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3D surface topology guides stem cell adhesion and differentiation

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Polymerized high internal phase emulsion (polyHIPE) foams are extremely versatile materials for investigating cell–substrate interactions in vitro. Foam morphologies can be controlled by polymerization conditions to result in either open or closed pore structures with different levels of connectivity, consequently enabling the comparison between 2D and 3D matrices using the same substrate with identical surface chemistry conditions. Additionally, here we achieve the control of pore surface topology (i.e. how different ligands are clustered together) using amphiphilic block copolymers as emulsion stabilizers. We demonstrate that adhesion of human mesenchymal progenitor (hES-MP) cells cultured on polyHIPE foams is dependent on foam surface topology and chemistry but is independent of porosity and interconnectivity. We also demonstrate that the interconnectivity, architecture and surface topology of the foams has an effect on the osteogenic differentiation potential of hES-MP cells. Together these data demonstrate that the adhesive heterogeneity of a 3D scaffold could regulate not only mesenchymal stem cell attachment but also cell behavior in the absence of soluble growth factors.

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1. Introduction

Cell interactions with their surrounding extracellular matrix (ECM) play an important role in regulating cellular functions as basic as proliferation [1,2] and as complex as stem cell differentiation [3–5]. It has become widely appreciated that the properties of this cell-ECM interface, including surface topography [6,7], hydrophobicity and hydrophilicity [8], and surface chemistry [4,7,9], must mimic those of native ECM to appropriately guide cell function. Spatial and temporal patterns of these cues are equally important in regulating function [10]. More importantly, the configuration of these physical cues, i.e. their topology, is critical in controlling cellular function by matching the endogenous topology of the cell membrane to enhance signaling and function. One way to understand this is the design of surfaces where chemistries are arranged in different patterns that create different topological cues to control cell adhesion and hence signaling. Previous studies have showed that patterns of click chemistry [11], photolithography (by UV crosslinking) [11–13], and alkanethiol self-assembled monolayers (SAMs) [14,15] all directed cell adhesion and lineage specification. For example, adhesive sites spaced 34 or 62 nm apart directed mesenchymal stem cells (MSCs) to mature into osteoblasts or adipose cells, respectively [16]. Yet ECM proteins such as fibronectin exhibit heterogeneously distributed cell binding sites [17].
that are exquisitely controlled at the nanometer length scale [18]. When ligand spacing is similarly disordered [19] with spacing >70 nm, stem cell adhesion and function improve [20]. However, ECM is a 3D network and these patterning methods are limited to planar substrates.

To overcome this challenge, high internal phase emulsion (HIPE) templated matrices have been used to different topological arrangements of ligand spacing in 3D foams [21,22]. The water-in-oil HIPE matrix can be stabilized by two amphiphilic block co-polymers: poly(1,4-butadiene)-b-poly(ethylene glycol) (PBD-PEO) and polystyrene-b-poly(acrylic acid) (PS-PAA) [21]. By using co-polymers with dissimilar hydrophobic blocks, their phase separation at the oil-water interface could be exploited upon polymerization of the HIPE template [23,24], resulting in foams surface functionalized in situ with domains of either PEO or PAA. This created topologically defined 3D foams with domains that were cell inert (PEO) or charged to facilitate protein deposition and thus cell adhesion (PAA). Adhesive domain areas ranged from tens to hundreds of square nanometers creating unique "surface topology." We previously determined that foams exhibiting smaller adhesive domains that were spaced hundreds of nanometers apart supported the most robust human mesenchymal progenitor (hES-MP) adhesion [21]. One drawback of these PAA/PEO foams was that they lacked appreciable interconnectivity that limits cell infiltration unlike the open lattice found in native ECM [17]. Consequently here, we synthesized foams with closed as well as open pore structures [25,26] to determine if scaffold morphology and pore interconnectivity regulated stem cell adhesion and differentiation.

2. Materials and methods

2.1. Scaffold preparation

All chemicals were purchased from Sigma Aldrich unless otherwise stated. Styrene and divinylbenzene (80% technical grade) monomers were passed through a column of activated basic alumina (Brockmann Activity I) to remove the inhibitor p-tert butylcatechol prior to use. The initiators K2S2O8 and azobisisobutyronitrile (AIBN; Fisher Scientific), tetrahydrofuran, block co-polymers poly(1,4-butadiene)-b-poly(ethylene oxide) (PBD-PEO, Mw = 14,700 g/M) and polystyrene-b-poly(acrylic acid) (PS-PAA, Mw = 18,600) (PolymerSource Inc., Montreal), and the surfactant Span 80 were all used as received and all copolymers have a reported polydispersity index (PDI) of 1.1–1.3. High internal phase emulsions were prepared in a method adapted from Viswanathan and coworkers [21]. Briefly, the surfactant was solubilized in the oil phase at 0.01 mol% (relative to the monomer) in all cases. For copolymers mixtures of PBD-PEO and PS-PAA, the surfactants were used in molar ratios 75:25, 50:50, and 25:75 as defined in Table 1. Resulting foams are herein referred to by their PEO molar content, e.g. 25% PEO will be PE025, except for pure PSS, which will be referred to as PAA100. THF (10 µl/mg) was used to dissolve PSS-PAA before its addition to the oil phase due to its poor solubility in styrene/divinylbenzene (St/DVB). The aqueous phase was added using a peristaltic pump at a rate of 10 ml/min, which was continuously stirred at 750 rpm. All emulsions had an aqueous phase volume fraction of 90%. For closed porous foams, the aqueous phase contained 0.1 w/v % radical initiator K2S2O8. For open porous foams, the oil phase contained 1 w/v % radical initiator AIBN, which was added immediately prior to emulsification. The resulting emulsions were polymerized at 50 °C for 24 h, Soxhlet extracted in isopropl alcohol for 24 h to remove unreacted monomers, and vacuum dried prior to use. Emulsions were stabilized with the surfactant Span 80 and were prepared using previously established protocols [27] as a control for the 3D foam architecture.

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<th>Foam compositions based on their mixture of PBD and PAA as well as the nomenclature used in the study.</th>
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2.2. 3D cell culture

Human Embryonic Stem cell derived Mesenchymal Progenitor cells (hES-MP™, Collectics, UK), which differentiate towards osteogenic, chondrogenic and adipogenic lineages [28], were used in all experiments. We have previously shown hES-MPs to express the bone markers alkaline phosphatase and mineralized matrix [29]. Cells were cultured in growth media containing Alpha modified Minimum Essential Medium (Alpha-MEM, Gibco, UK) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (Invitrogen, UK) and 10 ng/ml basic-fibroblast growth factor (b-FGF, Invitrogen, UK). Cells were maintained in a humidified 37 °C incubator at 5% CO2. Cultures were passaged at 70–80% confluence using Trypsin EDTA and used at passages between 5 and 10.

Scaffolds (1.2 cm in diameter and 3–5 mm in height) were sterilized for cell culture using 70% ethanol (EtOH) overnight and then washed three times with PBS to remove EtOH. To prevent scaffold buoyancy in the well plate, they were weighed down with custom dental grade stainless steel rings for the first week in culture. Scaffolds were washed once with media before seeding with hES-MP cells at a density of 100,000 cells/scaffold in 50 µl media. Cells were incubated at 37 °C for 90 min before adding 1 ml of media/well without b-FGF. After 24 h of seeding, scaffolds were transferred to a new well plate with subsequent media changes every 2–3 days. For experiments investigating mineral deposition, hES-MPs seeded on scaffolds were treated with 10 nM dexamethasone (Dex) 24 h after seeding. Cells used in the MTT, Alamar Blue, and Titertacs Apoptosis assays (all from Invitrogen) were all cultured for 7 days on the indicated substrates prior to use in the assay, which were all performed according to manufacturer instructions with positive and negative controls on tissue culture plastic.

2.3. Cell morphology by confocal microscopy

Cells were fixed and stained for nuclear and actin staining on days 7, 14, 21 and 28 as follows. Scaffolds were washed once with PBS and fixed with 3.7% formaldehyde for 40 min then permeabilized with 0.1% Triton X for 20 min. Cells were then incubated with Phalloidin-Texas Red (1:100 in PBS) and DAPI (25 µl) for 1 h on a plate rocker at room temperature. Unbound stain was washed with PBS three times and scaffolds were stored in PBS at 4 °C until imaged. Imaging was performed using an inverted Zeiss LSM 510 confocal laser scanning microscope with a 10× objective.

2.4. SEM imaging

Scaffolded foams were prepared for scanning electron microscopy (SEM) by placing each foam on an aluminum stub with an adhesive carbon pad. Samples were coated with 15 nm gold (Edwards S150B Sputter Coater) prior to SEM using a Philips XL20 scanning electron microscope at an accelerating voltage of 10 kV and spot size of 3.0 nm. Scaffolds and interconnect diameters were measured from scanning electron micrographs using ImageJ.

For biological SEM, cells cultured on foams for 28 days were fixed in 2.5% glutaraldehyde (1 ml/well) overnight. Scaffolds were then rinsed in PBS twice for 15 min each followed by dH2O once for 15 min. Cells were then incubated with Phalloidin-Texas Red (1:100 in PBS) and DAPI (25 µl) for 1 h on a plate rocker at room temperature. Unbound stain was washed with PBS three times and scaffolds were stored in PBS at 4 °C until imaged. Imaging was performed using an inverted Zeiss LSM 510 confocal laser scanning microscope with a 10× objective.}

2.5. Calcium staining by alizarin red S

hES-MP cells cultured on open porous scaffolds for 28 days were fixed in 3.7% formaldehyde for 30 min and washed with PBS once and in dH2O twice. Alizarin Red S (0.5 w/v%) in dH2O was adjusted to a pH of 4.1 using 0.1M NaOH. 1 ml was added to each scaffold and placed on a rocker for 30 min. Scaffolds were then washed three times in dH2O to remove unbound stain and left to air dry. Blank scaffolds were also treated with Alizarin Red to assess baseline staining. To quantify mineral deposition, 0.5 ml of perchloric acid (5% v/v in dH2O) was added to each scaffold to de-stain it. 1:1 v/v of absolute EtOH:hexamethyldisilazane for 1 h. This was followed by a hexamethyldisilazane wash twice, each for 10 min before samples were left to dry completely under vacuum. Samples were then sputter coated and viewed under SEM as previously described.

2.6. RNA isolation, cDNA synthesis, and qPCR microarrays

hES-MP cells were cultured on both closed and open porous foams for 7 days. Scaffolds were washed once with PBS and total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, UK). Reverse transcription was performed using 5 µg total RNA to obtain cDNA using the QuantTect Reverse Transcription Kit (Qiagen, UK). Genomic DNA was eliminated from RNA samples by incubating samples with Wipeout buffer for 2 min at 42 °C prior to reverse transcription. Resulting cDNA samples were then placed in a customized 384 well Taqman microarray (Applied Biosystems). For genes, see Supplementary Table 1 to detect gene expression. The array plate was analyzed by RT-PCR using the 7900HT RT-PCR system (Applied Biosystems). The reactions were amplified for 40 cycles. Ct values were acquired from amplification curves in triplicate, and relative fold change was computed using the ΔΔCt method [30]. Gene expression was expressed as a fold change of the cells cultured in each foam composition versus cells maintained on tissue culture.
Block copolymer functionalized polyHIPE foams were synthesized to generate either closed or open pore morphologies by the addition of either a water or oil soluble radical initiator, i.e. K$_2$S$_2$O$_8$ or AIBN, to created closed (Fig. 1A) or interconnected pores (Fig. 1B), respectively. Herein, open porous refers to foams with an interconnected 3D structure while closed porous refers to foams with a lack of interconnectivity between adjacent pores. Open porous foams functionalized with either PEO, PAA, or their mixtures exhibited pore sizes in the range of 40–80 μm (Supplemental Figure 1A and green arrow). Additionally, average interconnected pore diameters did not exhibit statistically significant changes with surface chemistry (Supplemental Figure 1B and red arrow). Relative to closed porous foams [21], average pore size diameters did not differ significantly. Roughness of the open porous foam surfaces also appeared lower and uniform relative to closed porous foams based on SEM images (Fig. 1). Thus, pore size and the level of interconnectedness within each foam type was independent of surface chemistry and pore size was independent of initiator.

Given previous segregation of copolymer mixtures in closed porous foams, we next investigated how similar segregation of the same copolymers in open porous foams, shown schematically in Fig. 2 as ‘topology,’ could affect adhesion. Human embryonic stem cell derived mesenchymal progenitor cells (hES-MP) were cultured for a period of 7 days in the absence of any specific growth factors. Cell viability was first assessed by MTT and alamar blue assays to determine cell viability (metabolic activity) and relative cell number as a function of copolymer composition and pore morphology. While MTT showed higher cell viability on open versus closed pore foams, mixed copolymer compositions, which had previously been identified to have adhesive nano-domains, e.g. PEO50 and PEO75 [21], retained the most cells independent of pore morphology (Supplemental Figure 2A). Alamar blue staining also confirmed higher cell viability on mixed versus single copolymer compositions for open pore foams (Supplemental Figure 2B); little apoptosis was detected for open pore foams and only a modest increase was noted for close pore (Supplemental Figure 3).

**Fig. 1.** 3D scaffold morphologies. Scanning electron micrographs of polyHIPE foams with (A) closed pore morphology and (B) interconnected open pore morphology for the indicated copolymer mixtures. Scale bars are 50 μm.
Irrespective of foam morphology and the initial amount of cell adhesion, hES-MPs proliferated and spread poorly on foams composed of PEO100 (Fig. 2, bottom), which is consistent with PEO inhibiting protein and cell attachment [31]. Poor cell spreading was also noted for uniformly adhesive PAA100 foams (Fig. 2, top), which is consistent with observations of reduced cell motility and proliferation on substrates with high ligand density [32,33]. In contrast hES-MP adhesion and spreading was the greatest on PEO75 and PEO50 open porous foams (Fig. 2, middle) corroborating the total cell number observed on these compositions by day 7 (Supplemental Figure 2A). Cells cultured on closed porous foams adhered to and spread on either the curved void surface or around the struts of the voids (solid arrows, Fig. 2) whereas cells cultured on open porous foams adhered and spread through the interconnects in a three-dimensional matrix (open arrows, Fig. 2). Since hES-MP cell adhesion and spreading was dependent on scaffold surface topology for open porous foams as it was on closed porous foams, these data imply a similar mechanism with the presence of adhesive nano-domains as was observed with closed porous foams [21].

Beyond 7 days, adhesion and spreading of hES-MPs on open and closed porous foams was also evaluated after 14, 21 and 28 days. Closed porous foams did not support cell proliferation at these time points. However hES-MP cells cultured on open porous foams exhibited cell proliferation across all the copolymer compositions at the specified time points (Supplemental Figure 4). This indicates that although differential cell adhesion occurred in the first week of culture, cells that were initially adherent but not well spread were still able to proliferate over long periods in culture.

Fig. 2. Differential cell adhesion on polyHIPEs. At the far left is a schematic of the surface topology on the pore walls of polyHIPE foams as a function of PEO molar ratio. Confocal images of filamentous actin (red) and nucleus (blue) staining of hES-MPs cultured on the foams (auto-fluorescence in green) for 7 days show differential adhesion and cell spreading on copolymer functionalized closed porous and open porous foams compared to Span 80 functionalized foams. Solid arrowheads indicate hES-MPs adhering and spreading around the pore struts of the closed porous foams. Open arrowheads indicate hES-MP spreading through the interconnected pores. Dashed lines indicate regions magnified in other images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Although the surface topology of adhesive domains regulated basic cell functions over 7 days in culture, it was not clear to what extent it would influence hES-MP differentiation over the same time course and beyond. Gene expression for different mesenchymal lineages (Supplemental Table 1) was measured by microarray of hES-MP cells cultured on PEO100, PEO75, PEO50, PEO25, and PAA100 open and closed porous scaffolds normalized to undifferentiated cells (Fig. 3A, B; Supplemental Table 2). While closed pore scaffolds generally exhibited larger changes of individual gene expression than open pore scaffolds, the average fold change for certain lineages was dependent on the scaffold’s adhesive surface topology and pore morphology (Fig. 3C); for closed porous foams, mRNA was up-regulated in hES-MP cells in a topology-dependent manner only for mRNA associated with osteogenesis. For open porous foams, osteogenesis was also topology-dependent but osteogenic genes increased with the PEO concentration, unlike closed porous foams. Correlation coefficients to directly compare gene expression between closed and open porous foams across all topologies was strongest for osteogenesis at \( r = 0.84 \), indicating opposing osteogenic gene regulation as a result of foam composition and surface topology.

To validate short-term gene expression, longer-term mineral deposition was assessed after 28 days of culture for open porous foams where cell confluence was independent of surface chemistry at that time point (Supplemental Figure 4); closed porous foams, lacking interconnectivity, inhibited cell viability over the same time course, and thus only cells cultured on open porous scaffolds were examined for mineralization. Cells treated with dexamethasone, a glucocorticoid which stimulates osteogenic differentiation and mineralization [34], deposited more calcium for all scaffold compositions. However without dexamethasone, cells on PEO50 and PEO75 open porous foams deposited the most calcium as measured by alizarin red absorbance (Fig. 4), indicating that copolymer composition and thus the surface topology of adhesive domains alone has the potential to support hES-MP cell differentiation. SEM images showed that cells became confluent on each scaffold (Fig. 5A), but hES-MP cells cultured on PEO75 and PEO50 scaffolds also secreted collagen within scaffold pores (Fig. 5B). High magnification images also showed mineral deposits on these foam compositions (Fig. 5A, arrows), again suggesting preferential osteogenic differentiation on scaffolds that display appropriate surface topology. In the presence of dexamethasone, mineralized layers were present on all surfaces regardless of surface chemical composition (Fig. 5C).

Surface topology of adhesive domains may be most biomimetic on PEO75 foams [21] and the open porous structure then may

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**Fig. 3.** Lineage specific gene expression. Lineage specific gene expression data as a function of foam PEO% represented as (A) heat maps comparing fold difference change of open and closed porous foams (B) mean fold change comparing open and closed porous foams. The correlation coefficient between the change of open and closed porous foams as a function of PEO% is noted. Data is mean ± SEM, \( n = 3 \). (C) Mean fold change of lineage specific genes comparing open and closed porous foams. The correlation coefficient between the fold change of open and closed porous foams as a function of PEO% is noted. Data is mean ± SEM, \( n = 3 \).
induce the most robust osteogenic response, but it is still not clear what signaling proteins regulate cell adhesion, spreading, and differentiation on the different surface chemistry and porous structures. Osteogenic gene expression (Supplemental Table 2) was assessed for its correlation with the mean expression of selected cell-matrix signaling genes for both open and closed porous scaffolds (Fig. 6A). Open porous scaffolds exhibited a higher correlation coefficient between average cell-matrix signaling gene expression and osteogenic gene expression. Direct comparisons by two-way ANOVA also confirm these trends, and so to identify specific signaling genes responsible for surface topology-induced osteogenesis, specific signaling gene changes were examined for their correlation with average osteogenic gene expression (Fig. 6B). Integrin-ECM interaction related genes, PTK2 (protein tyrosine kinase 2), also referred to as focal adhesion kinase (FAK), CDC42, and ROCK1 all exhibited the highest correlation coefficients implying a contractile role in surface topology-related signaling similar to other ECM cues including stiffness [35], topography [6], and shape [36].

4. Discussion

4.1. hES-MP adhesion to polyHIPE foams is dependent on surface topology rather than on topography

The onset of differentiation is linked to how and what cells adhere within their extracellular matrix. Matrix properties such as stiffness, topography and surface chemistry have been widely implicated in directing lineage specification [3,4]. While the spatial presentation of adhesive sites on 2D substrates regulates differentiation [16], surface topology’s role in 3D matrices has been less well investigated. In the present study, emulsion-templated polyHIPE foams provide a platform for exploiting block copolymer phase separation to generate 3D matrices with adhesive heterogeneity that better reflects the extracellular matrix [3]. Fine-tuning of emulsion parameters allowed further control over scaffold architecture, i.e. closed versus open porosity, to make scaffolds more permissive for
cell infiltration and longer-term differentiation assays required to assess surface topology's influence over stem cell fate. As first consideration for these scaffolds, topographical features are well known to regulate stem cell adhesion, cytoskeletal organization [37], signaling [38], and fate between osteogenic or adipogenic lineages [6,39]. In closed porous foams, surface topographical features appear mildly dependent on polymerization conditions, though all compositions have surface roughness below 50 nm [21]. In open porous foams, SEM analysis showed that foams did not exhibit substantially higher surface roughness than closed porous foams (Fig. 1). Previous reports indicate that features in excess of 70 nm promote cell adhesion, likely via integrin clustering [40], and differentiation [6,41]. Thus while foams do not have completely smooth surfaces, they may not have induced osteogenesis. The conditions that caused the roughness in closed porous foams may be associated with the internal phase water droplets having excess surface tension [42]; while this is normally dissipated by coalescence to form interconnecting pores, in this case, the excess surface tension could lead to the formation of finger instabilities at the oil/water interface that create surface topographies. However, the strongest evidence that topographical features were less influential is the observation of composition-dependent opposing responses for osteogenic genes in hES-MP cells grown in open and closed porous foams (Fig. 3). These data suggest that other HIPE properties, e.g., adhesive domains and pore interconnectivity, could possibly account for differential effects on differentiation, which is discussed below.

4.2. Osteogenic differentiation may be regulated by topology and porosity

Gene expression of hES-MP cells cultured on open and closed porous foams implicated osteogenesis as being dependent on pore morphology, but with a strongly negative correlation coefficient between the two foam structures. While neither program was highly upregulated, subsequent topology-dependent mineralization suggests the importance of the particular osteogenic genes that were highly up-regulated. One such candidate were the BMPs, which have been previously described as regulators of osteogenic differentiation [43,44]. We found that bone morphogenic protein 5 (BMP5) increased for both open and closed pore foams but specifically peaked at PEO75 for open pore foams. Additionally, osteopontin and TWIST-2 were up-regulated on closed pore foams, both peaking at approximately PEO25, suggesting that the porous structure as well as the composition dependent adhesive topology influences osteogenesis. TWIST-2 is known to be a critical early regulator for osteogenic differentiation by inhibition of RUNX2 [45], while osteopontin has shown to interact with specific integrins, which is important for osteogenic differentiation in mesenchymal stem cells. These results imply that the porous structure in addition to the topology may influence osteogenic differentiation as cells can better attach and spread over a larger volume and appear more 3D than in closed porous foams where cells may likely feel a more 2D.

Human Mesenchymal Stem Cell (hMSC) differentiation ultimately depends on stiffness-mediated, Rho-ROCK-induced contractility [35,36,46] and would therefore expect stiff polystyrene scaffolds used here to support osteogenesis independent of surface topology. However, cell contractility depends on sufficient cell adhesion to pull against the underlying matrix [36]. Furthermore, adhesion mediated activation of focal adhesion kinase (FAK) has been shown to be important in early commitment of osteogenic differentiation of hMSCs [47]. FAK activation has been implicated in triggering extracellular regulating kinase 1/2 (ERK1/2) signaling to induce osteogenesis of mesenchymal stem cells [48]. Yee et al. have hypothesized that increased adhesion via α5β1 integrins activated ERK1/2 signaling via FAK [49]. Taken together, our data suggest that differentiation is not just linked to stiffness-induced changes in cell morphology but rather to stiffness regulated integrin signaling and adhesive organization, much like occur in other 3D systems [50]. The wider implication of our data in the context of biomaterial design is that complex differentiation pathways may be triggered via structural organization of early adhesion events, i.e., adhesive heterogeneity.

5. Conclusions

We have demonstrated the use of emulsion templated foams containing heterogeneously spaced adhesive nano-domains in guiding mesenchymal stem cell adhesion and differentiation. Adhesive patches that better reflect the adhesive spacing found in ECM allowed for the greatest amount of cell spreading irrespective of scaffold architecture. Introducing porosity by varying polyHIPE polymerization parameters increased the likelihood of
osteospecific differentiation in a topology-dependent manner. In this study we use stiff polystyrene matrices; modifying stiffness by altering matrix chemistry independently of scaffold topology could further fine-tune the scaffold's properties to allow differentiation of mesenchymal stem cells towards other lineages.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.01.034.

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