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Oxygen Tension Differentially Influences Osteogenic Differentiation of Human Adipose Stem Cells in 2D and 3D Cultures

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ABSTRACT

Skeletal defects commonly suffer from poor oxygen microenvironments resulting from compromised vascularization associated with injury or disease. Adipose stem cells (ASCs) represent a promising cell population for stimulating skeletal repair by differentiating toward the osteogenic lineage or by secreting trophic factors. However, the osteogenic or trophic response of ASCs to reduced oxygen microenvironments is poorly understood. Moreover, a direct comparison between 2D and 3D response of ASCs to hypoxia is lacking. Thus, we characterized the osteogenic and angiogenic potential of human ASCs under hypoxic (1%), normoxic (5%), and atmospheric (21%) oxygen tensions in both 2D and 3D over 4 weeks in culture. We detected greatest alkaline phosphatase activity and extracellular calcium deposition in cells cultured in both 2D and 3D under 21% oxygen, and reductions in enzyme activity corresponded to reductions in oxygen tension. ASCs cultured in 1% oxygen secreted more vascular endothelial growth factor (VEGF) over the 4-week period than cells cultured in other conditions, with cells cultured in 2D secreting VEGF in a more sustained manner than those in 3D. Expression of osteogenic markers revealed temporal changes under different oxygen conditions with peak expression occurring earlier in 3D. In addition, the increase of most osteogenic markers was significantly higher in 2D compared to 3D cultures at 1% and 5% oxygen. These results suggest that oxygen, in conjunction with dimensionality, affects the timing of the differentiation program in ASCs. These findings offer new insights for the use of ASCs in bone repair while emphasizing the importance of the culture microenvironment. J. Cell. Biochem. 110: 87–96, 2010.

KEY WORDS: ADIPOSE STEM CELLS; 3D CULTURE; OSTEOGENESIS; OXYGEN; HYPOXIA

Approximately 10% of fractures are slow or nonhealing [Logeart-Avramoglou et al., 2005] and may be characterized by poor vascularization and a resulting hypoxic local microenvironment [Schleier et al., 2006]. The absence of a sufficient vasculature impairs native healing processes by limiting the availability of nutrients to resident progenitor cells and infusion of inductive factors from the systemic circulation that promote healing. In a rabbit fracture model, oxygen tension in the fracture callus at day 4 post-fracture was as low as 0.8% and remained as low as 3% in newly formed bone up to 17 days post-fracture [Brighton and Krebs, 1972]. Similarly, oxygen tension 3 days post-injury in a healing canine rib defect was reported at 1% oxygen [Komatsu and Hadjiargyrou, 2004]. Cell transplantation is a promising alternative to the "gold standard" of autologous bone grafting to stimulate bone repair. Bone marrow-derived mesenchymal stem cells (MSCs) are under widespread investigation for use in bone repair and regeneration, having demonstrated efficacy in both preclinical and clinical models [Caplan and Dennis, 2006; Sterodimas et al., 2009]. Adipose stem cells (ASCs) are another source of osteoprogenitor stem cells that exhibit multilineage potential similar to MSCs upon induction [Zuk et al., 2002; Gimble and Guilak, 2003; Mizuno, 2009]. The consequences of a low oxygen environment on cells are unclear but may include enhanced apoptosis, reduced proliferation, and inhibited differentiation; all of which ultimately impact tissue repair.

It has generally been shown that osteoblasts respond to hypoxia through hypoxia inducible factor-1α related pathways and downstream

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molecules that affect osteodifferentiation [Utting et al., 2006; Riddle et al., 2009] and enhance skeletal repair [Wang et al., 2007]. However, the data describing the effect of oxygen tension on the osteogenic differentiation of MSC-type populations are contradictory. Rat bone marrow-derived MSCs exhibited optimal differentiation and subsequent bone formation when cultured in 5% oxygen [Lennon et al., 2001]. Conversely, reduction in oxygen level was shown to inhibit various activities of murine ASCs, specifically their rate of proliferation and osteogenic differentiation [Malladi et al., 2006]. Even transient exposure to reduced oxygen inhibited osteoblastic differentiation of murine preosteoblasts, BMSCs, and ASCs compared to cells maintained in atmospheric oxygen tension [Salim et al., 2004]. Finally, human marrow-derived progenitor cells exposed to conditions ranging from 1% to 10% oxygen demonstrated increased proliferation with reduced osteogenic activity over 2 weeks versus cells cultured in ambient air [D'Ippolito et al., 2006; Raheja et al., 2009].

Beyond the oxygen microenvironment, other culture conditions such as dimensionality (i.e., 2D monolayer culture vs. 3D culture) can have profound effects on osteogenic differentiation. The expression of various genes characteristic of osteogenesis (e.g., alkaline phosphatase (ALP), osteocalcin, and bone sialoprotein) was distinctly different between rat MSCs cultured on alginate films in 2D or within alginate beads [Barralet et al., 2005]. Compared to monolayer culture, 3D systems are capable of increased ECM adsorption and retention due to increased available area. Furthermore, specific geometry of the substrate alone has been able to affect significant differences in MSC differentiation [Ruiz and Chen, 2008]. These data suggest that the cell culture geometry has a profound effect on cell behavior.

In this report, we provide evidence that the osteogenic capacity of ASCs is influenced to varying degrees by both oxygen tension and culture geometry. This study examines changes in biochemical markers, gene expression, and proteins secreted by ASCs as both dimensionality and oxygen tensions are varied. Oxygen tensions of 1% and 5% were chosen to represent conditions characteristic of diseased and healthy bone tissues, respectively, along with oxygen tensions of target sites for cell transplantation. Understanding the behavior of these cells under physiologic and diseased conditions will allow us to better predict their response to various engineered applications.

MATERIALS AND METHODS

CELL CULTURE

ASCs were obtained from lipoaspirates following informed consent and used as provided by the supplier (Zen-Bio, Inc., Research Triangle Park, NC). Lipoaspirates came from female donors with an average age of 41 years (n = 7, range: 27–51) and BMI of 25.2 (range: 22.5–28.2). Cells were expanded and maintained in monolayer culture using DMEM-F12 basal media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (J R Scientific, Woodland, CA), 1% penicillin–streptomycin (Mediatech, Manassas, VA), and 0.1% amphotericin B (Invitrogen) in a standard humidified incubator (37°C/5% CO_2/21% O_2). ASCs were used between passages 5 and 7.

CELL SEEDING

ASCs were seeded on tissue culture plastic (TCP) or poly(lactide-co-glycolide) (PLG) scaffolds for 2D and 3D studies, respectively. Scaffolds were fabricated from PLG (8515 DLG 7E, Lakeshore Biomaterials, Birmingham, AL) using a gas foaming/particulate leaching method as previously described [Davis et al., 2009; He et al., 2010]. This process generated scaffolds of 8.5 mm diameter × 1.5 mm thickness (total volume of 85 μl) and an average porosity of 95%.

For 2D experiments, ASCs were seeded near confluence (30,000 cells/cm^2) in basal media on TCP. ASCs were seeded on PLG scaffolds to study the osteogenic potential in 3D. Prior to seeding, scaffolds were sterilized using 95% ethanol for 30 min, then rinsed twice with PBS over 30 min. Sterilized scaffolds were then incubated for 30 min in basal media prior to static seeding with ASCs (1×10^6 cells/scaffold). Cells were allowed to attach for 2 h prior to culture in basal media for 24 h. After allowing cells to attach, scaffold seeding efficiency was measured 2 h later by quantifying total DNA content from cell lysates using the Quant-iT PicoGreen dsDNA kit (Invitrogen).

After culture in basal media for 24 h, the media for ASCs in either 2D or 3D culture was exchanged for media containing osteogenic supplements (10 mM β-glycerophosphate, 50 μg/ml ascorbate-2-phosphate, 10 nM dexamethasone (all from Sigma–Aldrich, St. Louis, MO), and 10 nM 1α, 25-$(OH)_2$ Vitamin D_3 (Biomol International, Plymouth Meeting, PA)). Cells were cultured in 1%, 5%, or 21% oxygen-regulated incubators for up to 4 weeks with media changes three times a week. Scaffolds were maintained on an XYZ shaker at 25 rpm to enhance nutrient transport.

As polyploidy can occur in culture during abnormal cell division, potentially skewing values for DNA content away from expected diploid values used in normalizing data, ASCs at passage 5–7 were seeded at 30,000 cells/cm^2 in 12-well dishes and cultured in 0% or 10% FBS in order to impair or promote ASC proliferation. After 1 week, cells were rinsed in PBS, lysed in passive lysis buffer, and DNA content was quantified using the Quant-iT PicoGreen dsDNA kit.

OSTEOGENIC POTENTIAL

ALP expression in 2D was qualitatively observed using a cytochemical stain [D'Ippolito et al., 1999]. Osteogenic potential of ASCs was assessed at 1, 7, 14, 21, and 28 days by rinsing cells or scaffolds in PBS, collecting cells in 500 μl passive lysis buffer (Promega, Madison, WI), and sonicating. Changes in ALP activity were determined quantitatively from the lysate using a routine PNPP colorimetric assay and subsequently measuring the absorbance at 405 nm as described [Davis et al., 2009] and normalized to total DNA content determined from the same cell lysate.

Extracellular calcium deposition was quantified after culture for 4 weeks. Mineral distribution in 2D was visualized with an Alizarin red stain as described [Stanford et al., 1995]. Calcium content was quantified using previously described methods [Davis et al., 2009]. Baseline calcium levels from cultured ASCs or scaffolds alone were determined for additional assay controls at the time of seeding.

For measurement of vascular endothelial growth factor (VEGF) protein secretion, media was exchanged with fresh media containing osteogenic supplements 24 h prior to sample collection for

quantification of secreted VEGF using a protein-specific ELISA kit (R&D Systems, Minneapolis, MN). VEGF accumulation over 24 h was normalized to DNA content.

CELL VIABILITY AND APOPTOSIS

Cell viability was assessed by measuring metabolic activity with alamarBlue. At each time point, alamarBlue stock solution (AbD Serotec, Raleigh, NC) was added to fresh media containing osteogenic supplements to make a 10% (v/v) solution. Media was then added to each sample and returned to their respective oxygen tensions. After 2 h, the absorbance of the media was measured at 570 and 600 nm, and dye reduction was quantified according to the manufacturer's instructions. ASC apoptosis was quantified using a commercial caspase-3/7 activity assay (Promega, San Luis Obispo, CA). Caspase activity in 2D cultures was determined according to the manufacturer's instructions. For 3D cultures, cells were first detached from scaffolds with trypsin (5 min) and resuspended at 100,000 cells/ml in osteogenic media. Caspase activity was detected from 100 μl of the resulting cell suspension per assay instructions. All assays were normalized to total DNA mass to account for differences in cell proliferation.

QUANTITATIVE PCR

ASCs or ASC-seeded scaffolds were briefly washed with PBS, total RNA was collected using RNeasy Mini kit (Qiagen, Valencia, CA), and 200–1,000 ng of total RNA was reverse-transcribed with Superscript II Reverse Transcriptase (Invitrogen). qtPCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) on a Mastercycler® realplex2 (Eppendorf, Westbury, NY); primers and probes for *BGLAP*, *COL1A1*, *VEGFA*, and *RUNX2* were purchased from Applied Biosystems. Amplification conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Quantitative PCR results were normalized to *RPL13* transcript level to yield ΔC_t. Fold change in expression was subsequently calculated using the formula $2^{-\Delta C_t}$ [Schmittgen and Livak, 2008].

STATISTICAL ANALYSIS

Results are expressed as mean ± standard error of the mean, assuming normal distribution of the data sets. Statistical analyses were performed between oxygen tensions using a Student–Newman–Keuls multiple comparisons test where probability values (P) for significance were calculated; $P < 0.05$ was considered statistically significant.

RESULTS

PROLIFERATIVE RESPONSE OF ASCs TO OXYGEN

The proliferation of ASCs in 2D correlated strongly with oxygen tension. The most striking response was the observed increases in proliferation over time for cells cultured in 21% oxygen (Fig. 1A). Despite seeding near confluence, cells at 21% oxygen exhibited a 66% increase in DNA content after 4 weeks over initial seeding. Furthermore, cells maintained in 21% oxygen exhibited more than a 2.5- and 2-fold increase in DNA content over cells cultured in 1% and 5% oxygen, respectively. Cells grown in reduced oxygen exhibited significant drops in total DNA content over the first week, after which DNA content for cells at 5% oxygen remained constant for the remainder of the experiment. ASCs at 1% oxygen exhibited an additional drop in DNA content after 3 weeks that remained steady for the remainder of the study.

ASCs exhibited minimal proliferation in 3D culture. While ASCs were efficiently seeded on polymeric scaffolds (91.4 ± 7.8%, n = 4), scaffolds exhibited significant reductions in DNA content after 1 week in culture for all oxygen tensions (Fig. 1B). Steady declines in DNA content were observed in 5% and 21% conditions over 4 weeks with no significant differences between oxygen tensions. Significantly less DNA was observed for constructs cultured in 1% oxygen after 1 and 2 weeks. ASC-containing scaffolds cultured in 1% oxygen exhibited reduced cell adhesion or viability compared to cells maintained in 5% or 21% oxygen, as demonstrated by lower DNA content over the first week in culture. We observed sloughing of cells from scaffolds when cultured in 1% oxygen, but similar behavior was not detected for cells at 21% oxygen. The remaining

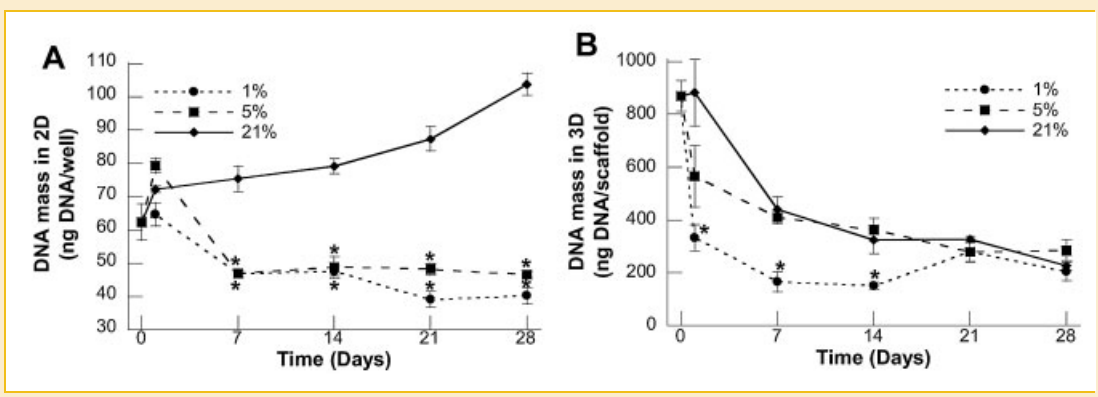

Fig. 1. ASC proliferation was measured by quantifying total DNA mass in 2D and 3D cultures. Total mass of DNA collected from (A) 2D and (B) 3D cultures at 1%, 5%, and 21% oxygen. Data are mean ± SEM; n = 5 for 2D, n = 4 for 3D. *$P < 0.05$ versus 21% oxygen.

adherent cells cultured in 1% oxygen actively proliferated, indicated by increased DNA content at weeks 3 and 4. After 4 weeks in culture, no significant differences in DNA content were observed between oxygen tensions.

CONTRIBUTION OF OXYGEN AND DIMENSIONALITY TO CELL VIABILITY AND APOPTOSIS

The contribution of oxygen tension to cell viability exhibited similar trends in 2D and 3D, although the magnitude of dye reduction was significantly different (Fig. 2A,B). Specifically, cells cultured in 1% oxygen commonly exhibited the greatest alamarBlue reduction, while cells in 21% oxygen demonstrated the lowest levels. Additionally, temporal trends in alamarBlue reduction could be observed in both 2D and 3D for nearly all conditions. Cell viability appeared to peak at 14 days for cells in 2D culture in 1% and 5% oxygen, while cells in 21% oxygen were consistently lower and more stable. In 3D, cell viability continued to increase over time for 1% and 5% oxygen, while ASCs in 21% oxygen exhibited consistent levels of cell viability after 2 weeks.

When examining apoptosis, cells cultured in 21% oxygen commonly exhibited the highest levels of caspase activity, while cells in 1% oxygen commonly demonstrated the least activity. In 2D culture, ASCs in 1% oxygen possessed decreasing caspase activity values over time, while cells in 5% and 21% exhibited relatively stable values over the 28-day study (Fig. 2C). In 3D, temporal trends in caspase activity were more evident, with the highest caspase levels observed for cells at 1 day, regardless of oxygen tension, and these values continued to decrease over 4 weeks (Fig. 2D).

Abnormal cell division during prolonged periods of cell culture can result in polyploidy, and the ability to reliably normalize these data to DNA content depends on the bulk of ASCs being diploid. Upon quantifying DNA content for ASCs grown in 0% or 10% FBS, we detected similar values of DNA mass per cell (0.00129 ± 0.00029 vs. 0.0010 ± 0.00040 ng DNA/cell, respectively; $n = 6$; $P = \text{NS}$), confirming that these cells are primarily diploid and not polyploid.

INFLUENCE OF OXYGEN AND DIMENSIONALITY ON OSTEOGENESIS

We monitored changes in ALP production and activity as an indicator of early osteoblastic differentiation of ASCs as a function of oxygen tension. The intensity of ALP cytochemical stains increased with oxygen tension in 2D (Fig. 3A), and these qualitative observations were in agreement with the biochemical quantification of intracellular ALP activity (Fig. 3B). Cells cultured in 21% oxygen exhibited the highest ALP activity relative to 5% or 1% oxygen when cultured in either 2D or 3D (Fig. 3B,C), and ASCs at 1% oxygen consistently generated the lowest ALP activity.

Culture geometry also influenced ALP activity. At 21% oxygen, ALP activity was ~10 times greater for cells in monolayer culture

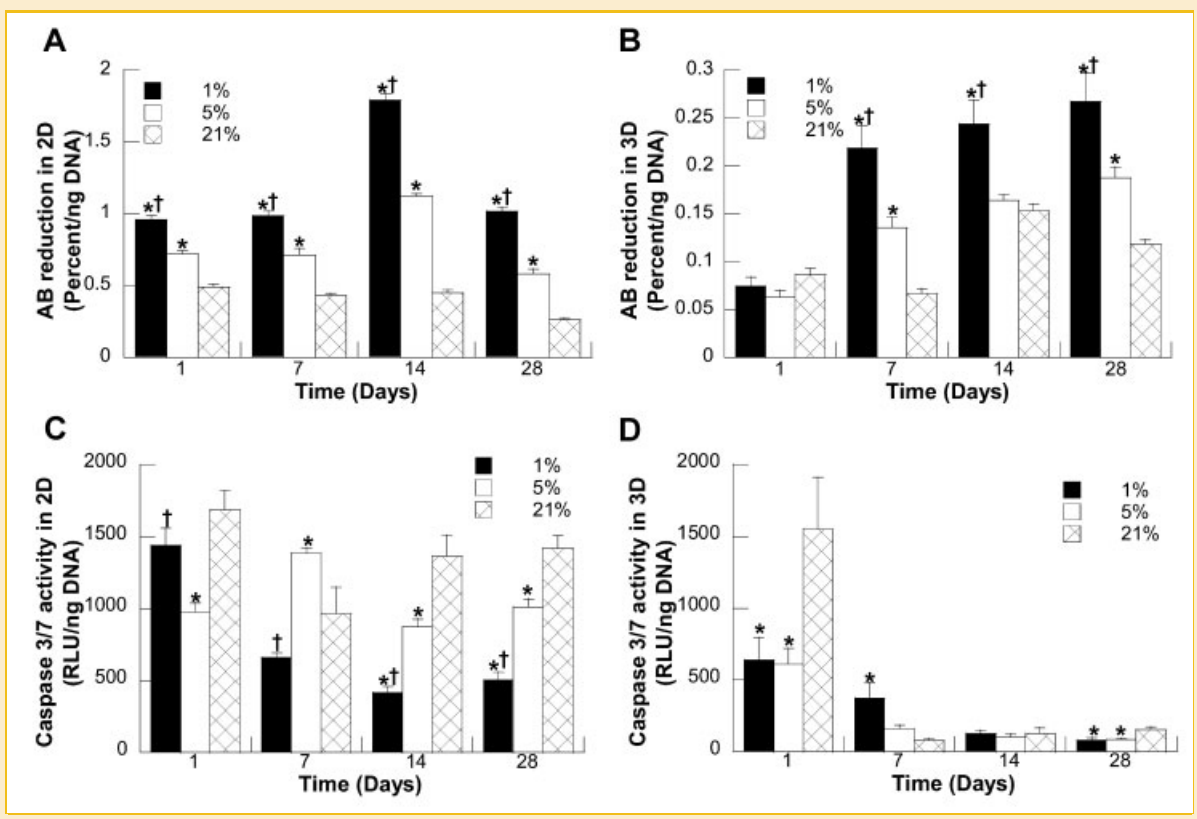

Fig. 2. Metabolic activity measured by alamarBlue (AB) reduction in 2D (A) and 3D (B). Apoptosis quantified by caspase-3/7 activity in ASCs over 4 weeks in 2D (C) and 3D (D). Data are mean ± SEM; n = 3. *$P < 0.05$ versus 21% oxygen; †$P < 0.05$ versus 5% oxygen.

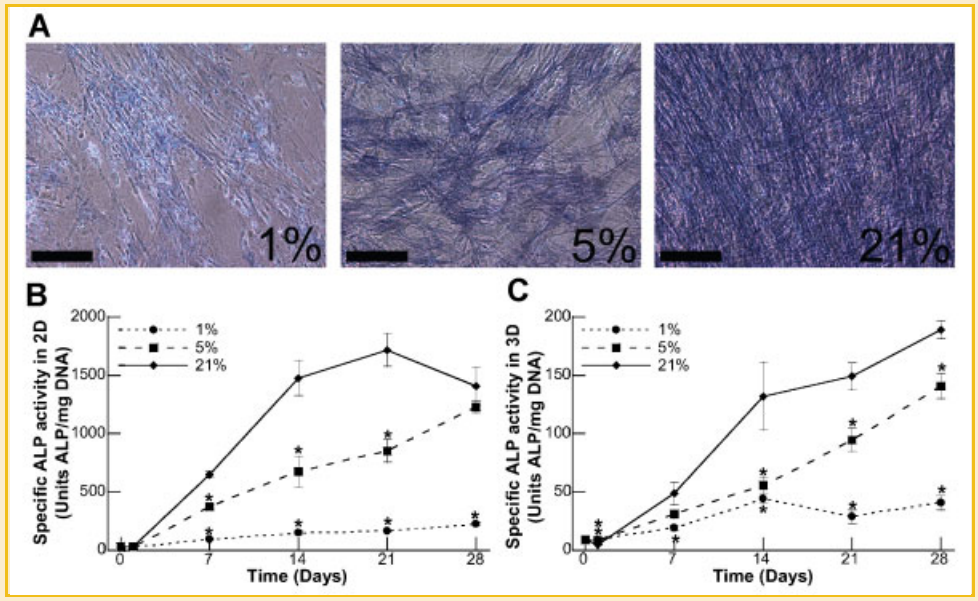

Fig. 3. A: Biochemical stains of alkaline phosphatase activity in ASCs cultured in 2D at 1%, 5%, and 21% oxygen after 4 weeks in culture. Images are taken at 10× magnification; scale bars represent 250 μm. Alkaline phosphatase activity for ASCs cultured in various oxygen tensions over 4 weeks in (B) 2D and (C) 3D cultures. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

compared to cells cultured in 3D scaffolds at each time point examined (Fig. 3B,C). Similarly, ALP activity was higher in cells cultured in 2D compared to 3D under both 5% and 1% oxygen, although these differences, as well as actual activity, were lower than cells cultured in 21% oxygen.

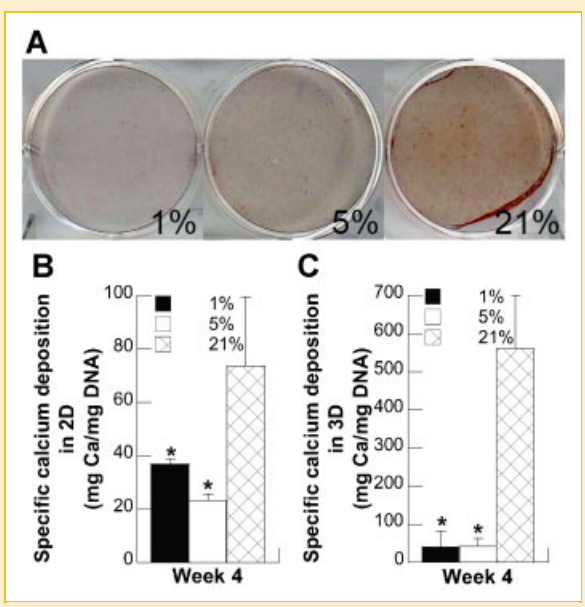

Fig. 4. A: Calcium deposition was observed in ASCs cultured at 1%, 5%, and 21% oxygen in 2D for 4 weeks by alizarin red staining. Quantification of secreted calcium by ASCs in (B) 2D and (C) 3D culture after 4 weeks. Data are mean ± SEM; n = 5 for 2D ALP, n = 3 for 2D calcium, n = 4 for 3D. $^{*}P < 0.05$ versus 21% oxygen. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The osteogenic response of ASCs to altered oxygen and substrate geometry was also assessed through deposition of extracellular calcium. Alizarin red staining of 2D samples confirmed significantly less calcium deposition for cells cultured in 1% and 5% oxygen compared to cells in 21% oxygen (Fig. 4A). These qualitative observations of calcium secretion were confirmed by calcium extraction and quantification. ASCs secreted significantly less calcium when cultured in 1% and 5% oxygen compared to 21% oxygen in both 2D and 3D cultures, mirroring trends in ALP expression (Fig. 4B,C). In contrast to ALP activity, wherein activity was higher for cells cultured in 2D versus 3D, calcium deposition was reduced in 2D at all oxygen tensions. Deposition of calcium by ASCs in 2D at 21% oxygen was eight times lower than those in 3D. No significant differences were observed between calcium deposition for ASCs in 1% and 5% oxygen, regardless of culture dimensions.

EXPRESSION OF GENES ASSOCIATED WITH OSTEOGENESIS

Expression of the osteogenic marker genes *RUNX2*, *COL1A1*, and *BGLAP* (osteocalcin) was analyzed by qtPCR from RNA lysates collected under 2D or 3D conditions over 4-week culture at 1%, 5%, or 21% oxygen. We first examined expression of *RUNX2*, a transcription factor required for osteoblastic differentiation and bone development [Ducy et al., 1997]. There was a steady increase in *RUNX2* expression in cells cultured on 2D in 21% oxygen through 2 weeks of culture, after which time expression decreased (Fig. 5A). Cells cultured in 2D at 5% or 1% oxygen demonstrated a steady increase in *RUNX2* expression through week 3 when they reached peak levels of expression. Interestingly, where *RUNX2* expression decreased with prolonged culture in 21% oxygen, it maintained high levels of expression through week 4 in cells cultured at 1% or 5% oxygen. In contrast to the ability of oxygen to influence *RUNX2* expression for cells cultured in 2D, we observed no statistically

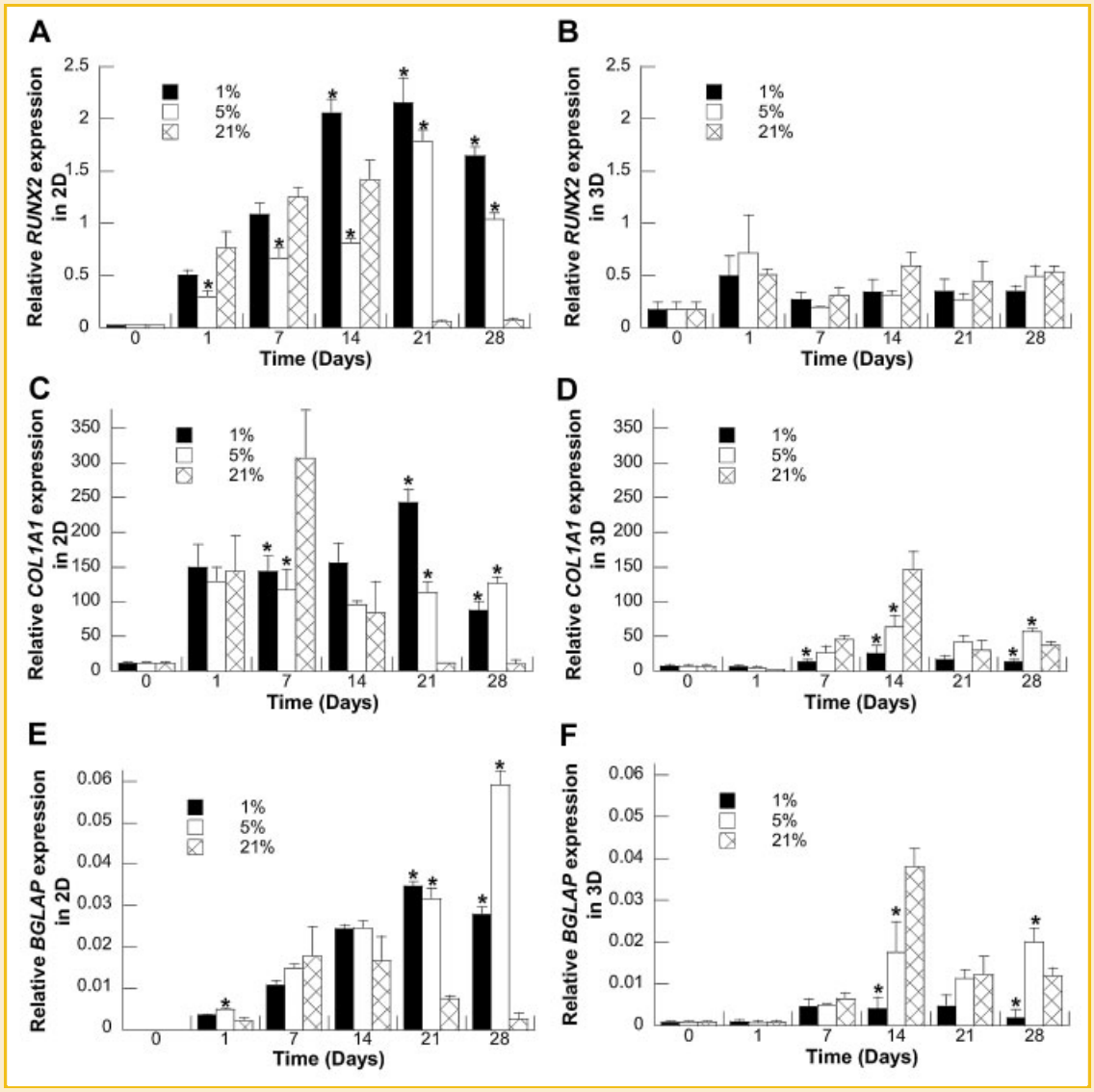

Fig. 5. Quantitative PCR results for genes monitored in ASCs over 4 weeks in 2D and 3D culture: *RUNX2* (A,B), *COL1A1* (C,D), and *BGLAP* (E,F). Values reflect fold change in the target mRNA expression over RPL13. Data are mean ± SEM; n = 3. $^{*}P < 0.05$ versus 21% oxygen.

significant influence of oxygen tension upon *RUNX2* transcript for cells cultured in 3D scaffolds (Fig. 5B). Overall expression of *RUNX2* at each time point and oxygen tension was higher in 2D compared to 3D.

Type I collagen is the primary organic component of bone and forms the bulk of the osteoid matrix; *COL1A1* encodes for two of the three fibrils that compose collagen I filaments [Kocher and Shapiro, 1998]. The influence of oxygen tension upon *COL1A1* transcript was similar to that upon *RUNX2* and *BGLAP*, with cells cultured in 21% oxygen in 2D expressing *COL1A1* more rapidly than cells cultured in 5% or 1% oxygen, only for expression to decrease with prolonged culture (Fig. 5C). In comparison, cells cultured at 5% or 1% oxygen in 2D exhibited lower levels of expression than 21% oxygen but without showing significantly decreased expression after 4 weeks. *COL1A1* expression in 3D was highest in 21% oxygen increasing through week 2 (Fig. 5D). In comparison, expression levels in 3D at 5% and 1% oxygen displayed lower magnitudes but had similar temporal profiles. Overall levels of expression in 3D were lower than that in 2D at each time point.

Osteocalcin, encoded by the gene *BGLAP*, is a noncollagenous protein important for calcium binding and osteoid mineralization [Nacamuli et al., 2003], and serves as a delayed marker of osteogenic differentiation [Weinreb et al., 1990]. *BGLAP* revealed similar temporal patterns and magnitudes of expression in response to altered oxygen tension and substrate geometry as compared to *COL1A1*. *BGLAP* expression in 2D-cultured cells at 21% oxygen increased up to 2 weeks (Fig. 5E), and declined thereafter; cells cultured in 2D under 1% or 5% oxygen also increased *BGLAP* expression throughout the duration, and expression was significantly higher after 3 and 4 weeks of culture than in cells maintained at 21% oxygen. *BGLAP* expression in 3D closely followed that of *COL1A1* both in relative magnitudes and

temporal expression (Fig. 5F). As with *RUNX2*, these data suggest that reductions in oxygen tension slow the course of osteogenic differentiation.

ANGIOGENIC POTENTIAL

VEGF is a potent proangiogenic molecule that induces migration and proliferation of endothelial cells and stimulates neovascularization [Ferrara and Gerber, 2001]. It also exerts pleiotrophic effects upon osteoblasts, stimulating their proliferation and migration [Mayr-Wohlfart et al., 2002]. Thus, VEGF secretion by ASCs was monitored due to its proangiogenic and osteoinductive properties. VEGF secretion in 2D-cultured cells was highest in cells cultured at 1% oxygen at all time points examined; there was no statistical difference in VEGF secretion between cells cultured at 5% and 21% oxygen (Fig. 6A). Similarly, cells cultured in 3D secreted highest levels of VEGF when cultured in 1% oxygen, and 5% oxygen culture also increased VEGF secretion compared to 21% oxygen control, although secretion decreased with time in culture compared to 2D (Fig. 6B). Increased VEGF secretion under reduced oxygen conditions was mirrored by enhanced *VEGFA* transcript in 2D culture (Fig. 6C). The transient ability of hypoxia to stimulate VEGF secretion at 1% and 5% oxygen in 3D culture was also observed in levels of *VEGFA* transcript: there was a large, albeit not statistically significant, effect of hypoxia on *VEGFA* levels after 1 day of culture at 1% or 5% oxygen (Fig. 6D). With continued time in culture, *VEGFA* transcript continued to increase at 1% oxygen compared to 21%; 5% oxygen exhibited a trend for increased *VEGFA*, but this was without statistical significance.

DISCUSSION

Our data demonstrate that among the oxygen tensions examined, ASCs differentiate and mineralize the extracellular environment most effectively in atmospheric oxygen. Markers of the osteoblastic phenotype including ALP and calcium increased with oxygen tension and over the duration of the study. Osteogenic marker genes, *RUNX2*, *COL1A1*, and *BGLAP*, were expressed at earlier time points in atmospheric conditions and later for normoxic and hypoxic conditions in 2D. In ASCs cultured in 3D, lower magnitudes of gene expression were detected compared to ASCs in 2D culture and differences in temporal expression were not observed between oxygen tensions. Differentiation of ASCs were inhibited in 3D culture when compared to 2D culture. Conversely, the proangiogenic response of ASCs to the local oxygen microenvironment was enhanced under hypoxia, with distinct temporal differences in both protein and gene expression of VEGF between 2D and 3D culture.

ASCs were chosen for this experiment because they represent a promising alternative to MSCs as a source of osteoprogenitors for use in bone repair and regeneration. In order to capitalize on the

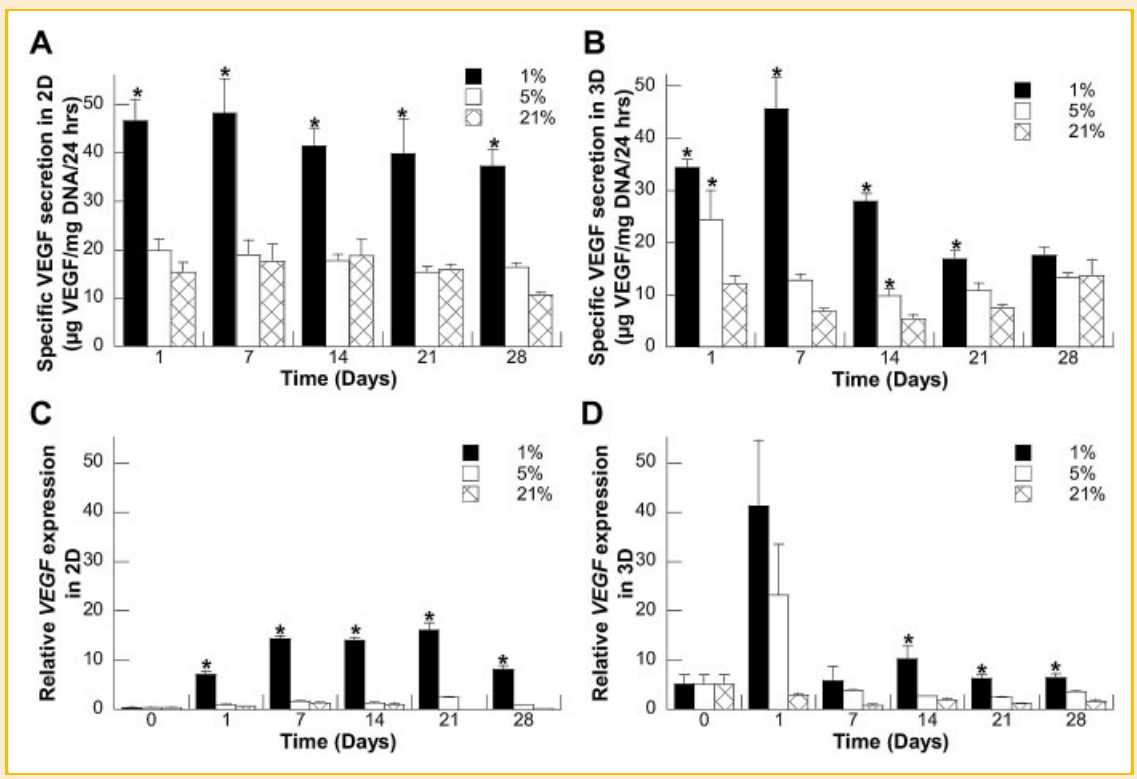

Fig. 6. VEGF secretion was quantified for ASCs cultured in (A) 2D and (B) 3D. Expression of $VEGF_{165}$ mRNA was quantified for ASCs cultured in (C) 2D and (D) 3D. Data are mean ± SEM; n = 5 for 2D VEGF protein, n = 4 for 3D VEGF protein, n = 3 for VEGF mRNA. $^{*}P < 0.05$ versus 21% oxygen.

potential efficacy of ASCs for use in cell-based therapies of bone repair, it is necessary to understand how they behave in physiologically relevant microenvironments characteristic of both healthy and diseased bone. Whereas MSCs effectively synthesize osteoid tissue in vitro and in vivo [Kassem et al., 2004], their use may be limited because of concerns involving MSC yield. Indeed, MSCs form only a small fraction (0.001–0.01%) of the total nucleated cell population within bone marrow, thereby requiring extensive expansion through in vitro cell culture techniques [Logeart-Avramoglou et al., 2005]. Furthermore, MSC frequency declines with age and disease [Stolzing et al., 2008]. In contrast to MSCs, ASCs collected via lipoaspiration yields a much larger fraction (0.5–5%) of the total stromal volume fraction (high density pellet formed from lipoaspirates) [Peroni et al., 2008]. In conjunction with increased availability of adipose tissue, this increased yield potentially translates to shorter delays between harvesting of autologous tissue, expansion, and ultimately, transplantation into host tissue.

We initially examined the influence of altered oxygen microenvironment upon ASC proliferation and found there to be a significant impact in both 2D and 3D culture. ASCs seeded near confluence in 2D and cultured in atmospheric oxygen continued to proliferate over 4 weeks, likely as a function of sufficient oxygen transport via diffusion through the media to the cell layer. However, cells cultured in 1% or 5% oxygen demonstrated reductions in DNA content, and presumably cell number, which may be a function of cell death or loss of adhesion to the culture dish. Indeed, routine media changes promoted the loss of cell clusters for cells cultured in 1% or 5%, but not 21%, oxygen. While we did not test lower cell densities, it is expected that ASC proliferation is affected in part by cell seeding density alone [Lund et al., 2009]. The response of ASCs to physiological oxygen tensions when cultured at lower densities may allow for increased proliferation, and the synergistic contribution of cell number and oxygen tension merits further study. When ASCs were cultured on 3D scaffolds, we observed significant drops in DNA content within the first week for all oxygen conditions. Furthermore, a significant reduction in DNA content was observed in cells cultured at 1% oxygen for 7 or 14 days compared to 5% and 21% oxygen. This is most likely due to cell death resulting from lower levels of oxygen within scaffolds as a result of diffusion limits. In the absence of perfusion, diffusion dictates the level of oxygen available to cells seeded on scaffolds [Malda et al., 2007]. Such observations have been previously reported in MC3T3-E1 preosteoblasts cultured on PLG scaffolds [Shea et al., 2000]. Our data related to cell viability and apoptosis, together with changes in DNA mass over time, suggest that the extended culture of ASCs under these conditions may preselect for cells that are more robust and can participate in osteogenesis while residing in challenging microenvironments.

ALP activity is high in cells undergoing early osteodifferentiation and is maintained until maturation into osteocytes [Piattelli et al., 1997]. ALP is thought to facilitate mineralization of organic matrix through calcium binding, generation of free phosphates, or degrading mineralization inhibitors [Anderson, 1989]. Consistent with previous results using MSCs [Raheja et al., 2008], ASCs in both 2D and 3D cultures demonstrated strong positive correlations between oxygen tensions and ALP activity. Of special interest is that ALP activity in cells cultured under 1% or 5% oxygen was inhibited compared to cells cultured at 21% oxygen, and not eliminated. Calcium deposition reflected trends observed with ALP activity. Significantly more calcium was detected in 21% oxygen over those in 1% and 5% oxygen. These data indicate that reductions in pericellular oxygen overwhelm the ability of osteoinductive chemicals to commit ASCs toward the osteogenic lineage and supports other literature suggesting that reduced pericellular oxygen maintains the pluripotency of stem cells or delays their differentiation.

The expression of genes implicated in osteogenesis—*RUNX2*, *COL1A1*, and *BGLAP* (osteocalcin)—was consistently higher in cells cultured in 2D compared to 3D cultures at each oxygen tension. These observations suggest that osteogenic differentiation occurs more robustly and at an earlier time point in 2D compared to 3D cultures. That is, in the absence of any difference in pericellular oxygen, 2D culture drove osteogenesis more effectively than the 3D culture geometry. Furthermore, while all genes peaked in their levels of expression, cells at lower oxygen tensions, especially in 2D culture, exhibited delayed expression of osteogenic genes. These findings suggest that the applicability of ASCs for tissue engineering applications may benefit from preconditioning cells in 21% oxygen to differentiate cells prior to implantation. Kärner et al. [2008] recently observed similar behavior with osteodifferentiated human embryonic stem cells, wherein osteogenic markers were detected earlier in 2D compared to 3D cultures. It may be possible that while ASCs are more differentiated in 2D, they are unable to efficiently mineralize their surroundings due to the lack of special geometry available in 3D. Alternatively, osteogenic differentiation may be attenuated in 3D because of structural cues from the scaffolding material not present in 2D culture. For example, increased substrate modulus in 2D culture may cue ASCs toward an osteogenic phenotype, whereas ASCs in 3D may perceive the lower scaffold modulus as reduced necessity to build bone.

We showed that, like ALP activity, calcium deposition was positively correlated with increasing oxygen tension. Highest levels were detected in 21% oxygen for both 2D and 3D cultures. However, ASCs in 3D measured the highest levels of calcium despite heightened expression of osteogenic markers in 2D. These differences may be attributed to better entrapment of deposited calcium in 3D substrates in comparison with 2D surfaces. Shear caused by media changes and PBS rinses over cell layers in 2D culture may have removed a fraction of deposited calcium. Calcium embedded within 3D scaffolds would have been better protected from such forces. Furthermore, in contrast to a tissue culture treated surface, surfaces of PLG scaffolds have great affinity to bind minerals [Davis et al., 2009]. Nucleation points in scaffolds can leach minerals from the media in the absence of cells [Yefang et al., 2007], potentially obfuscating quantified levels of calcium.

ASCs produce cytokines regulating a plethora of cellular actions, including the release of the pro-angiogenic factor VEGF,

whose actions recruit and stabilize developing vasculatures [Kim et al., 2007; Rubina et al., 2009]. ASCs exposed to hypoxia at 1% oxygen enhance VEGF secretion, which functions in a paracrine fashion to promote the survival and proliferation of microvascular endothelial cells [Rehman et al., 2004]. Consistent with these data, we demonstrate enhanced VEGF synthesis and secretion by ASCs cultured at 1% oxygen compared to 5% and 21% oxygen in 2D as well as 3D culture conditions coupled with increasing VEGF secretion correlating with reductions in oxygen. These data are in agreement with other studies characterizing the angiogenic potential of cells of the osteogenic lineage in 3D. MC3T3-E1 preosteoblasts exhibited heightened angiogenic response and delayed osteogenesis in 3D culture compared to 2D culture [Jarrahy et al., 2005]. Interestingly, where 1% oxygen culture induced VEGF secretion and synthesis in both 2D and 3D, there was a distinct temporal profile for VEGF synthesis and secretion associated with each culture geometry. Under 2D culture, cells cultured at 1% oxygen sustained higher VEGF secretion, as well as *VEGFA* synthesis, over the course of the 4-week experiment. In contrast, the influence of 1% oxygen upon VEGF for cells cultured in 3D was transient, peaking after 7 days and decreasing thereafter. This may reflect differences in oxygen availability between the two culture geometries and cellular adaptation to chronic hypoxia. Finally, MSCs are also recognized as perivascular cells whose physiologic function includes secretion of trophic factors to stimulate angiogenesis [Caplan, 2009]. Early expressions of both VEGF mRNA and protein in 2D and 3D cultures, particularly in response to hypoxia, may indicate an early physiologic response to recruit proangiogenic elements prior to osteogenic differentiation.

This study demonstrates the importance of both the oxygen microenvironment and the culture geometry upon the osteogenic differentiation of ASCs. Reduced levels of oxygen severely diminished the ability of ASCs to undergo osteogenic differentiation. Despite such inhibition, osteogenic differentiation in 2D culture was significantly enhanced over that in 3D. Given the hypoxic nature of bone defects [Schleier et al., 2006], ASCs may require additional osteoinductive factors, such as bone morphogenetic proteins, to differentiate. In light of these data, it may also be beneficial to differentiate ASCs ex vivo in atmospheric oxygen to initiate the osteogenesis program prior to implantation. Jeon et al. [2008] have demonstrated that undifferentiated ASCs could still form bone when transplanted on a bioceramic-containing polymer scaffold, but to substantially reduced degrees than cells stimulated by BMP-2. Despite a reduced osteogenic potential, concurrent upregulation of VEGF production could potentially lead to angiogenesis, thereby enhancing osteogenesis. High levels of VEGF secretion, especially under hypoxia, advocates cell-based therapies as an alternative to recombinant protein delivery. Furthermore, the early production of angiogenic molecules followed by the later production of osteogenic molecules closely mimics a physiologic wound healing response. This temporal expression of biological markers observed in 3D culture emphasizes the importance of dimensional cues in regulating ASC development. These observations will provide researchers a better basis on which to develop and use ASCs in the future.

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