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# Stiffness of Extracellular Matrix Components Modulates the Phenotype of Human Smooth Muscle Cells in Vitro and Allows for the Control of Properties of Engineered Tissues

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**Abstract**

Smooth muscle cells (SMCs) play a significant role in the pathogenesis of atherosclerosis. 2D cultures elucidated valuable information about the interaction between SMCs and extracellular matrix (ECM) components. However, 3D constructs better represent the native vascular environment. Furthermore, a limited number of studies addressed the effect of ECM stiffness on SMCs phenotype. We investigated the effect of stiffness of different ECM substrates by modulating their concentrations, including the effect on morphology, proliferation, expression of the contractile protein α-smooth muscle actin (α-SMA) and deposition of collagen type I (Col I) and collagen type III (Col III) proteins. At low concentrations of Col I gels and Col I gels supplemented with 10% fibronectin (Fn), SMCs exhibited non-elongated, 'hill-and-valley' shape and large mean cellular area, indicating a hypertrophic morphology, characteristic of the synthetic phenotype. However, with increasing concentration, mean cellular area and proliferation relative to cells cultured in 2D dropped. Whole protein secretion into the culture media and deposition of Col I and Col III generally decreased with increasing stiffness. Moreover, percentage of α-SMA+ SMCs decreased with increasing gel concentration, pointing to a shift towards the synthetic phenotype. Supplementing Col I with 10% Laminin (Ln) maintained higher cellular area and aspect ratio at all gel concentrations and did not change α-SMA expression significantly, compared to Col I alone or Col I + Fn. Overall, these results demonstrate that ECM components and stiffness could provide the tools to modulate the phenotype and function of SMCs in vitro, which allows for the control of properties of engineered tissues.



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*Keywords:* Smooth muscle cells; Collagen type I, Extracellular Matrix, Stiffness

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## 1. Introduction

Atherosclerosis is a major cause of cardiovascular diseases, which are leading morbidities worldwide [1]. The disease involves a highly sophisticated cascade of events that are orchestrated by complex interactions between dysfunctional endothelium, smooth muscle cells (SMCs) and inflammatory cells such as monocytes [2]. Under homeostatic conditions, SMCs reside in the medial layer of blood vessels and exhibit a contractile phenotype that help in regulating blood pressure and flow through vasoconstriction and vasodilation [3]. SMCs also play a significant role in the pathogenesis of atherosclerosis. They possess remarkable plasticity, which can be observed during phenotypic switching from the contractile phenotype to a synthetic or non-contractile phenotype, in response to atherogenic stimuli such as lipids [2]. Synthetic SMCs possess enhanced migratory and proliferative capacity, which allows for their transmigration from the media towards the intima where they contribute to plaque formation[4].

The phenotype of SMCs is modulated by environmental cues including surrounding extracellular matrix (ECM)[5]. The effects of ECM components such as collagen, laminin (Ln) and fibronectin (Fn) on the phenotype of SMCs have been investigated by some research groups. Fn, a glycoprotein in blood plasma and a primary component of the ECM, has been reported to promote a synthetic phenotype of SMCs [6-8]. In atherosclerotic lesions, Fn translocates, down the concentration gradient, from blood plasma across the permeable, compromised endothelium towards the intima. Collagen type I (Col I) was shown to promote the synthetic phenotype [9, 10]. Atherosclerotic lesions are characterized by high levels of fibronectin and collagen type I [2, 11]. Conversely, Ln, a basal membrane glycoprotein that is abundant in healthy blood vessels, was shown to retain cells in their contractile state [6, 7, 12]. Seemingly, previous studies that investigated the influence of Fn and Ln on SMCs phenotype are very limited and were conducted in 2D where SMCs were seeded on top of ECM component rather than in 3D [6-8].

Such setups do not provide models that closely represent the cellular microenvironment. This is due to the remarkable differences in cell physiological behavior when cultured in 2D, as compared to cells cultured in a more representative microenvironment [13, 14], such as being encapsulated within an assortment of available natural ECMs. Different natural substrates such as Col I, fibrin and gelatin of varying concentrations, have been used to recreate the 3D atherosclerotic microenvironment [15-22]. However, such studies did not consider the effect of stiffness on SMCs phenotype. Alternatively, some studies have investigated the effect of stiffness of biosynthetic substrates such as polyacrylamide and polyethelyneglycol on the phenotype of SMCs [23, 24]. Cells sense the mechanical properties of the ECM through integrin, which anchor the cytoskeleton of cells to surrounding ECM. Therefore, our hypothesis is that changes in stiffness of ECM alter SMCs behavior [24]. Here, we aim to examine the influence of stiffness of Col type I, supplemented with Fn or Ln in 3D, by modulating its concentration, on the phenotype of human SMCs in vitro.

## 2. Methods

### *2.1. Cell culturing*

Human smooth muscle cells were purchased from ATCC (PCS100012) cryopreserved at passage 2. Further culturing was carried out up to passage 7, which was used for all experiments in this study. Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM-F12) supplemented with 5% fetal bovine serum (FBS), 1% penicillin-streptomycin antibiotic, 50µg/ml ascorbic acid, 10mM L-glutamine and one vial of endothelial cell growth supplement reconstituted in 1ml phosphate buffer saline (PBS), was used to culture cells.

### *2.2. Fabrication of cell-embedded 3D gel constructs*

SMCs were homogeneously suspended at a density of 25,000-50,000 cells/ml in a cold solution of Col I from bovine, supplemented with 9 % 10X PBS, 2.3% 1M NaOH, 1.5% HEPES buffer, 10 % full media, 2.53 – 60.61 % stock Col I (9.9 mg/ml) and 74.67 -16.59 % deionized water. Solutions were kept cool during fabrication to prevent

quick gelation of Col I before actual seeding. The gels were allowed to polymerize at 37°C (15 – 20 min). Gel constructs were supplemented with 10% Ln (0.5 mg/ml) or 10% Fn (0.5 mg/ml). Upon gelation, media was added to the constructs to maintain necessary hydration. Col I was diluted accordingly to make final concentrations of 0.25, 2.00 and 6.00 mg/ml. Media was changed for all conditions every 2 days. Experiments were stopped when cells seeded on tissue culture plates at a density of 4000 cells/cm2 reached approximately 80% confluence. To isolate cells from gel constructs, gels were digested using 0.1% collagenase type I (Worthington Biochemical Corp.) for 2 hours at 37°C with frequent gentle shaking to facilitate dissolution, transferred to conical tubes and centrifuged at 1000rpm for 5 minutes.

### *2.3. Immunofluorescence Labeling*

For immunofluorescence labeling, cells were fixed using 2% PFA for 30 minutes followed by permeabilization using 0.2% triton X-100 in PBS and blocked with 10% FBS for 30 minutes at room temperature (RT) to prevent non-specific binding. Subsequently, samples were incubated with primary antibody (anti-α-smooth muscle actin (α-SMA, ab5694) 1:200 in 0.2% triton X-100 in PBS and 1% FBS (solution X), or anti-collagen I (ab34710), or anti-collagen III (ab7778) 1:250 in solution X for 2 hours at RT. Samples were then washed with PBS, followed by incubation with Alexa Fluor (AF) 568-conjugated secondary antibody (1:200 or 1:250 in solution X, A10042, Life Technologies) for 1 hour at RT. Nuclei were counterstained with DAPI and cytoskeleton was stained with AF 488-cojugated Phalloidin (A12379, Life Technologies).

### *2.4. Microscopy*

Images for quantification were obtained using an epifluorescence microscope (Axiovert Z10, Carl Zeiss Microscopy GmbH, Göttingen, Germany). 3D volumetric images were taken using an upright laser scanning confocal microscope (Olympus FV1000). 488nm laser was used to detect cytoskeleton filaments and 405 nm excitation to detect DAPI. Emission wavelengths were set to 500-520 nm for AF 488 channel and 420-470 for DAPI channel.

### *2.5. Flow Cytometry and Bradford Assay*

Proliferation of SMCs was assessed by counting isolated cells from gels by a flow cytometer (BD Accuri C6, corporation, USA). Cell count for all conditions was normalized by cell count in 2D. Although high proliferation capacity is a characteristic of synthetic SMCs, it does not exclusively dictate the phenotype. Therefore, we quantitatively assessed the morphology of cells. Morphology of SMCs within gels was measured by taking bright field images for all conditions at multiple imaging planes. Individual cells were isolated manually from acquired images and analyzed for area and aspect ratio, the latter computed through the division of the major axis by the minor axis of a bounding ellipse for each cell, using FIJI software.

Whole protein analysis was done using Bradford assay, where culture media was replaced by serum-free media overnight for all conditions and for gels void of cells, which served as a control. This allowed us to eliminate the effect of any proteins that could leach out from the gel into the media.

Expression of the contractile protein α-SMA was assessed by isolating the cells from gels as described above and performing immunostaining. Live/dead assay was conducted on live SMCs and dead SMCs, killed with 0.1% triton X-100, seeded directly on tissue culture plate, and negative control is human umbilical endothelial cells (HUVECs). This was used for gating in order to exclude dead cells and debris from the analysis. Cells grown on tissue culture plate were detached using 0.05% Trypsin-EDTA solution for 5 minutes at 37°C. In addition, expression of Col I and Col III were assessed in the same way by incubating the cells with the appropriate antibodies. All samples were subsequently resuspended in PBS and run on a flow cytometer. For detection of live cells, green fluorescence emission for c`alcein was measured using 530 nm bandpass filter and ethidium homodimer-1 expressed on dead

cells was measured using a 585 nm bandpass filter. Detection of α-SMA, Col I and Col III was done using 488 nm laser and detection filter with 585/40 nm. The level of expression of α-SMA, Col I and Col III was further verified by measuring the intensity of fluorescence images of isolated cells seeded on glass slides and incubated at 37°C overnight. Mean intensity values for each marker were obtained using Fiji.

### *2.6. Statistical Analysis*

All analyses were performed on GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA). Parametric one-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparisons test was performed to compare between the means of conditions and a level of significance of α=0.05 was employed. $p$-values < 0.05 were considered significant. All data are reported as mean ± standard error of the mean (SEM).

## 3. Results

At the cessation of experiment (7 days), SMCs were extracted from gels by collagenase digestion and isolated for counting using flow cytometry. Cell number decreased by 44.9% then by 72.4% in Col I gels, as their stiffness were increased via collagen concentration increment from 0.25 mg/ml to 2.00 mg/ml and subsequently 2.00 mg/ml to 6.00 mg/ml. Following the same comparison, but between Col I gels supplemented with Fn (Col I + Fn), the cell number decreased by 27.1% and 24.6% and with addition of Ln (Col I + Ln) in the gels the decrease was 66.1% and 80.2%, respectively (Fig. 1A). None of the comparisons were statically significant (Fig. 1A). Protein secretion by SMCs encapsulated within Col I gels were analyzed using Bradford Assay. Comparing the softest and stiffest gels, 0.25 mg/ml vs. 6.00 mg/ml, whole protein levels within the media decreased by 71.4%, 22.9%, and 90.4% in Col I, alone, Col I + Ln, and Col I + Fn, respectively, (Fig. 1B).

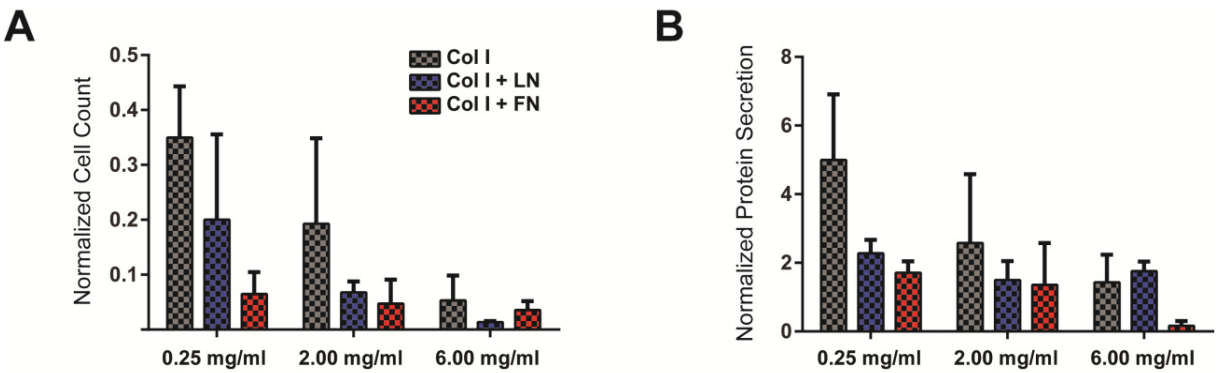


Fig. 1. Smooth muscle cell phenotype assessment through cellular proliferation and protein secretion. Cell proliferation in different 3D gel conditions relative to cells grown in 2D on tissue culture plates (Panel A). 7 days post seeding, cells were isolated from gels and counted using flow cytometry (n = 2, ± SEM). Whole protein secretion analysis relative to cells grown in 2D on tissue culture plates. 7 days post seeding culture media was replaced by serum-free media overnight and collected the next day for Bradford analysis of whole protein (Panel B, n = 2, ± SEM).

Mean cellular area of SMCs decreased when embedded in increasing gel stiffness by 8.2% (0.25 mg/ml *vs*. 2.00 mg/ml) and 53.0% (2.00 mg/ml *vs*. 6.00 mg/ml), $p < 0.05$ (Fig. 2A). Supplementing Col I with Fn resulted in reduced mean cellular area with increased Col I stiffness also, by 37.8% (0.25 mg/ml *vs*. 2.00 mg/ml) and 31.6% (2.00 mg/ml vs. 6.00 mg/ml), $p < 0.0001$. With the addition of Ln within Col I, mean cellular area increased by 12.1% (0.25 mg/ml *vs*. 2.00 mg/ml) and 3.9% (2.00 mg/ml vs. 6.00 mg/ml). Ln maintained a higher mean cellular area even in 6.00 mg/ml gels compared to Col I alone or Col I + Fn with percentage difference 57.2% and 62.7%, respectively, $p < 0.05$. Mean aspect ratio of cells cultured within Col I alone and Col I + Fn decreased with

increasing gel concentration, while adding Ln increased mean aspect ratio from 0.25 mg/ml to 6.00 mg/ml by 67.1%, $p < 0.01$. SMCs in Col I + Ln vs. SMCs in Col I + Fn maintained a higher mean aspect ratio, even at 6.00 mg/ml, 195.1% and 89.9%, respectively ($p < 0.0001$) (Fig. 2A). Phalloidin stains qualitatively shows that area and aspect ratio of cells decreased with increasing stiffness for Col I (Fig. 2B first row), and Col I + Fn (Fig. 2B second row), while cells embedded in gels with Ln maintained elongated morphology reflected by high aspect ratio values (Fig. 2C third row).

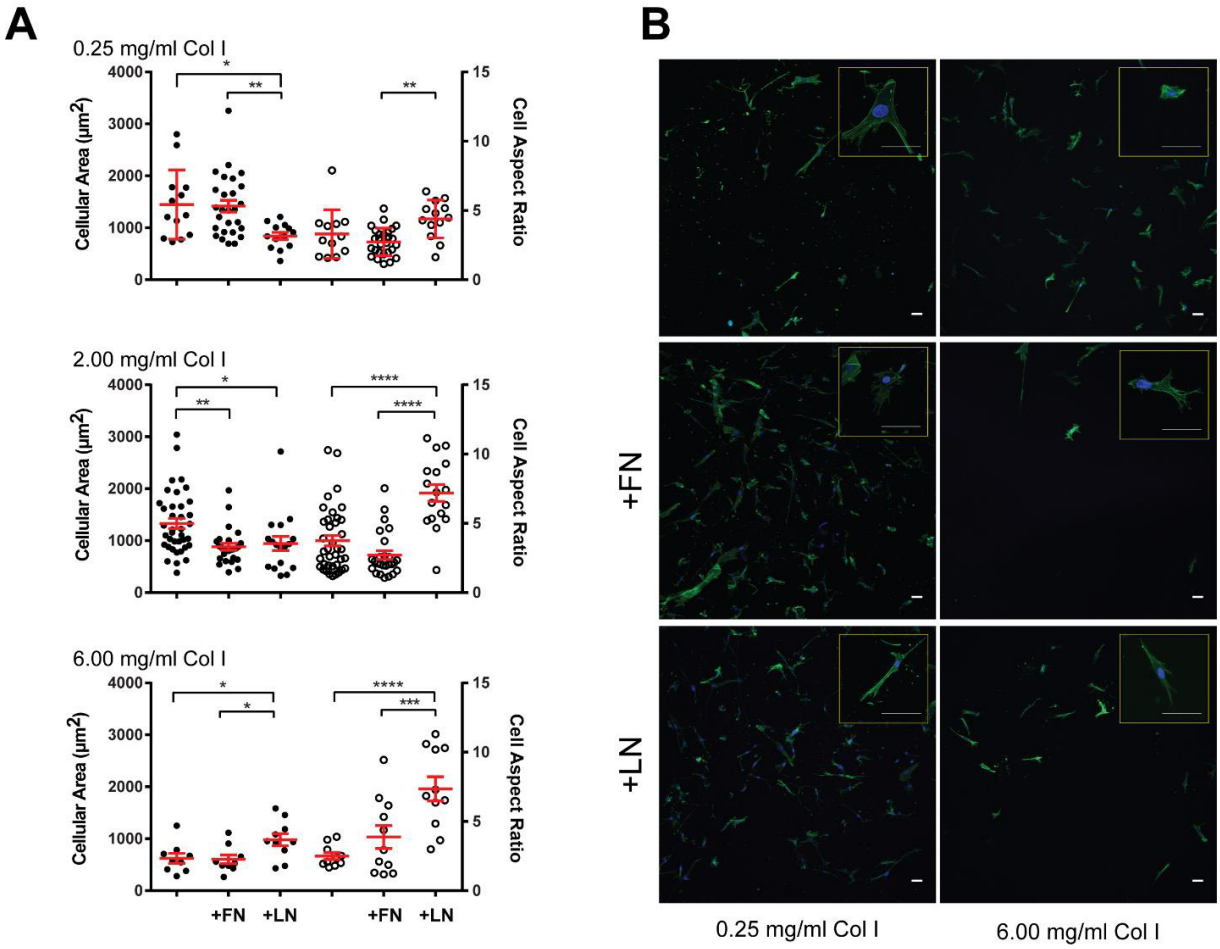


Fig. 2. Smooth muscle cell phenotype assessment through cellular morphology. Bright field images were obtained at fixed exposure time and analysed for cellular area and aspect ratio using Fiji. ($n \geq 9, \pm$ SEM). Mean cellular area (left Y-axis) and mean aspect ratio (right Y-axis) of cells embedded in different concentrations of gels (Panel A). Superimposed Z stacks of cytoskeleton in 0.25 mg/ml and 6.00 mg/ml of the three examined gels. Cells were immunostained using 488 AF-conjugated Phalloidin and imaged using confocal microscopy. Inset shows representative single cell (Panel B). (Scale bar: 50 μm)

Flow cytometry results revealed a decline in percentage of α-SMA+ cells for SMCs embedded in Col I and Col I + Fn with increasing concentration. SMCs in Col I + Ln maintained a population of α-SMA+. Percentage of Col I+ and Col III+ SMCs increased with increasing stiffness of Col I gels, while 2.00 mg/ml and 6.00 mg/ml Col I + Ln reduced the number of Col I+ SMCs (Fig. 3 first row).

Fluorescence intensity analysis (Fig.3 second row), showed that cells grown within Col I of increasing stiffness (0.25 mg/ml *vs*. 6.00 mg/ml) exhibited a 29.4% reduction in α-SMA expression. Col I + Ln generally caused an increased expression of α-SMA, ~1.3 and ~1.7 folds higher compared to Col I and Col I + Fn respectively at 6.00

mg/ml. Col I + Fn has SMCs that expressed the lowest levels of α-SMA in all gel concentrations, exhibiting significant differences compared to gels with Ln at all concentrations ($p < 0.05$). Moreover, 2.00 mg/ml and 6.00 mg/ml Col I + Fn, resulted in significantly lower expressions of α-SMA when compared to control cells seeded in 2D ($p < 0.001$).

Deposited Col I by SMCs grown in Col I gels decreased with gel stiffness by 31.6% (0.25 mg/ml *vs*. 2.00 mg/ml), then increased by 41.5% (2.00 mg/ml *vs*. 6.00 mg/ml) ($p < 0.05$). Adding Ln to Col I caused embedded SMCs to deposit the lowest amount of Col I at all concentrations compared to Col I alone Col I + Fn. Interestingly, gels with Ln did not exhibit significantly different expression of Col I when compared to cells in 2D, even at 6.00 mg/ml. Deposited Col III by cells cultured in Col I gels increased by 53.2% from 0.25 - 2.00 mg/ml and by 10.6% from 2.00 - 6.00 mg/ml, $p < 0.05$. Expression of Col III in cells grown in Col I + Fn peaked at 2.00 mg/ml, $p < 0.0001$. Adding Ln to the gel decreased expression of Col III with increasing gel concentration, where it exhibited expression levels lower by 49.4% and 12.9% compared to Col I and Col I + Fn respectively, $p < 0.0001$.

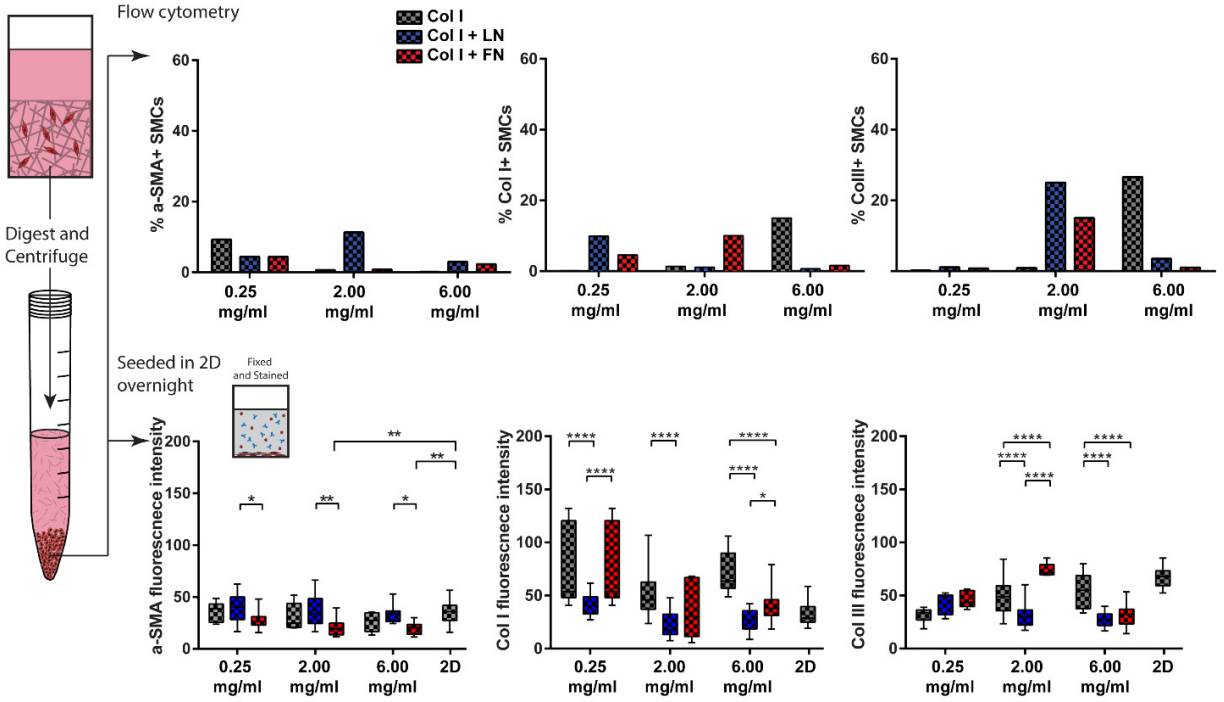


Fig. 3. Smooth muscle cell phenotype assessment through cellular protein markers. Flow cytometric analysis for percentage of α-SMA+, Col I+ and Col III+ SMCs in all gel conditions (Top row). Recovered cells from gels were immuno-labelled and run on flow cytometer. Live/Dead stains were used to exclude debris and dead cells from the analysis (n = 1). Bottom row. Fluorescence intensity levels of α-SMA, Col I and Col III, in all gel conditions and concentrations. Recovered cells from gels were seeded overnight on a multi-well glass slide and stained against α-SMA, Col I and Col III ($n \geq 18$, ± SEM) (Bottom row).

## 4. Discussion

When SMCs are cultured on 2D Col I, Fn or Ln matrices, some changes in cell phenotype have been documented including downregulation of contractile protein markers and increased proliferation rate[6, 8-10]. Studies that investigated 3D natural ECM components mostly examined Col I only, and at a constant gel concentration[22]. Here we investigated the effects of varying the concentration, thus stiffness, of 3D Col I as well as Ln and Fn, on the phenotype of SMCs.

At low concentrations of Col I alone, SMCs exhibited non-elongated, 'hill-and-valley' shape and large mean cellular area, indicating a hypertrophic morphology, characteristic of the synthetic phenotype. However, with increasing gel concentration, mean cellular area decreased, which can be due to the higher cross-links present in the collagen matrix, possibly preventing cells from normal spreading. Another effect of increasing the stiffness of Col I gels is the reduced proliferation of embedded SMCs relative to cells cultured in 2D. This also might be caused by the higher cross-links present in the matrix, which in turn might hinder cell-cell contact, thus reducing proliferation. Whole protein secretion into the culture media decreased with increasing stiffness, and this was contrary to levels of deposition of Col I and Col III proteins. Whole protein includes collagens, proteoglycans and other ECM components [2], which some might have increased but the majority decreased, resulting in an overall reduction of protein secretion. In stiffer Col I gels, cells are constrained in space which might be signaling them to act as non-elongated (synthetic) phenotype, depositing more Col I and Col III. Moreover, SMCs grown in Col I gels exhibited lower expression of α-SMA with increasing gel concentration, as demonstrated by fluorescence intensity, pointing to a shift towards the synthetic phenotype.

Similar to Col I, SMCs cultured in Col I + Fn showed a hypertrophic morphology at low stiffness gels. However, with increasing gel stiffness, mean cellular area, aspect ratio and proliferation decreased. Fibronectin possesses binding sites for collagen [25], so the more Col I is present in the matrix, the more cross-links will be formed, which might prevent normal cell spreading and proliferation within the matrix. Whole protein secretion and deposition of Col I and Col III generally decreased with increasing stiffness. This can be due to low number of repeats in flow cytometry analysis, which does not sufficiently reflect % of SMCs that are positive for a particular protein. At all concentrations, cells grown in Col I + Fn gels demonstrated higher Col I and Col III deposition and lower a-SMA levels compared to Col I + Ln gels, indicating that Fn can promote the synthetic phenotype.

In contrast to Col I alone or Col I + Fn gels, SMCs grown in Col I + Ln exhibited increased cellular area and aspect ratio with increasing stiffness. This indicates that the basal lamina protein supports spreading of cells and maintains an elongated morphology regardless of gel concentration. Changing the stiffness of Col I + Ln gels did not cause significant changes in α-SMA levels. In contrast, whole protein secretion, deposition of Col I and Col III and proliferation relative to cells grown in 2D decreased with increasing stiffness. Moreover, supplementing the gel with Ln increased percentage of α-SMA+ SMCs at any given gel concentration, compared to Col I alone or Col I + Fn gels, which could mean that Ln promotes a shift towards the contractile phenotype even in stiff gels.

In conclusion, analytical methods can be further optimized by increasing number of samples used in flow cytometry. Also, increasing initial cell density is crucial to obtain more results. However, this was a limitation in our study because increasing cell density above 50K/ml caused the gel to shrink due to cells contraction. This can be overcome by using higher passages of SMCs as they adopt a synthetic non-contractile phenotype. To increase sample size, multi-color immunostaining should be utilized. In addition, a Golgi Apparatus blocker (Brefeldin A) can be used to ensure retaining of intracellular secretions. Overall, this study provides insights into potential effects of stiffness of ECM components in 3D on the phenotype of SMCs. Results presented here demonstrate that ECM components and stiffness could provide the tools to modulate the phenotype and function of SMCs *in vitro*, which allows for the control of properties of engineered tissues. This is particularly important in modelling vascular diseases such as atherosclerosis, which requires careful selection of SMCs phenotype to closely mimic a lesion.

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