Published in final edited form as:

J Tissue Eng Regen Med. 2008 June ; 2(4): 210–220. doi:10.1002/term.85.

Collagen nanofibres are a biomimetic substrate for the serum-free osteogenic differentiation of human adipose stem cells

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Abstract

Electrospinning has recently gained widespread attention as a process capable of producing nanoscale fibres that mimic native extracellular matrix. In this study, we compared the osteogenic differentiation behaviour of human adipose stem cells (ASCs) on a 3D nanofibre matrix of type I rat tail collagen (RTC) and a 2D RTC collagen-coated substrate, using a novel serum-free osteogenic medium. The serum-free medium significantly enhanced the numbers of proliferating cells in culture, compared to ASCs in traditional basal medium containing 10% animal serum, highlighting a potential clinical role for *in vitro* stem cell expansion. Osteogenic differentiation behaviour was assessed at days 7, 14 and 21 using quantitative real-time RT-PCR analysis of the osteogenic genes collagen I (*Coll I*), alkaline phosphatase (*ALP*), osteopontin (*OP*), osteonectin (*ON*), osteocalcin (*OC*) and core-binding factor- α (*cbfa1*). All genes were upregulated (>one-fold) in ASCs cultured on nanofibre scaffolds over 2D collagen coatings by day 21. Synthesis of mineralized extracellular matrix on the scaffolds was assessed on day 21 with Alizarin red staining. These studies demonstrate that 3D nanoscale morphology plays a critical role in regulating cell fate processes and *in vitro* osteogenic differentiation of ASCs under serum-free conditions.

Keywords

adipose stem cells; serum-free medium; nanofibres; electrospinning; bone tissue engineering

1. Introduction

The replacement or restoration of function to traumatized, damaged or lost bone is an increasingly significant clinical problem. It is currently estimated that over 800 000 bone-

grafting operations are performed annually worldwide and these numbers will continue to grow as the life expectancy of the population increases (Olivier *et al.*, 2004). Although autogenous bone procedures and various allogeneic and synthetic materials are used clinically with varying success, the development of new tissue-engineering strategies for bony repair has become a major research thrust in orthopaedic bioengineering. In recent years, stem cell technologies and scaffold biomaterials have advanced in exciting ways to bring regenerative medicine and bone tissue engineering closer to widespread clinical application.

While the future of stem cell-based tissue engineering shows promise, prospects for clinical translation would be aided by a serum-free culture medium that still maintains the plasticity of the stem cells. Recent studies suggest that, while certain adult stem cell populations are inherently non-immunogenic, exposure to animal serum (such as fetal bovine serum), commonly used to maintain and expand cells in culture, may elicit a significant immune response. Serum proteins can be internalized into cells and are not necessarily depleted by simple washing techniques (Spees *et al.*, 2004). Moreover, the US Federal Drug Administration (FDA) requires reduction of animal serum exposure for cell cultures intended for human transplantation before clinical use (Kim *et al.*, 2005). Thus, the need exists for culture medium conditions that obviate the dependency of stem cells on animal serum to grow and differentiate in culture.

A variety of stem cells have all been considered for cell-based tissue-engineering therapies, including connective tissue stem cells from periosteum, meniscus and dura mater (Petrie *et al.*, 2007; Cowan *et al.*, 2004), both prenatal and postnatal stem cells derived from amniotic fluid and umbilical cord blood, respectively (Miao *et al.*, 2006; Park *et al.*, 2006), dermal fibroblast stem cells (Hee *et al.*, 2006), bone marrow-derived mesenchymal stem cells (BMSCs; Petite *et al.*, 2000; Gronthos *et al.*, 2003; Gomes *et al.*, 2003) and adipose stem cells (ASCs; Zuk *et al.*, 2002; Katz *et al.*, 2005; Gimble and Guilak, 2003). Among these cells, ASCs have been shown to differentiate readily into osteoblast-like cells *in vitro* (Strem *et al.*, 2005; Gabbay *et al.*, 2006) and repair bony defects *in vivo* (Cowan *et al.*, 2004; Lendeckel *et al.*, 2004). Additionally, a typical harvest yield is approximately 40-fold greater than what is achievable through a single bone marrow aspiration, making ASCs attractive for autologous therapeutic use (Strem *et al.*, 2005).

One potentially successful approach for the creation of effective scaffolding materials for bone regeneration is to mimic the natural extracellular matrix (ECM), thus providing the necessary chemical and mechanical cues that drive cellular differentiation. This concept of biomimicry is focused on the creation of the natural environmental niche that challenges cells to do what they do best (Benyus, 1997). In this way, we may be able to capitalize on the cells' innate abilities to respond to their environment without the necessity of genetic or pharmacological manipulations. Recently, several investigators have employed electrospinning as a technique capable of producing nanoscale fibres that mimic the ECM for tissue-engineering applications (Matthews *et al.*, 2002; Buttafoco *et al.*, 2006; Li *et al.*, 2002, 2006). Electrospinning is a process that uses an electric field gradient to pull a charged polymer solution towards a grounded collector, resulting in a non-woven mesh of nanofibres. In native bone, the organic components of the ECM are approximately 90% type I collagen and 10% proteoglycans and other non-collagenous proteins. To date, collagen type I from calfskin and types I and III from human placenta have been successfully electrospun from solutions dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP; Matthews *et al.*, 2002). For bone tissue-engineering applications, the implications of collagen nanofibre technologies as biomimetic substrates could be profound.

Both the selection of an appropriate scaffold biomaterial and serum-free culture conditions are critical considerations for the clinical translation of ASC-based technologies for skeletal repair. In this study, we investigated the differentiation patterns of ASCs on a three-dimensional (3D) nanofibrous matrix of type I collagen and a two-dimensional (2D) collagen-coated substrate, using a novel serum-free osteogenic medium. Osteogenic differentiation of ASCs *ex vivo* was quantified using real-time RT-PCR for several bone markers, including collagen I, osteopontin, osteocalcin, osteonectin, alkaline phosphatase and core-binding factor- α .

2. Materials and methods

2.1. Materials

The following materials were purchased from Gibco-Invitrogen (Grand Island, NY, USA): DMEM/F12 medium, FBS, antibiotic/antimycotic and MEM non-essential amino acids. Trypsin-EDTA and L-glutamine were purchased from Cellgro-Mediatech (Herdon, VA, USA). The following components were purchased from Sigma-Aldrich: ITS+3, fatty acid supplement, hydrocortisone, β -estradiol, progesterone, dexamethasone, ascorbate-2-phosphate, and β -glycerophosphate. All other materials used are specified below.

2.2. Cell isolation and culture

Adipose tissue was obtained from two female patients (aged 39 and 40 years) undergoing elective surgical procedures (lipectomy, panniculectomy) in the Department of Plastic Surgery at the University of Virginia, in accordance with the University of Virginia Human Investigation Committee. Cells were isolated using methods previously described (Katz *et al.*, 2005; Zuk *et al.*, 2001). Briefly, harvested tissue was washed several times and enzymatically digested for approximately 30–60 min until smooth. A cell pellet was isolated via centrifugation, filtered to remove debris, and washed with lysis buffer to remove contaminating erythrocytes. The cellular solution was then refiltered, centrifuged and resuspended in serum-free DMEM/F12 and 1% antibiotic/antimycotic. The cell pellet was subsequently divided and plated into the following culture conditions: DMEM/F12 + 10% FBS, AR8, or AR8 + 0.5% human serum (HS; BioWhittaker/Cambrex). ASCs were maintained at 37 °C with 5% CO₂ and fresh medium was exchanged every 3 days. Cells were either passaged subconfluently, using 0.5% trypsin-EDTA, or used for experiments up to passage 5. The ASC serum-free medium formulation (AR8) was developed in the laboratory of Dr. Adam J. Katz and contains the following components: DMEM/F12, L-glutamine, dexamethasone, ascorbate-2-phosphate, ITS+3, fatty acid supplement, MEM non-essential amino acids, β -estradiol, progesterone, hydrocortisone, EGF, PDGF- $\beta\beta$, SCGF- β , TNF α , IL-1 β (previous five from Fitzgerald Industries Int., Inc. Concord, MA), antibiotic/antimycotic, and optional human serum (Parker *et al.*, 2007).

2.3. Cell cycle analysis

Cell cycle analysis was performed using the Guava[®] Personal Cell Analysis (PCA) system (Guava Technologies Inc., Hayward, CA, USA) for data acquisition and ModFit[™] software (Verity Software House) for analysis. ASCs were cultured in DMEM/F12 + 10% FBS, AR8, and AR8 + 0.5% HS and samples were collected at passage numbers 0–5. For each sample, a cell pellet containing 1 million ASCs was obtained and resuspended in 1 ml PBS. The sample was centrifuged and the PBS was aspirated, leaving about 100 μ l residual PBS. Ice-cold 70% ethanol (1 ml) was added dropwise, vortexing continuously. The samples were then refrigerated overnight prior to staining. To stain, the samples were washed with PBS twice, using centrifugation, and the final cell pellets were resuspended in 1 ml Cell Staining Solution (Guava Technologies). Samples were incubated in the dark at room temperature for 30 min before acquisition on the Guava PCA using the Guava Cell Cycle software module.

Raw data were imported into ModFit™ and analysed to report percentages of ASCs in cell cycle phases G₀/G₁, S or G₂/M.

2.4. Cell proliferation assay

Cell proliferation was quantified using the MTS CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. ASCs were plated at 10 000 cells/cm² in a 96-well tissue culture plate (Nuncbrand) in DMEM/F12 + 10% FBS, AR8 and AR8 + 0.5% HS. Following 48 h in culture, the existing medium was removed and replaced with fresh medium plus MTS solution (2 : 10 MTS : medium ratio) and incubated for exactly 2 h at 37 °C. The reaction was stopped with sodium dodecyl sulphate (Fisher Scientific, Fair Lawn, NJ, USA) in the amount of one-quarter of the total reaction volume. Absorbance was read using a spectrophotometer at 492 nm and converted to total cell number using a standard curve.

2.5. Rat tail collagen isolation

Rat tail collagen was isolated from the tail tendon of Sprague-Dawley male retired breeders by the method previously described by Elsdale and Bard (1972). Briefly, the skin was separated from the tail and discarded; the remaining pieces of the tail were segmented and dissolved in 0.5 M acetic acid at 4 °C for 24 h. The dissolved solution was then centrifuged at 1500 r.p.m. for 5 min. The pellet was discarded and sodium chloride was slowly added to the supernatant to a maximum concentration of 10% w/v while stirring at 600 r.p.m. at 4 °C. The solution was then centrifuged at 6000 r.p.m. for 20 min. The mixture separated into three layers; the top two layers were removed and the pellet was resuspended in a small amount of deionized water. The pellet solution was then centrifuged for 10 min at 6000 r.p.m. The supernatant was discarded and the pellet was resuspended in a small amount of deionized water, frozen, and lyophilized for 3 days to remove the liquid.

2.6. Fabrication of nanofibrous meshes

Nanofibre scaffolds of rat tail collagen were fabricated using the electrospinning technique. Rat tail collagen (RTC) was dissolved at concentrations in the range 5–9% w/v in 1,1,1,3,3,3 hexafluoro-2-propanol (HFP; Fisher Scientific), at 4 °C to avoid heat denaturation of the collagen. The polymer solutions were then loaded into a 5.0 ml glass syringe fitted with an 18-gauge blunt-ended stainless steel needle mounted to a syringe pump (Aladdin; World Precision Instruments Inc.) and dispensed at a flow rate of 1 ml/h. The solution was charged upon leaving the needle by applying a voltage (20 kV) via a lead attached to the syringe needle from a high-power voltage supply (Gamma High Voltage Research Inc.). A grounded collector plate covered with aluminium foil was placed a specified distance away (10–30 cm), creating an electric field between the syringe tip and the collector plate, in the direction of gravity. Glass coverslips (Fisherbrand, diameter 22 mm, Fisher Scientific) were plasma-treated using the Lectro-Treat 3-D Surface Treater (Lectro Engineering Co., St. Louis, MO, USA) and placed on the foil, opposite the syringe needle. In this manner, collagen nanofibres were electrospun directly onto the glass coverslips. Nanofibre samples were crosslinked and sterilized under UV light for 24 h.

2.7. Preparation of RTC-coated coverslips

The measured collagen concentration per coverslip, 1 mg/cm², was kept constant between the coated and spun substrates. RTC was dissolved in 0.5 M acetic acid and poured into several Petri dishes containing the glass coverslips to achieve a final concentration of 1 mg/cm². The Petri dishes were left uncovered in a sterile cell-culture hood overnight to allow the acetic acid to evaporate, depositing the collagen onto the glass substrates. The coverslips

were then sterilized under UV light for 24 h and subsequently washed twice with sterile water to remove any residual acetic acid.

2.8. SEM preparation

Acellular nanofibre samples were removed from the centre of the electrospun collection area and mounted on scanning electron microscope (SEM) mounts with carbon adhesives. For cellular samples, nanofibres were fixed at 4 °C in 1% glutaraldehyde for 1 h and 3% glutaraldehyde overnight and then dehydrated through increasing concentrations of methanol before being mounted for SEM. Both samples were sputter-coated with gold using a BAL-TEC SCD005 Sputter Coater for 200 s and imaged using a 15 kV accelerating voltage on a JEOL 6400 SEM with Orion 6400 image processing.

2.9. Nanofibre characterization

A parametric study of rat tail collagen in HFP was performed over varying concentrations and collecting distances while maintaining a constant driving voltage of 20 kV and flow rate of 1 ml/h. Samples were prepared for SEM as detailed above. SEMs were taken at magnifications of $\times 500$, $\times 1000$ and $\times 3000$. Nanofibre diameters were analysed using SEM images at $\times 3000$ magnification in ImageJ software (National Institutes of Health, MD, USA). For each SEM image, 65 diameter measurements were randomly recorded using the 'Measure' tool in ImageJ and averaged together. Bead area density was chosen as a metric to characterize uniformity in nanofibres. Beads are created under undesirable processing parameters that result in a discontinuity in the electrospinning jet. Bead area density was calculated by measuring bead diameters at the centre of each bead, also using the Measure tool in ImageJ and SEM images at $\times 3000$. Assuming all beads were circular in shape, individual bead areas were calculated and added together to yield a total surface area covered by beads. The 'Threshold' tool in ImageJ was used to transfer the image to black and white in order to approximate the total surface area of collagen, including both fibres and beads. Total collagen area divided by bead area resulted in the bead area density per image.

2.10. Cell seeding onto nanofibrous meshes

For seeding of nanofibre constructs, ASCs were removed from their respective culture flasks with 0.5% trypsin/EDTA and replated onto dry nanofibre meshes in AR8 medium at a cell density of 25,000 cells/cm². Because nanofibres were electrospun directly onto glass coverslips, we used a minimal amount of vacuum grease to adhere each coverslip to the bottom of a Petri dish (21.5 cm² total area); three coverslips were placed in each Petri dish and cultured under the same conditions (triplicate). Cell suspension volume at time of seeding was kept at a minimum (~200 μ l per coverslip) to optimize ASC attachment. Cell-seeded constructs were placed in the incubator for approximately one hour, at which point the dishes containing the constructs were flooded with additional AR8 medium.

2.11. Assessment of filopodia extension

Scanning electron micrographs were taken of ASCs cultured on both 2D and 3D substrates after 7 days in culture in AR8 serum-free osteogenic medium (SFOM). SEMs taken at $\times 1000$ were analysed using Image J (NIH). Briefly, the length of filopodia, in pixels, was calculated using the Measure tool and then converted to μ m. Filopodia extensions to other cells were not counted; only those extensions that had one end fixed to the cell body and the other end in free space were analysed. Four samples were analysed per substrate group.

2.12. Osteogenic induction of ASCs

Two days post-seeding, AR8 was exchanged with a novel osteogenic formulation (day 0), promoting the differentiation of ASCs to a bone-like phenotype. The novel osteogenic medium was composed of AR8 supplemented with 0.1 μ M dexamethasone, 50 μ M ascorbate-2-phosphate, and 10 mM β -glycerophosphate. All medium was exchanged every 3 days. Osteogenic differentiation was carried out for a total of 21 days, with assessment of osteogenic differentiation at days 7, 14 and 21.

2.13. Alizarin red staining

Alizarin red (AZ; ACROS Organics) was used to assess the presence of calcified matrix in the differentiating ASCs. Following 21 days in culture, cells were fixed with 70% ethanol at 4 °C for 1 h and then stained with 40 mM Alizarin red solution in deionized water for 10 min at room temperature. After washing five times with deionized water, the samples were imaged using the Axioskop 40 (Carl Zeiss).

2.14. RNA isolation and real-time PCR

Cell/scaffold composites at each time point (7, 14, 21 days) were screened by quantitative real time RT-PCR for expression of genes implicated as possible differentiation markers of osteoblastic cell populations. Each Petri dish containing three collagen nanofibre substrates or three collagen-coated substrates was washed completely with PBS to remove all residual medium and the cells were disrupted from collagen substrates with the addition of Qiagen cell lysis buffer containing β -mercaptoethanol. Total RNA was isolated following the protocol outlined in the RNA isolation kit from Qiagen (Valencia, CA, USA) and quantified using a spectrophotometer. Reverse transcription of the RNA was performed in a 20 μ l reaction using the iScript cDNA synthesis kit from Bio-Rad Laboratories (Hercules, CA, USA) containing 5 $\times$ iScript reaction mix, iScript reverse transcriptase and nuclease-free water. The complete mixture was incubated for 5 min at 25 °C, 30 min at 37 °C and 5 min at 90 °C. Real-time PCR was then performed, employing the iQ SYBR Green Supermix (Bio-Rad). To minimize pipetting errors, a master mix of iQ SYBR Green Supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 50 U/ml iTaq DNA polymerase, 6 mM MgCl₂, SYBR Green I, 20 nM fluorescein, and stabilizers) and forward and reverse primers were prepared (see Table 1 for primer sequences). The primers were used at a concentration of 500 nM each. The template DNA was added to aliquots of the master mix for reproducibility of replicate samples, for a total reaction volume of 50 μ l. PCR analysis was performed in a MyIQ Thermocycler (Bio-Rad). Typical cycle parameters were: 95 °C 3 min hot start, 40 cycles of 95 °C 10 s, 55 °C 45 s, 1 min 95 °C, 1 min 55 °C and 80 cycles of 55 °C 10 s. SYBR Green I fluorescence intensity was measured at the end of each extension. Melting curve analysis was used to check product specificity. The relative gene expression level was normalized by that of the housekeeping gene (18s RNA).

2.15. Statistical analysis

Results are presented as mean $\pm$ SEM. Comparisons were made using Minitab 15 Statistical Software by one-way ANOVA, followed by Tukey's test for pairwise comparisons. Significance was asserted at $p < 0.05$.

3. Results

3.1. Cell cycle analysis of ASCs

Cell cycle was used to measure changes in cell cycle kinetics (replication and division of chromosomes) under varying culture conditions. The results showed that a significantly

greater percentage of ASCs were actively dividing (S + G₂ phase) when cultured in AR8 compared to control medium DMEM + 10% FBS (Figure 1). Furthermore, this result was not passage number-or donor-specific (Sefcik *et al.*, 2005). Thus, AR8 is able to maintain and expand ASCs in culture to an equal or greater degree than animal serum-containing conditions, an important consideration for obtaining clinically relevant numbers of cells for transplantation.

3.2. Serum-free media screening

ASCs were plated at a high cell density on tissue culture polystyrene (TCP) in AR8, AR8 + 0.5% HS, or DMEM + 10% FBS, and total cell number was assessed after 48 h in culture. AR8 culture resulted in significantly fewer ASCs attached to TCP after 48 h compared to DMEM + 10% FBS control or AR8 + 0.5% HS (* $p < 0.01$). Statistical analysis by one-way ANOVA also revealed a significant difference between DMEM + 10% FBS and AR8 + 0.5% HS (# $p < 0.05$). As shown in Figure 2A, ASCs spontaneously formed multicell aggregates when cultured in AR8 on TCP at high cell density. Further interrogation revealed that these cell aggregates were viable and could be disaggregated and replated in AR8 + 0.5% HS (Figure 2B). The reduction seen in ASC attachment, as well as the spontaneous cell aggregation that occurs with high density culture in AR8 on TCP, supports the need for extracellular matrix proteins to promote cell attachment in the absence of serum.

3.3. Nanofibre parametric study

To resolve the issues of ASC aggregation at high cell densities under serum-free conditions, nanofibre substrates of collagen type I were chosen to serve as a biomimetic extracellular matrix. To this end, electrospinning rat tail collagen was evaluated at several different collagen concentrations and collecting distances for a constant driving voltage of 20 kV and flow rate of 1 ml/h. Average fibre diameter decreased with decreasing solution concentration (Figure 3A). The literature suggests that a quadratic relationship exists between average fibre diameter and solution concentration (Gu *et al.*, 2005), a notion that was supported by our data ($R^2 = 0.8684$). In this study, increasing the solution concentration from 8% to 9% nearly doubled the average fibre diameter (Figure 4). Fibre diameter was also less variable for all concentrations tested at shorter collecting distances (Figure 3B). Furthermore, bead area density was analysed to characterize the uniformity of the electrospun fibres. Bead area density and solution concentration resulted in a near-perfect quadratic fit (Figure 5A; $R^2 = 0.9994$). Similar to the trend seen with diameter, bead area density was also minimized at shorter collecting distances. The results of the parametric study revealed that uniform fibres (reproducible diameter, lowest bead area density) could be electrospun under the following conditions: 8% w/v RTC solution in HFP, 1 ml/h flow rate, 10 cm collecting distance and 20 kV driving voltage. These conditions resulted in a non-woven nanofibre mesh with an average fibre diameter of 168 nm and a bead area density of about 1% (Figure 4A). The average thickness of the collagen nanofibre meshes (not including the coverslip) was measured using microcalipers to be $178 \pm 46 \mu\text{m}$.

3.4. ASC filopodia extension on collagen substrates

After 7 days in culture in AR8 SFOM, ASCs on 2D collagen coatings and 3D collagen nanofibres were prepared for SEM to assess any differences in cellular morphology. Scanning electron micrographs revealed a stark contrast in the length of filopodia extensions from ASCs cultured on the two substrates. Further quantification demonstrated that filopodia length was significantly longer on ASCs cultured on 3D nanofibres compared to ASCs cultured on 2D coatings (Figure 6). Interestingly, the collagen nanofibres were not visible in these SEMs captured after approximately 9 total days in media. Although UV cross-linking is a less harsh alternative to chemical treatments and has been shown to only denature collagen at higher temperatures (>50 °C; Sionkowska, 2005), additional cross-

linking mechanisms should be investigated further. However, care should be taken in utilizing harsh chemical solvents, such as glutaraldehyde, that may denature the collagen or prove to be cytotoxic to cells if not completely washed off (Barnes *et al.*, 2007).

3.5. Osteogenic differentiation of ASCs on RTC substrates

ASCs cultured in AR8 SFOM on both the 3D RTC nanofibres and the 2D RTC coating stained positively for calcified matrix deposition with Alizarin red on day 21 (Figure 7). Osteogenic gene expression of ASCs was detected using real-time quantitative RT-PCR for six osteoblastic markers after exposure to AR8 SFOM for 7, 14 and 21 days. ASCs cultured on 3D collagen nanofibres exhibited greater gene expression levels across all six bone markers tested by day 21, compared to 2D coating (Figure 8), as indicated by a fold change (3D/2D) >1. There was no statistically significant difference in gene expression across time-points (e.g. day 14 vs. day 21). Osteopontin gene expression on day 21 was significantly greater on 3D nanofibres vs. 2D coating (* $p < 0.05$). The expression of 18s RNA was used to normalize gene expression levels.

4. Discussion

The expansion and culture of ASCs *in vitro* in completely serum-free conditions may greatly improve the clinical translation of these cells. However, when serum is removed from culture, cells often undergo growth arrest, thereby limiting their expansive potential or jeopardizing their viability. To this end, we characterized the cell cycle kinetics of ASCs in our serum-free media formulation, AR8, and compared the results with ASCs cultured in AR8 supplemented with only 0.5% human serum, an amount easily obtained through an autologous blood draw, or standard control medium (DMEM/F12 + 10% FBS). Cell cycle analysis revealed that ASCs cultured in AR8 were not only viable and proliferating, but there were actually significantly greater numbers of cells entering mitosis compared to ASCs cultured in the other media formulations (Figure 1). Furthermore, the cell cycle has been shown to be an important consideration for stem cell differentiation, specifically for haematopoietic stem cells (Colvin *et al.*, 2004). The authors have suggested that there may exist certain windows in the cell cycle, termed 'differentiation hot spots', in which cells are particularly susceptible to entering the differentiation process and that these windows may in fact occur during the S/G₂ phase. The potential role AR8 may play in increasing the differentiation ability of ASCs by altering cell cycle kinetics remains to be seen.

In our earliest work with ASCs, we found a cell seeding density of 20 000 cells/cm² to be most favourable for the osteogenic differentiation of the cells (unpublished data). However, seeding at cell densities >10 000 cells/cm² in AR8 resulted in spontaneous aggregation and the formation of cellular masses. Interestingly, these cellular aggregates were viable after 24 h and could be disaggregated, replated and expanded in culture with the addition of 0.5% human serum. The inability of ASCs to remain in monolayer culture at high cell densities in AR8 suggests that ASCs are dependent upon the extracellular matrix proteins found in serum for their attachment to tissue culture polystyrene. This is not surprising, as many groups have reported on the dependence of matrix proteins for cellular adhesion *in vitro* (Mooney *et al.*, 1995; Barshavit *et al.*, 1991; Ogle *et al.*, 1989; Reyes *et al.*, 2007).

In the studies presented, we used electrospinning to synthesize a biomimetic 3D substrate by mimicking the nanometer-scale fibre diameter of native bone matrix. Electrospinning is an increasingly popular technology in tissue engineering because both the solution properties and process control variables can be tailored to create a substrate of a specific size, composition and porosity. Process parameters can be easily adjusted to yield nanofibres in the micrometre–nanometre range, and the density of the nanofibre mesh can be varied to provide different size pores for cell ingrowth. Isolation of type I collagen from rat tails

allowed for the collection of a large volume of collagen in an inexpensive manner that could then be utilized for large-scale experiments, minimizing lot variability. Characterization of the rat tail collagen nanofibres produced by electrospinning allowed for the creation of uniform nanofibres to act as a biomimetic ECM for the *ex vivo* culture of ASCs. Our parametric analysis revealed a quadratic relationship between average fibre diameter and solution concentration. Gu *et al.* (2005) and Fridrikh *et al.* (2003) have also reported on a quadratic relationship between these two variables for the electrospinning of polyacrylonitrile (PAN), poly(caprolactone) (PCL) and poly(ethylene oxide) (PEO). In contrast, others have reported that both a power law (Deitzel *et al.*, 2001) and a linear relationship (Boland *et al.*, 2001) exist between fibre diameter and concentration. It has been suggested that either the presence or absence of splitting the electrospinning jet could result in the different relationships observed for different polymer/solvent systems (Gu *et al.*, 2005; Doshi and Reneker, 1995). Bead area density also followed a quadratic fit to solution concentration; this parameter was minimized in these studies to achieve uniform fibre diameters. To date, the presence of beads on nanofibres has been viewed as the result of suboptimal parameters in the electrospinning process. However, it is unknown whether or not beading may play a role in modulating the differentiation process and should be investigated further.

Cell–ECM interactions have been studied extensively and have been shown to modulate shape and alignment, cytoskeletal organization, gene expression, cell fate processes and gap-junctional communication for a variety of cell types (Adams and Watt, 1993; Chen *et al.*, 1997; McBeath *et al.*, 2004; Schiller *et al.*, 2001). Certain extracellular matrix components can provide stimulatory or inhibitory influences on native cells, including circulating stem cells or resident osteoblasts, via local niche elements (Scadden, 2006). Osteogenesis is one process that is highly dependent on the structural and chemical cues received from the ECM. For example, osteoprogenitor cells bind to a well-organized structure of type I collagen fibrils in the ECM to regulate differentiation towards a mature osteoblast phenotype (Franceschi and Iyer, 1992). We found that ASC cellular structure, specifically the length of filopodia, was significantly altered when the cells were cultured on 3D substrates of type I collagen nanofibres (Figure 6). Changes in cell shape have been previously suggested to modulate the extent of differentiation in pre-osteoblastic cells (Spiegelman and Ginty, 1983; Thomas *et al.*, 2002; Shih *et al.*, 2006). Additionally, McBeath *et al.* (2004) have demonstrated that mechanical cues, such as patterned cell culture substrates, can influence the lineage commitment of mesenchymal stem cells, suggesting that cell shape and cytoskeletal mechanics are integral drivers of stem cell differentiation and developmental processes (Chen *et al.*, 1997). Thus, the difference in filopodia length observed between ASCs cultured on 2D vs. 3D substrates may modulate ASC lineage commitment and explain the resultant increase in osteogenic differentiation of the cells cultured on 3D nanofibres.

3D culture systems will likely play a key role in the future of regenerative medicine as we try to mimic *in vivo* settings. In this study, we compared electrospun 3D type I collagen nanofibre constructs to 2D collagen coatings in order to examine the effects of nanoscale geometry on ASC differentiation with identical substrate composition. Our studies suggest that nanoscale morphology plays a critical role in modulation of cell fate processes and *in vitro* osteogenic differentiation of ASCs. Quantitative RT–PCR results showed that all genes were upregulated (>one-fold) in ASCs cultured on 3D nanofibres compared to 2D collagen coatings by day 21 (Figure 8). However, statistical analysis revealed that only osteopontin expression was significantly ($p < 0.05$) enhanced on the 3D nanofibre scaffolds. Additionally, ASCs cultured in AR8 SFOM on both the 3D RTC nanofibres and the 2D RTC-coating stained positively for calcified matrix deposition with Alizarin red on day 21, as expected (Figure 7). Although mineralization is indirectly related to the temporal expression profiles of individual gene products (Puchtler *et al.*, 1969), qualitative

differences in the staining patterns of Alizarin-positive material were observed. Other investigators have recently taken notice of the potential benefit of utilizing 3D culture systems to drive cellular differentiation. Gabbay *et al.* (2006) most recently examined the effects of a 3D substrate on the differentiation behaviour of ASCs. Cells grown in a bovine collagen gel showed greater expression of all osteogenic markers (cbfa-1, ALP, ON, OP, Coll-I, JNK2) than did the ASCs grown on tissue culture polystyrene. In addition, Shih *et al.* (2006) have recently demonstrated that a non-woven nanofibrous mesh of type I collagen altered the cytoskeleton of human bone marrow-derived MSCs (BMSCs), perhaps explaining the changes observed in cell fate processes, including increased proliferation rates and cellular differentiation towards an osteogenic lineage. However, the authors did not observe any differences in osteogenic gene expression (osteopontin, osteocalcin, osteonectin, type I collagen) of BMSCs cultured on nanofibres of varying diameters (50–200 nm, 200–500 nm, 500–1000 nm), suggesting the importance of a 3D morphology regardless of scale. While our gene expression results support the use of a 3D nanofibre mesh to enhance the differentiation potential of ASCs, RTC fibres were not morphologically distinct or apparent after approximately 9 days in media, and had likely been remodelled or obscured by new matrix synthesized by the cells. Because cells were seeded onto dry collagen nanofibre meshes in a minimal amount of medium, initial cell attachment occurred on the 3D morphology. Quantitative PCR results thereby suggest that the initial structural cues sensed by the ASCs were robust, upregulating osteogenic gene expression after 21 days in culture (Figure 8). The effects of chemical cross-linking on collagen nanofibres to preserve fibre integrity and the subsequent effects of these bona fide nanofibres on cellular differentiation should be investigated further.

5. Conclusions

For bone tissue-engineering applications, the implications of a biomimicking collagen nanofibre matrix could be profound. In this study, the role of a 3D collagen nanofibre matrix was two-fold: first, the 3D nanofibres provided the extracellular matrix protein component necessary for ASC adhesion in AR8 at high cell densities; and second, the 3D architecture provided the structural and chemical cues suggested to regulate the differentiation of osteoprogenitor cells, resulting in enhanced ASC differentiation towards an osteoblastic phenotype. The serum-free medium AR8 and the novel osteogenic formulation we present here bear direct relevance to the clinical applications of ASCs for skeletal repair.

Acknowledgments

This study was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (Grant No. AR-052352-01A1) and Whitaker Foundation faculty start-up funds to E.A.B. as well as the National Institute of Dental and Craniofacial Research (Grant No. DE-010369-08) to R.C.O. L.S.S. was supported by a National Science Foundation Graduate Research Fellowship.

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Figure 1. Cell cycle analysis of ASCs cultured in AR8, AR8 + 0.5% HS, or DMEM + 10% FBS. Significantly more ASCs are actively dividing when cultured in AR8 vs. standard control medium (DMEM + 10% FBS). * $p < 0.05$

Figure 2.

Assessment of cell viability and morphology of ASCs cultured in AR8, AR8 + 0.5% HS, or DMEM + 10% FBS. (Top) ASCs were seeded at 10 000 cells/cm² and total cell number was quantified after 48 h. **p* < 0.01 compared to AR8; #*p* < 0.05 compared to AR8 + 0.5% HS. (Bottom) ASCs were seeded at 20 000 cells/cm² in AR8. Cell aggregation occurred within 24 h of cell seeding (A). Cell aggregates were viable and could be disaggregated and replated in AR8 supplemented with 0.5% HS (B), demonstrating the importance of extracellular matrix proteins found in serum. Scale bar = 200 μm

Figure 3.

Characterization of nanofibre diameter at a constant voltage of 20 kV and flow rate of 1 ml/h. Average nanofibre diameters are plotted against RTC concentrations (A) and various collecting distances (B). The results suggest a quadratic relationship between average fibre diameter and concentration ($R^2 = 0.8684$)

Figure 4.

Scanning electron micrographs of electrospun rat tail collagen nanofibres at 10 cm collecting distance, 1 ml/h flow rate and 20 kV driving voltage for 8% and 9% concentrations (w/v) of RTC in HFP. Increasing the concentration by only 1% nearly doubled the average fibre diameter. (A) 8% RTC, average diameter 168 ± 70 nm; (B) 9% RTC, average diameter 345 ± 149 nm. Scale bar = 1 μ m

Figure 5. Characterization of bead area density at a constant voltage of 20 kV and flow rate of 1 ml/h. Bead area density is plotted against RTC concentration (A) and collecting distance (B). A near-perfect quadratic relationship exists between bead area density and concentration ($R^2 = 0.9994$)

Figure 6.

Quantitative analysis of ASC filopodia extension on 2D collagen coating vs. 3D collagen nanofibres after 7 days in SFOM. Filopodia length is significantly greater on ASCs cultured on 3D nanofibres (* $p < 0.01$), as demonstrated in representative scanning electron micrographs of the ASC leading edge on 2D coating (A) and 3D nanofibres (B). Scale bar = 5 μm

Figure 7.

Osteogenic differentiation of ASCs on 3D RTC nanofibres and 2D coating is exemplified by Alizarin red staining. Undifferentiated ASCs on RTC coating show no staining for calcified matrix deposition by Alizarin red (A). ASCs differentiated in AR8 SFOM for 21 days stain red for calcified matrix on RTC coating (B) and nanofibre mesh (C). Scale bar = 50 μm

Figure 8. Quantitative real-time RT-PCR results of osteogenic gene expression of ASCs cultured for 21 days in AR8 SFOM. Data represent the average fold change in gene expression of ASCs differentiated on 3D RTC nanofibres compared to 2D collagen coatings. All genes were upregulated (>one-fold) on the nanofibre samples by day 21 (* $p < 0.05$, 3D vs. 2D)

Table 1Nucleotide sequences of human primers used to amplify genes of interest [Frank *et al.* 2002]

Gene of Interest	Forward Primer (5' – 3') Reverse Primer (5' – 3')	Annealing Temp. °C
18s	CGGCTACCACATCCAAGGAA GCTGGAATTACCGCGGCT	58.1
Alk Phos	GACCCCTTGACCCCCACAAT GCTCGTACTGCATGTCCCCT	58.1
Collagen I	CAGCCGCTTCACCTACAGC TTTGTATTCAATCACTGTCTTGCC	56.5
Osteocalcin	GAAGCCCAGCGGTGCA CACTACCTCGCTGCCCTCC	59.3
Osteonectin	ATCTTCCCTGTACACTGGCAGTTC CTCGGTGTGGGAGAGGTACC	59.3
Osteopontin	CTCAGGCCAGTTGCAGCC CAAAAGCAAATCACTGCAATTCTC	56.5
Cbfa1	GCCTTCAAGGTGGTAGCCC CGTTACCCGCCATGACAGTA	56.5