript III First-strand Synthesis SuperMix according to manufacturer’s instructions (Invitrogen). Real-time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) with Power Sybr Green PCR Master Mix (Applied Biosystems). The cycle threshold was normalized to GapDH and compared to a relative standard curve for each primer set. Primer sequences and concentrations are given in Supplementary Table S1.

**Scaffold preparation**

3-dimensional sheets of PCL lattice comprised of 300 μm struts spaced 500 μm apart in a 0, 60, 120 degree repeating pattern with 85% porosity and a height of 9 mm were produced through fused deposition modeling as previously described (Porter et al., 2007; Peister et al., 2009) with a height of 9 mm. Cylinders of a 6 mm diameter were cut from the sheet with a dermal biopsy punch (Miltex, York, PA). The cylinders were incubated in 5 M NaOH for 2 h at 37 °C to clean and partially degrade the scaffold surface, thereby increasing surface roughness. The scaffolds were then washed 3× in excess sterile water and sterilized through 70% ethanol evaporation.

To produce a collagen network throughout the pores of the PCL lattice, 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 9 parts sodium bicarbonate and 250 μl was placed in a custom mold. The PCL cylinder was then placed in the mold/collagen and the collagen allowed to gel for 30 min at room temperature, and then cooled to ~80 °C for 2 h. The PCL/collagen was then placed in a lyophilizer 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). To maintain scaffold orientation during culture, the cylinders were placed in a holder consisting of a sterile 3/4” Teflon disk with 4 stainless steel pins surrounding the cylinder.

**Cell seeding onto PCL/collagen scaffolds**

AFS cells and MSCs were expanded as described above. Six million cells were resuspended in 150 μl of modified Chang media or CCM and slowly placed drop-wise on the top of the scaffold. The media were readily absorbed by the collagen mesh, with minimal pooling at the bottom. The cell/scaffold was placed in the 37 °C incubator for 1 h to promote cell attachment, after which time 4 ml of modified Chang media or CCM was carefully added to each well. The scaffolds were cultured under static conditions for 3 days to allow the cells to attach to the scaffold.

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After 3 days, the media was carefully aspirated and osteogenic media was added to each osteogenic sample and control samples were cultured in CCM. At this time, the scaffolds were placed on a rocker plate to increase media perfusion through the scaffold (Belly Button® orbital shaker, 7.5 rpm, minimal pitch, Stovall, Greensboro, NC). The media was changed every 2–3 days for 15 weeks (Peister et al., 2008).

The seeding efficiency was determined at day 3, prior to the placement in osteogenic media. The scaffolds were washed 2× with PBS to remove unattached cells. The scaffolds were then immersed in 1 ml of trypsin–EDTA for 5 min at 37 °C. The trypsin was inactivated with 5 ml complete culture media and the cells pelleted by centrifugation at 450×g for 10 min. The cell pellets were resuspended in 3 ml CCM, stained with trypan blue and counted in duplicate on a hemocytometer. N=4.

Microcomputed tomography scanning

At 3, 5, 10, and 15 weeks, the scaffolds were removed from the Teflon holders and placed in a sterile polysulfone sample holder. Mineralization of the scaffolds was quantified using the VivaCT (Scanco Medical, Switzerland) at a 21.5 voxel resolution. Samples were evaluated at a threshold of 80, a filter width of 1.2, and filter support of 1. For each scaffold, a measurement of the volume filled with hydroxyapatite was determined. The samples were then removed aseptically from the sample holder and returned to the Teflon holder for further culture. N=12.

Calcium assay

At 15 weeks in either osteogenic or control media, the scaffold was dissected into 4 sections by slicing in a custom matrix so that each scaffold was cut vertically across the diameter of the cylinder and horizontally to produce 4 sections of equal size. ¼ of the scaffold, ½ of the top half of each scaffold, was used to determine the calcium within the matrix. 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 were mixed with 300 μl of calcium reagent (Arsenazo III, Diagnostic Chemicals Limited) and the absorbance determined at 615-nm with a spectrophotometer. N=6.

DNA assay

The PicoGreen DNA quantification kit (Invitrogen) was used to following the protocol recommended by the manufacturer to determine at 15 weeks the relative amount of DNA within scaffolds from the different experimental groups. Lambda DNA standards were produced from 1 μg to 1 ng. The cell lysates were diluted 1:10 in Tris–EDTA buffer. 100 μl of the PicoGreen working solution and 100 μl of each sample were placed in triplicate, in black 96-well plates. After a 5-minute 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=6.

Cell viability staining

Cell viability at 15 weeks was determined using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells according to manufacturer’s instructions (Molecular Probes, Invitrogen). Briefly, scaffolds were cut longitudinally and washed 3 times in excess PBS and then incubated for 45 min in 4 μM Calcein AM and 4 μM Ethidium homodimer-1 with gentle shaking. The scaffolds were then washed 3 times with PBS and analyzed using a confocal microscope (LSM 510 UV, Carl Zeiss, Thornwood, NY). Micrographs were taken on the exterior surfaces: top, bottom, and outside surfaces as well as the cut surface to check the viability at the core of the scaffold.

Histology

For histological examination, scaffolds were removed from culture at 15 weeks and fixed in 4% paraformaldehyde. An ethanol gradient (30 min in 70%, 1 h in 90%, 95% and 100% ethanol) was used to dehydrate the samples. They were next processed three times through xylenes for 40 min each, infiltrated with methylmethacrylate (MMA) for 3 h and embedded in MMA containing 3% PEG softener. Five micrometer sections were cut with an osteomicrotome (SM2500; Leica Microsystems, Wetzlar, Germany), stretched flat with 70% ethanol onto a polylysine coated microscope slide (Lomb Scientific), overlayed with a plastic film and slides were clamped together before being dried overnight at 50 °C. Sections were then stained using combined von Kossa and van Giesen stains to visualize the mineralised bone and connective tissue respectively (Reichert et al., 2010).

Data analysis

Data are reported as mean±SE and statistical analyses was performed using Graphpad Prism 5 software using a general linear model (ANOVA) and Tukey’s post-hoc analysis with Bonferroni adjustment or pair wise comparisons; with p<0.05 considered significant.

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2011.03.001.

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References


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