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Behavior of Human Mesenchymal Stem Cells in Fibrin-Based Vascular Tissue Engineering Constructs

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Abstract—A limitation of current tissue engineering vascular graft technology is the provision of an expandable, autologous cell source. By harnessing the multipotency of mesenchymal stem cells (MSC), it is hoped that functional vascular cells can be produced. To date, a range of 2D and 3D environments have been investigated for the manipulation of MSC differentiation pathways. To this end, this study aims to test the hypothesis that MSC seeded in various fibrin gel environments will exhibit evidence of a smooth muscle cell (SMC) phenotype. Initially, a range of cell-seeding densities were screened for 2D and 3D fibrin constructs, where it was observed that a seeding densities of 500,000 cells/mL facilitated gel compaction without degradation or loss in cell viability. Additionally, positive expression of CD49, CD73, CD105 markers and negative expression of hemopoietic stem cell-associated CD34 and CD45 indicated that MSC phenotype was retained within the fibrin gel. Nonetheless, a decrease in the gene expression of α-smooth cell actin and calponin was observed for MSC cultured in static 3D fibrin gels. Although a slight recovery was observed after 24 h mechanical stimulation, the fold-change remained significantly lower than that observed for cells cultured on 2D tissue culture plastic. While MSC differentiation toward a SMC appears possible in both 2D and 3D environments, scaffold architecture and mechanical stimulation undoubtedly play an important role in the creation of a functional SMC phenotype.

Keywords—Mesenchymal stem cell, Fibrin scaffold, 2D and 3D environments, Biomechanical stimulation, Hoop strain, Differentiation, Smooth muscle cell.

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ABBREVIATIONS

MSC	Mesenchymal stem cell
α-SMA	α-Smooth cell actin
SMC	Smooth muscle cell
DMEM	Dulbecco's modified Eagle's medium
TBS	Tris-buffered saline
TEVG	Tissue-engineered vascular graft

INTRODUCTION

The potential offered by mesenchymal stem cells (MSC) as a cell source for vascular tissue engineering applications has been well documented.[1,5,6,16,35] Bone marrow-derived cells have been shown to adopt smooth muscle cell (SMC) characteristics in the presence of a variety of biochemical factors.[11,16–18,23,39] Similarly, the role of mechanical signaling in MSC differentiation toward SMCs has also been investigated in 2D systems.[12,20,22,28,29] In 2D uniaxial strain systems, cells reorientate in a direction perpendicular to that of principal strain.[20] Conversely, *in vivo*, SMCs are found to orientate circumferentially, along the axis of principal strain (Figs. 1a, b). The significance of maintaining the correct strain field is highlighted in a study by Li *et al.*, where the expression of SMC-associated markers was seen to diminish once the cells had reoriented in a direction perpendicular to that of principal strain.[22]

In an attempt to mimic the mechanical constraints imposed in 3D cultures, Kurpinski seeded MSC on micropatterned silicone substrates, forcing them to align parallel to the direction of uniaxial strain.[21] This resulted in increased expression of SMC-associated calponin. Additionally, Nieponice *et al.* studied, and

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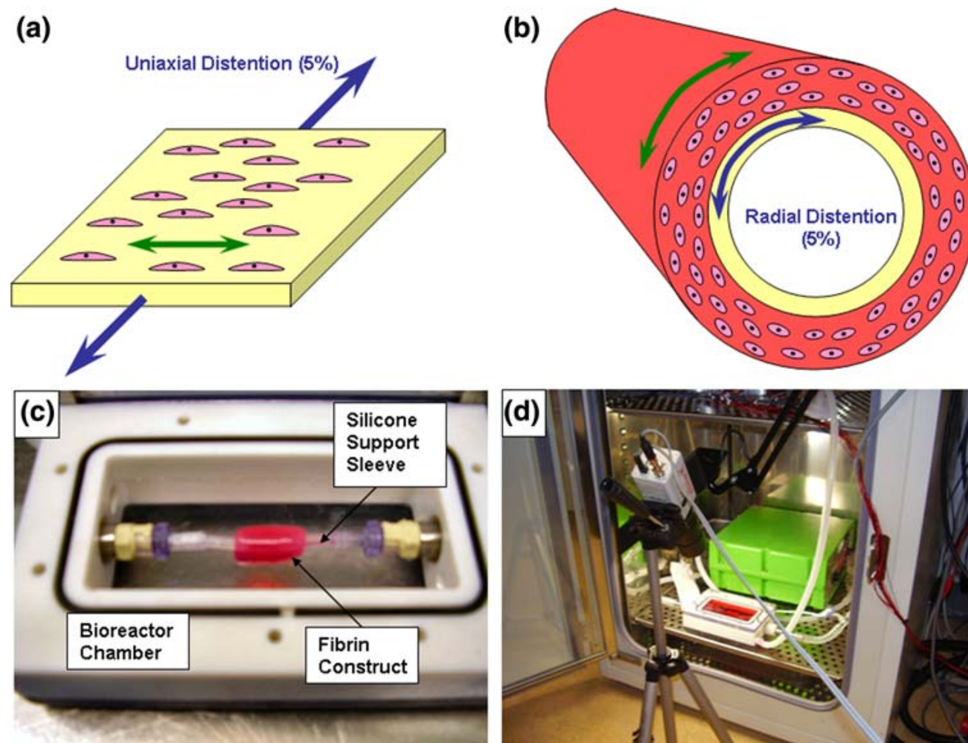

FIGURE 1. Experimental setup. (a) Schematic of uniaxial strain method used in other studies for monitoring the response of cells to cyclic strain. Note that cells align perpendicular to the direction of principal strain. (b) Schematic of the experimental setup used here, which more closely replicates the strain fields experienced by cells in the medial layer *in vivo*—cells align parallel to the direction of hoop strain. (c) Picture of the fibrin construct *in situ*. (d) Picture of the bioreactor setup inside the incubator.

showed qualitatively, the effect of dynamic conditioning of MSC seeded in 3D constructs.[26]

The objective of this study was to examine the effect of 2D and 3D environments on MSC phenotype and in particular SMC-associated markers. It was first necessary to choose an optimal scaffold composition and cell-seeding technique at an appropriate MSC-seeding density for this application. The unique potential of fibrin as a scaffold has been well documented.[2,9,13,19,23,33,37] The necessity to optimize the concentrations of fibrin scaffold components[7,14] and fibrinolytic inhibitors[15] has been highlighted previously. As a result, the seeding technique and the fibrin component concentrations used in this study were based on those previously optimized for tissue engineering vascular grafts.[13,34,37,40] MSC-seeded scaffolds were examined for a range of seeding densities in terms of gel compaction, MSC viability, metabolic activity, multipotency, and collagen formation. Thereafter, changes in gene expression of SMC-associated markers, including alpha-smooth muscle actin (α-SMA) and calponin, were examined using real-time quantitative PCR (qPCR). Furthermore, to replicate the *in vivo* biomechanical environment, MSC-seeded tubular fibrin gels were mechanically conditioned for 24 h and subsequent changes in SMC phenotype examined.

MATERIALS AND METHODS

Cell Source

Bone marrow aspirates were obtained from the iliac crest of normal donors. All procedures were performed with informed consent and approved by the Clinical Research Ethical Committee at University College Hospital, Galway, Ireland. MSC were isolated and expanded in culture as described previously by direct plating.[25] All MSC preparations were characterized for surface expression of CD14, CD34, CD45, CD73 (BD Pharmingen, UK) and CD105 (Serotec, UK) using a FACs ARIA sorter (Becton–Dickinson, UK). FACS analysis indicated the presence of a uniform population of cells negative for CD14, CD34, CD45 ($<2\%$) and positive for CD73 and CD105 ($>95\%$).[25] Cell multipotency was confirmed through adipogenic, osteogenic, and chondrogenic assays. Cultures were passaged at 4 to 6 day intervals and expanded to passage 4 for experimentation.

Fibrin Gel Characterization Study

The fibrin gel composition used in this study was based on concentrations optimized for tissue-engineered vascular graft (TEVG) applications, established

by Ye *et al.*[40] Fibrinogen (Sigma F4883, from human plasma; plasminogen free) was dissolved in Tris-buffered saline (TBS) and dialyzed with a cut-off membrane of 12,400 MW against TBS overnight at 37 °C. To reduce component volume and to increase the number of 3D replicates, optimization studies were performed in hemispherical constructs.[3,38] Gels were constructed so that final concentrations were 3.33 mg/mL fibrinogen, 4 U/mL thrombin, and 2.5 mM $CaCl_2$ in TBS at initial densities of 100,000–2,000,000 cells/ml. Gels were allowed to polymerize for 30 min prior to the addition of MSC medium containing aprotinin (0.1 TIU/mL).

Gel Compaction Measurement

Gel compaction was measured using the method previously described by Tuan *et al.*[38] Height measurements were taken daily over a 9-day time period.

Cell Viability

A Live/Dead® Viability/Cytotoxicity kit (Molecular Probes, UK) was used to visualize cell viability. Calcein acetoxmethyl (Calcein AM, 0.05%) and Ethidium homodimer-1 (EthD-1, 0.2%) stain healthy cells green and the nuclei of dead cells red, respectively. Gels were soaked in D-PBS for 30 min to remove phenol red and serum, prior to 30 min incubation in the cell assay solution. Samples were examined under a fluorescent microscope (Olympus 1X71, Olympus Biosystems GMBH, Germany).

Metabolic Activity

Cell metabolic activity was measured using an Alamar Blue™ assay by measuring the fluorescence intensity (530 nm excitation/590 nm emission) on a microplate fluorescence reader (FLX800, Biotek Instruments Inc.). This was carried out for a range of cell densities for 24 h, 48 h, and 7-day time points.

MSC Phenotype

To confirm that MSC phenotype was retained within the fibrin construct prior to mechanical stimulation, a cell outgrowth study was performed. Fibrin constructs cultured for 7 days were mechanically disrupted and digested in trypsin (Langanbach Services, Ireland) overnight at 4 °C. After 24 h, an equal volume of trypsin inhibitor (Langanbach Services, Ireland) was added to neutralize the solution, followed by further digestion in collagenase (Langanbach Services, Ireland) for 15 min. The resultant solution and remaining undigested constructs were transferred to fresh culture flasks and cultured in complete MSC medium in preparation for flow cytometry analysis. Expression levels of MSC-associated CD49, CD73 (BD Pharmingen, UK), CD105 (Serotec, UK) and HSC (hemopoietic stem cell)-associated CD34 and CD45 (BD Pharmingen, UK) were examined as an indication of cell phenotype post-fibrin culture. IgG1 and IgG2b were used as isotype controls to ensure false positives were not obtained.

Histological Staining for Collagen Formation

Constructs were histologically stained using a modified version of the Masson's Trichrome method to examine collagen formation. Slides were stained with hematoxylin, Biebrich Scarlet-acid Fuchsin, Phosphotungstic acid/Phosphomolybdic acid, Aniline Blue, and 1% acetic acid solutions. Samples were imaged at various magnifications using an inverted Olympus IX71 microscope (Olympus Biosystems GMBH, Germany).

Gene Expression Quantification

Total RNA Isolation

RNA was extracted from cells seeded on TCP using the Trizol method (Gibco BRL) according to manufacturer's instructions. For cells seeded in fibrin constructs, constructs were initially washed for 15 min in D-PBS on a shaker. Thereafter, they were transferred to a 15 mL centrifuge tube containing 1 mL of Tri reagent. Constructs were homogenized for 30 s using a handheld rotor–stator homogenizer, TissueRuptor (Qiagen). The lysate was then centrifuged and the supernatant was transferred to a fresh Eppendorf. The concentration and purity of the RNA were determined spectrophotometrically, using the Nanodrop (ND1000 Spectrophotometer).

cDNA Synthesis and Real-Time RT–PCR

cDNA was transcribed with Improm-II Reverse Transcription System (Promega) using 100 ng total RNA and random hexamers according to manufacturer's instruction. mRNA levels were assessed by real-time quantitative PCR (qPCR) using an ABI Prism 7000 Sequence Detection System and 7000 System SDS Software (Applied Biosystems, USA). TaqMan® Gene Expression Assays were used to test expression of α-SMA (HS00426835_gl) and calponin (CNN) (HS00154543_ml). Quantification was based on the relative expression of the target genes vs. glyceraldehyde-3-phosphate dehydrogenase (assay ID Hs 99999905_m1) as a reference gene using the 2-$\Delta\Delta$Ct method.[24]

Mechanical Conditioning

Construct Mold Design

Fibrin constructs for biomechanical stimulation were prepared using tubular molds. The construct molds consisted of flat-bottomed 10 mL polystyrene tubes (I.D. = 15 mm) coated with a silicone-based lubricant to aid mold release. Each mold contained a silicone insert which anchored a 3.2-mm diameter stainless steel mandrel, which was inserted via a center-bored hole in the tube cap. A silicone tubular sleeve (I.D. = 3.35 mm, O.D. = 4.65 mm, VWR) was placed on the outside of the mandrel, which was also lubricated. This was used to provide a substrate onto which the fibrin gel could contract and to facilitate sterile transfer of the construct into the bioreactor (Fig. 1c).

Fibrin Tubular Construct Preparation and Conditioning

Tubular fibrin gels were prepared at the concentrations described previously using an optimized seeding density of 500,000 cells/mL (~5 mm thick). Gels were allowed to polymerize for 30 min. They were then carefully transferred to flat-bottomed 50 mL tubes still anchored to the silicone support sleeve. Gels were allowed to contract for 7 days in medium containing aprotinin (0.1 TIU/mL) and were subsequently transferred to the bioreactor chamber (Figs. 1c, 1d). Constructs were subjected to 24 h of dynamical conditioning (5% radial distention at a frequency of 1 Hz). The pulsatile distention of the silicone support sleeve was achieved by placing a mechanical constrictor distal to the chamber in the flow loop. Pulsatile flow was provided by a peristaltic pump (Watson Marlow, 520U). Radial distention was recorded using a video extensometer, in an adaption of a bioreactor system described previously by the author.[27,31] As a method of control, fibrin gels were also maintained under static conditions ($n = 3$). Thereafter, gene expression was examined using the methods described above.

Statistical Analysis

Where appropriate, results were represented as means ± SE. A paired Student's t-test was used for the qPCR analysis, comparing static controls to conditioned samples. A p-value of less than 0.05 was considered statistically significant ($*p < 0.05$). For all experiments, $n = 3$ biological replicates, unless otherwise stated.

RESULTS

Fibrin Gel Characterization Study

Construct Compaction Study

Cell compaction was observed in MSC-seeded hemispherical constructs over a 9-day period in the presence and absence of aprotinin (Fig. 2). MSC were found to remodel the fibrin gel in a cell density-dependent manner. The presence of aprotinin was found to decrease the level of compaction observed. At the lower density of 100,000 cells/ml, very little compaction was observed in MSC-seeded constructs. A contributing factor may be the lack of synergistic gel contraction due to the sparse cell distribution, observed in Fig. 3b. At densities of 500,000 and 1,000,000 cells/mL, gel compaction was observed without construct disintegration, i.e., the gels loosing their mechanical integrity and breaking up.

Cell Viability

A Live/Dead cell viability assay was performed to ensure that cells embedded in the fibrin gel were viable (Fig. 3). These fluorescent images taken after 1 and 7 days of culture (initial density = 500,000 cells/mL) show cells have maintained a spherical morphology after their first day in culture; this is reflected at all cell densities studied.

Metabolic Activity

Cell metabolic activity was observed in fibrin constructs using an Alamar Blue™ Assay over a 7-day time period (Fig. 4). While a limitation exists in that metabolic activity is recorded on a "per construct"

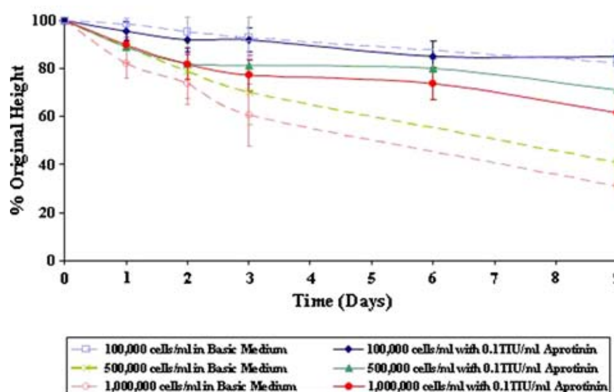

FIGURE 2. Gel compaction study. Measurement of the level of compaction that occurs in the vertical axis of gels compared to their initial height over a 9-day period. Compaction is recorded for constructs in the presence and absence of a fibrinolytic inhibitor, at a range of initial seeding densities ($n = 6$).

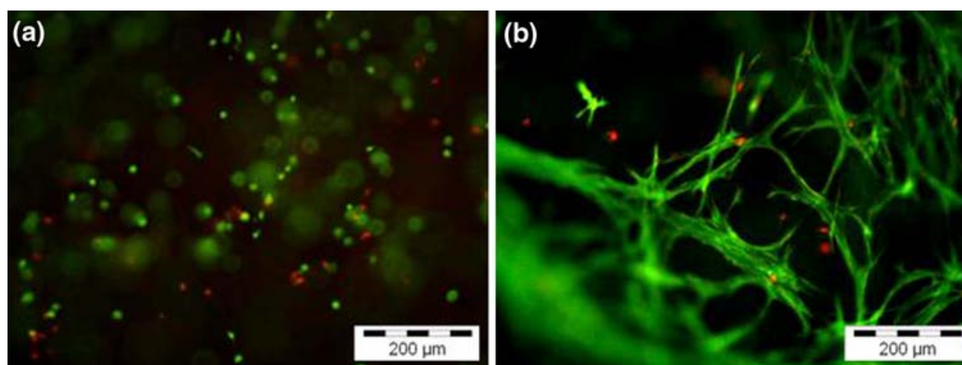

FIGURE 3. MSC viability in fibrin gel. A viability/cytotoxicity assay stains viable cells green while a red fluorescent dye infiltrates the membranes of damaged or dead cells. Hemispherical MSC-seeded fibrin construct cultured for (a) 1 day and (b) 7 days are shown (initial cell density = 500,000 cells/ml) (n = 3).

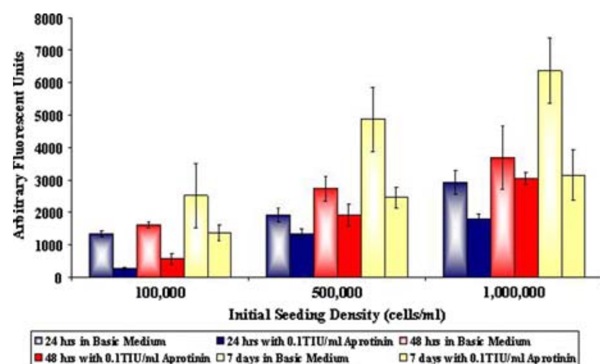

FIGURE 4. MSC metabolic activity in fibrin gel. Comparison of the relative metabolic activity of MSC, seeded at different densities in fibrin gels after 24 h, 48 h, and 7 days, both in the presence and absence of a fibrinolytic inhibitor (n = 6).

basis rather than a ''per cell'' basis, interesting observations can still be made by comparing cells in constructs at each time point in the presence or absence of aprotinin. The presence of aprotinin appears to reduce levels of MSC metabolic activity; increased construct remodelling is observed in the presence of aprotinin (Fig. 4).

MSC Phenotype

In Fig. 5, FACS analysis is illustrated by plotting the number of events against the intensity of the relevant fluorescent marker on a log scale. From this data it is deduced that the cell population studied is CD49^{+}, CD73^{+}, CD105^{+}, and CD 34^{-} and CD45^{-}, showing positive staining for MSC-associated markers and negative staining for HSC markers, respectively, in all post-fibrin cultures. Cells were not available in large enough numbers to perform differentiation assays post-fibrin culture; however, FACS analysis performed on constructs grown in the presence and absence of aprotinin compare favorably with cells cultured under standard culture conditions.

Histological Staining

MSC-seeded constructs were histologically stained using the Masson Trichrome technique to show evidence of collagen formation (Fig. 6). The hemispherical constructs at 7 days (Figs. 6a, 6c) showed almost complete compositional homogeneity, suggesting that limited remodeling of the fibrin has taken place at this stage. While the cells may be active in the remodeling process, they are yet to start synthesizing proteins to produce their own extracellular matrix. However, after 21 days of culture (Figs. 6b, 6d), constructs showed significant remodeling with a larger proportion of collagen visible.

Comparison of SMC-Associated Gene Expression in 2D and 3D Cultures

MSC like other cells are subjected to vastly different mechanical constraints in 2D and 3D culture. MSC seeded on TCP (2D) and MSC embedded in fibrin gels (3D) were cultured for 7 days. Figure 7 compares the expression of SMC-associated genes in MSC seeded on TCP and in fibrin gels at this time point. The expression of α-SMA and calponin decreased 70-fold and 30-fold, respectively, in MSC in the fibrin gels compared to TCP controls.

Changes in SMC-Associated Gene Expression in Response to Mechanical Stimulation

SMC-associated gene expression was compared between MSC seeded on TCP, in static fibrin gel and dynamic fibrin gel cultures. As shown in Fig. 7, dynamic culture results in a 4-fold increase in expression of α-SMA and a 2-fold increase in expression of

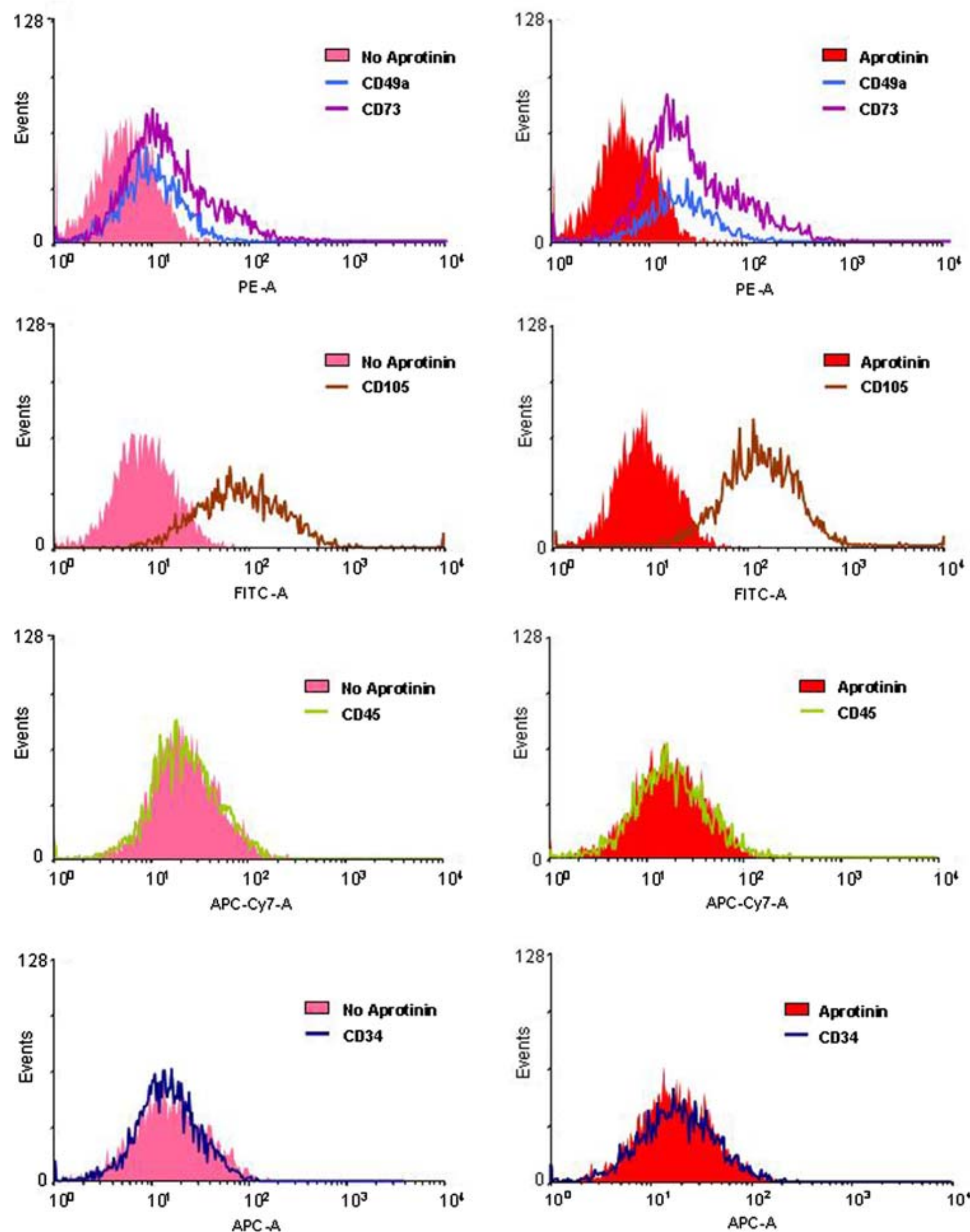

FIGURE 5. Comparison of MSC-associated markers in cells taken from fibrin outgrowth cultures in the presence and absence of aprotinin, a fibrinolytic inhibitor. Flow cytometry results where the presence of MSC-associated markers is analyzed. Expression levels of markers are compared to the non-specific binding of control cells (shown in pink where aprotinin was not present and red where aprotinin was present in culture) in their respective channels: CD49a, CD73 in PE; CD105 in FITC; CD45 in APC-Cy7, and CD 34 in APC (n = 3).

calponin. These expression levels are much lower than that observed for TCP.

DISCUSSION

The differentiation of MSC toward a SMC phenotype is likely to be dependent on a number of key factors including cell phenotype, cell density, scaffold selection, biochemical signaling, and biomechanical stimuli. While it can be difficult to study these often-interdependent factors in isolation, simplistic 2D and 3D tissue-engineered constructs can offer suitable environments for investigating their effects on differentiation.

Here, MSC were seen to remain viable and metabolically active in the 3D environment. Using real-time qPCR, the effects of the 2D and 3D fibrin gel environment on two well-established SMC markers were

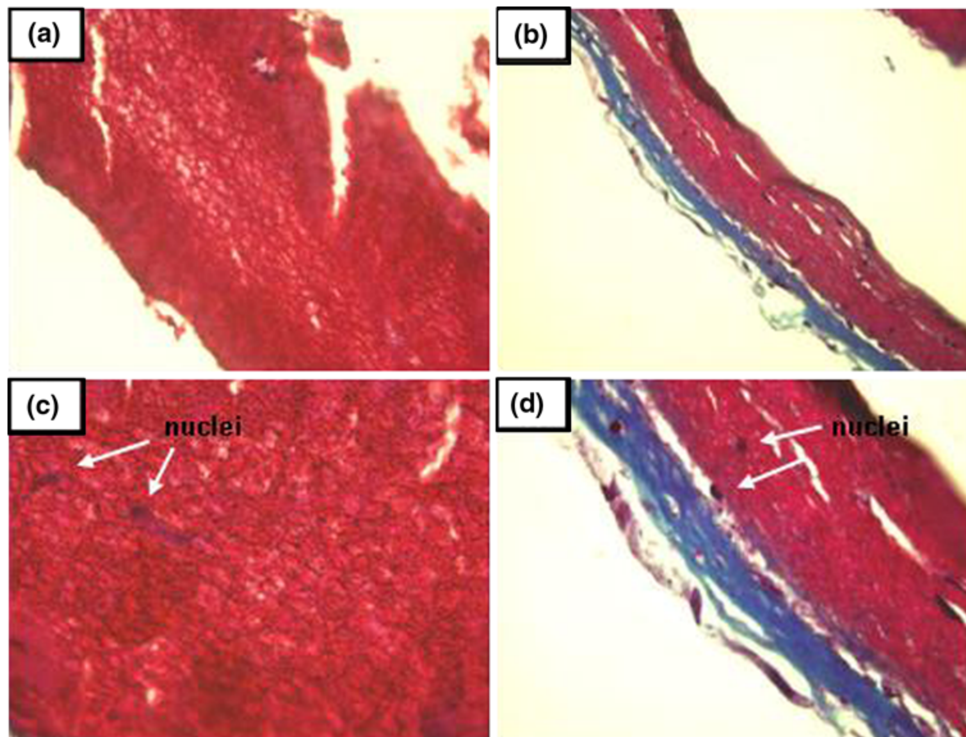

FIGURE 6. Histological staining of fibrin constructs. Histological sections of hemispherical constructs after 7 days in culture (a, c) and after 21 days in culture (b, d) at low (40×; a, b) and high (100×; c, d) magnifications. Sections were stained by Masson's Trichrome technique. Nuclei are show in black, fibrin in red, and collagen in blue. Initial MSC seeding density was 500,000 cells/mL.

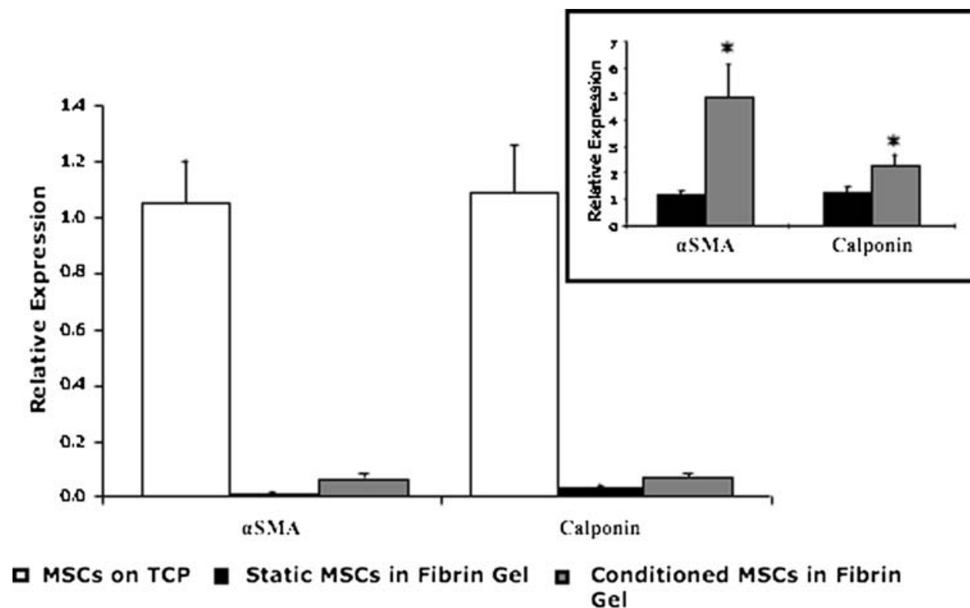

FIGURE 7. SMC-associated gene expression measured using qRT-PCR. Changes in gene expression of SMC-associated α-SMA and calponin after 7 days of culture on TCP, culture in static fibrin gels and in fibrin gels which are subjected to physiological levels of radial distention ($n = 4$). Dynamically conditioned vessels are subject to 24 h stimulation after 7 days of static culture ($n = 3$, $*p < 0.05$).

investigated. A significant decrease in levels of α-SMA and calponin was observed when the MSC were cultured in the 3D fibrin gel environment, which complements the findings of Park *et al.* in a similar study.[29] Tuan *et al.* have reported that fibroblasts, seeded at similar densities onto TCP and in fibrin gels,

maintain similar cell numbers after 9 days in culture.[38] Therefore, this phenomenon is unlikely to be due to drastically different levels of cell–cell interactions. It is apparent that cells find it easier to produce focal adhesions in 2D structures[8]; thus, by increasing the presence of integrin binding sites, the actin signaling pathway is stimulated.[4,10] The difference in substrate stiffness is likely to play a role in actin fiber concentration. Cell focal adhesions have been shown to be regulated by substrate flexibility.[30] These changes in cell structure depending on the local substrate and strain fields suggest that mechanical stimulation in of cells in 2D is not always particularly relevant to what occurs *in vivo*.[8] It is possible that cells that appear to differentiate toward a SMC phenotype on standard tissue culture plastic may lose this expression when they are transferred to a more elastic 3D environment, thereby illustrating the need for more physiologically relevant 3D vascular construct culture models.

Macroscopically, vessel walls are said to experience tensile hoop strain ranging from 2 to 18%, depending on the location within the vasculature.[32] The level of mechanical strain experienced by the cell in complex strain fields is difficult to estimate by experimental methods.[36] At present, computational models are being used to offer insights into the forces transmitted to individual cells embedded in mechanically stimulated scaffolds. It is hoped that by combining the efforts of predictive computational methods, and experimental models such as this, a greater understanding of the influence of mechanical strain on MSC differentiation will be obtained. In this study, when the gene expression levels of cells from static and dynamic cultures were compared experimentally, it was found that both α-SMA and calponin expression increased after 24 h exposure to physiological levels of tensile hoop strain. However, the resultant increase in SMC-associated gene expression was still significantly lower than MSC cultured on TCP. This corresponds with Park *et al*. where similar upregulations in α-SMA and SM22α (both ~2-fold) were observed after 24 h on uniaxial stretch.[28] They observed a subsequent drop in levels after a further 24 h of conditioning. It is hypothesized that this downregulation was in response to cell realignment in the direction of minimal strain, perpendicular to the direction of uniaxial stretch. However, this does not occur *in vivo*, as cells align themselves along collagen fibers in the same direction as principal strain. Nieponice *et al*. has illustrated this qualitatively, where MSC were found to align parallel to the direction of principal strain in 3D constructs subjected to uniaxial stretch for 6 days.[26] While this form of mechanical stimulation is only representative of pure radial THS, it provides a method in which 3D constructs can be evaluated more simplistically. The gene expression results presented here complement the immunofluorescence results shown by Nieponice *et al*.[26]

In summary, fibrin gels offer a suitable environment to observe the differences in MSC differentiation toward a SMC phenotype in 2D and 3D environments. In this study, it was revealed that at a chosen cell seeding density, MSC viability, and phenotype were maintained. However, there was a notable difference in the gene expression of two well-established SMC markers in 2D, 3D static, and 3D dynamic environments.

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