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Authors
Wong, Lian
Kumar, Ashwath
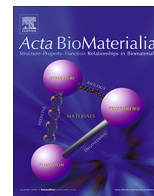Gabela-Zuniga, Basia
et al.

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Substrate stiffness directs diverging vascular fates

Lian Wong [a], Ashwath Kumar [b], Basia Gabela-Zuniga [c], Je Chua [c], Gagandip Singh [d],
Cassandra L. Happe [e], Adam J. Engler [e], Yuhong Fan [b,f], Kara E. McCloskey [a,g,∗]

[a] Graduate Program in Biological Engineering and Small-scale Technologies, University of California, Merced, United States
[b] School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA 30332, United States
[c] Bioengineering Department, University of California, Merced, United States
[d] Biological Sciences Department, University of California, Merced, United States
[e] Bioengineering Department, University of California, San Diego, United States
[f] Parker H. Petit Institute for Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, GA 30332, United States
[g] Materials Science and Engineering Department, University of California, Merced, United States

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ABSTRACT

Embryonic stem cells (ESC) are excellent cell culture systems for elucidating developmental signals that may be part of the stem cell niche. Although stem cells are traditionally induced using predominately soluble signals, the mechanical environment of the niche can also play a role in directing cells towards differential cell lineages. Interested in diverging vascular fates, we set out to examine to what extent mechanical signaling played a role in endothelial cell and/or smooth muscle fate. Using chemically-defined staged vascular differentiation methods, vascular progenitor cells (VPC) fate was examined on single stiffness polyacrylamide hydrogels of 10 kPa, 40 kPa and >0.1 GPa. Emergence of vascular cell populations aligned with corresponding hydrogel stiffness: EC-lineages favoring the softer material and SMC lineages favoring the stiffest material. Statistical significance was observed on both cell lines on almost all days. Transcriptome analysis indicated that the populations on the varying stiffness emerge in distinct categories. Lastly, blocking studies show that αvβ1, and not αvβ6, activation mediates stiffness-directed vascular differentiation. Overall, these studies indicate that softer materials direct VPCs into a more EC-like fate compared to stiffer materials.

Statement of Significance

Although stem cells are traditionally induced using predominately soluble signals, the mechanical environment of the niche also plays a role in directing cell fate. Several studies have examined the stiffness-induced cell fate from mesenchymal stem cells (MSCs) and undifferentiated embryonic stem cells (ESCs). This is the first study that rigorously examines the role of matrix stiffness in diverging vascular fates from a purified population of vascular progenitor cells (VPCs). We show that the emergence of endothelial cell (EC) versus smooth muscle cell (SMC) populations corresponds with hydrogel stiffness: EC-lineages favoring the softness material and SMC lineages favoring the stiffest material, and that αvβ1 activation mediates this stiffness-directed vascular differentiation.

1. Introduction

In vivo, cells are closely integrated with their cellular microenvironment - a complex system composed of soluble signals, cell-to-cell interactions, and extracellular matrix (ECM). The cells, soluble signals and ECM proteins combine to provide mechanical support, as well as signals that influence cell behavior and even cell fate. Although it is well-known that matrix elasticity influences 2D cell migration [1], matrix elasticity has recently been shown to provide a distinct signal that can exert effects on the lineage specification of progenitor cells [2]. Subsequently, numerous reports suggest that differentiation of epidermal stem cells [3], osteoblasts [4], and neuronal cells [2,5] can be regulated by tuning substrate stiffness to *in vivo* like conditions. Specifically, mesenchymal stem cells (MSCs) differentiate towards adipocytes on less stiff matrices, while bone differentiation occurs on stiff matrices [2,6].

Although not conducted using embryonic stem cells (ESCs), or vascular progenitor cells (VPCs), studies in MSCs support the hypothesis that stiffness can direct vascular fate. For example, comparing stiffer (8–15 kPa) and softer (2–5 kPa) nanofiber matrices, MSCs exhibited a shift from smooth muscle actin-expressing cells to Flk-1-expressing cells [7]. However, Flk-1 expression is a known VPC marker that can further differentiate into both ECs and SMCs [8], so it is unclear if an EC-specific lineage could be obtained. Stiffness-regulated smooth muscle fate has also been suggested in MSCs where, stiffer (15 kPa) substrates induce greater expression of SMC marker expression and softer substrates (1 kPa) induce chondrogenic and adipogenic marker expression [9]. However, some suggest that softer nanofibers (2 kPa) direct MSCs towards an EC fate, and it is unclear to what extent the nanofibrous architecture, relative to stiffness, directed the cell fate specification [10].

Despite the lack of evidence directly showing that low stiffness materials can direct EC fate from VPCs, a number of studies indicate that softer materials cause mature ECs to internalize vascular endothelial growth factor (VEGF) [11], upregulate the production of VEGF [12] and GATA2 [13]. In this turn, substituting for Rho/ROCK signaling [12]. Based on these studies, we speculate that softer substrates will direct more VPCs towards EC lineages compared to vascular SMC lineages.

Complicating this presumption is the definition of what is "soft" and "stiff" relative to the vascular system. For example, *ex vivo* blood vessel stiffness varies significantly between healthy and diseased endothelium and between arterial and venous ECs. Porcine aorta, mouse arteries, and bovine carotid artery stiffnesses have been reported at 5–8 kPa [14], 5 kPa [15], and 2.5 kPa [16], respectively. Conversely, atherosclerotic ApoE-null mice have arteries with increased stiffness, e.g. 28 kPa [15]. These broad ranges suggest that atherosclerosis conditions can vary widely and that recapitulating endothelial microenvironment is an important factor in disease hallmarks. Along with the need to control stiffness of the endothelial microenvironment is the need to employ a material with a suitable range of matrix elasticity, e.g. polyethylene glycol (6–26 kPa) [17], alginate (0.1–30 kPa) [18], or PDMS (0.1–2700 kPa) [19] and appropriate surface chemistry. Polyacrylamide (PA) can mimic most physiological conditions over three orders of magnitude (0.1 kPa–200 kPa by varying its bis-acrylamide cross-linker [1]. This provides significant mechanical control which, along with its simple surface chemistry for ligand attachment and suitable optical properties, makes PA hydrogels a suitable alternative to conventional tissue cultures substrates.

With defined parameters and materials, we sought to study how stiffness affects ESC-derived VPC fate. Using a mouse ESC line that expresses a GFP reporter under Tie-2 and a RFP reporter under α-smooth muscle actin, we examined the role of stiffness in the diverging fate of Flk-1^{+} VPCs on mechanically patterned hydrogels, i.e. Zebraxis, which have strips that vary in stiffness, and single stiffness surfaces (10 and 40 kPa). Lineage commitment of Flk-1^{+} cells towards EC or SMC lineages was observed over 3–10 days. That data show that low stiffness hydrogels direct more VPCs into the endothelial lineage and that high stiffness hydrogels direct more VPCs towards the SMC fate. We also identified specific integrin signaling pathways that appear to be directing stiffness-mediated vascular differentiation.

2. Methods

2.1. Mouse embryonic stem cell culture

Murine R1-ESC (ATCC) and A3-ESC (derived and characterized in-house [20]) were maintained on 0.5% gelatin coated plates in serum-free medium containing Knockout Dulbecco's Modified Eagle Medium (KO-DMEM; Invitrogen), 15% Knockout Serum Replacer (KSR; Invitrogen), 1X Penicillin-Streptomycin (Invitrogen), 1X Non-essential Amino Acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM 2-mercaptoethanol (Calbiochem), 2000 Units/ml of leukemia inhibitory factor (LIF-ESGRO; Chemicon), and 10 ng/ml of bone morphogenetic protein-4 (BMP-4; R&D Systems). Full media changes occurred every other day and cells were passaged every four to five days.

2.2. Polyacrylamide hydrogel fabrication

Zebraxis hydrogels were made according to the methods previously published [21]. Briefly, acrylamide was polymerized on aminosilanized 25 mm or 18 mm diameter coverslips. A solution containing the cross linker N,N′ methylene-bis-acrylamide, acrylamide, 1/100 vol 10% ammonium persulfate, and 1/1000 vol of N,N,N,′,N′-Tetramethylethylenediamine was mixed. Because cell migrated to the stiffer (elevated) strip on all Zebraxes hydrogels, the methods altered to examine stiffness-regulated signaling on only single stiffness hydrogels.

For single stiffness, two different combinations of acrylamide and bis-acrylamide were used to make 10 and 40 kPa substrates [22]. 25 μL (25 mm diameter coverslip) or 20 μL (18 mm diameter coverslip) of the mixed solution was placed between the aminosilanized treated coverslip and a Rain-X treated glass slide. 50 μg/mL fibronectin was chemically crosslinked to the substrates using photo activating cross linker Sulfo-SANPAH (Pierce).

2.3. Characterizing material properties of polyacrylamide hydrogels

Indentation experiments were performed with a commercial Atomic Force Microscope (AFM; NTEGRA Vita, NT-MDT). A spherical borosilicate glass bead with a diameter of 10 μm was used (SNL-10, Bruker). Young's modulus of elasticity, E, was calculated using Nanoindentation of Soft Elastic Materials on UIUC's Nanohub web-based calculation platform, where force curves were obtained by the deflection of the AFM cantilever as it was unloading from the sample [23]. Poisson's ratio was assigned a value of 0.5 [24].

All cell culture materials were treated with 50 μg/ml fibronectin. While we did not explicitly measure the density of fibronectin in this study, we have previously reported that no composition dependent differences were found in the capture of protein on polyacrylamide hydrogels [25]. Moreover, specific surface capture concentrations have been previously reported for this stiffness-controlled system and cell attachment did not vary with substrate stiffness patterns [21]. Moreover, previous studies have shown that swelling between regions of different substrate stiffness within polyacrylamide (PA) hydrogels is minimal when at high bis-acrylamide crosslinker concentrations, e.g. 0.4% w/v as in this manuscript [21,26]. In general, high bis-acrylamide minimizes swelling, as it "locks" polymer changes in place.

2.4. Induction of mouse embryonic stem cells to VPCs

For dissociation, R1-ESC were harvested from 0.5% gelatin coated dishes using TrypLE (ThermoFisher) while A3-ESC were disassociated from the MEF layer and purified through a gravity separation prior to plating on 100 mm tissue culture treated plates (Corning) coated with 0.050 mg/mL fibronectin (Corning). Cells were then fed with our induction medium containing alpha-MEM (Cellgro), 20% knockout serum replacement (ThermoFisher), 1X penicillin–streptomycin (ThermoFisher), 1X nonessential amino acids (ThermoFisher), 2 mM L-glutamine (ThermoFisher), 0.05 mM 2-mercaptoethanol (Calbiochem), 5 ng/mL BMP-4 (Peprotech), and 30 ng/mL or 20 ng/mL of VEGF (Peprotech) for R1 and A3, respectively. Cells were cultured for 2 days (R1) or 3 days

(A3) as previously optimized for generating the greatest number of Flk-1+ cells [27]. Adherent cells were harvested using Cell Dissociation Buffer (ThermoFisher) and sorted based on expression of Flk-1 (PerCP, Biolegend) using fluorescent activated cell sorting (BD, ARIA II).

The purified Flk-1+ cells were cultured onto fibronectin-coated Zebraxis hydrogels, single stiffness PA hydrogels or on our tissue culture plastic control (Fig. 1). To control for cell density, and because cell proliferation was greater on the stiffer materials, VPCs were plated at a higher cell density, 20,000 cells/cm^2 on the softer hydrogel compared with 10,000 cells/cm^2 on the stiffer materials. The VPC outgrowths were observed for the next 10 days in maturation medium consisting of 70% alpha-MEM (Mediatech), 30% DMEM (Invitrogen), 2X Nutridoma CS (Roche), 1X penicillin–streptomycin (Invitrogen), 1X nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.05 mM 2-mercaptoethanol (Calbiochem), and supplemented with 30 ng/mL VEGF and 5 ng/mL BMP4 for R1-ESC or 10 ng/mL VEGF and 10 ng/mL bFGF for A3-ESC (as previously optimized [17]).

2.5. Immunofluorescence

ESC-R1 and ESC-A3 Flk-1+ purified cells were grown for 3–10 days and then fixed with 4% paraformaldehyde (Tousimis) for 5 min at room temperature. Cells were placed in a solution of 0.7% Triton X-100 (Fisher), 5% donkey serum (Fitzgerald), and 1% bovine serum albumin (Sigma) for another 5 min at room temperature to be permeabilized and block non-specific binding of the antibody. Primary antibodies CD31 PE (rat, BD Biosciences) and calponin-1 (mouse CNN1, Sigma) were used in a dilution of 1:200 and 1:30000, respectively in 1% bovine serum albumin. Secondary FITC anti-mouse (Abcam) was used in a dilution at 1:300. Cells were stained for 1 h at room temperature for both primary and secondary incubation. During secondary incubation, DAPI was added to stain the nucleus.

2.6. RNA-Seq

NEBNext rRNA depletion kit (NEB) was used to remove ribosomal RNA before preparation of sequencing libraries using the NEBNext Ultra II RNA library prep kit for Illumina (NEB). Sequencing was performed by Georgia Tech Molecular Evolution Core with NextSeq High Output run systems (Illumina). Raw sequence reads were examined for quality using FastQC [28]. The reads were subsequently trimmed to remove adaptors and filtered for low quality bases using Trim Galore [29,30]. Clean sequence reads were aligned to mouse genome, mm9, using STAR aligner [31]. Gene counts were called using HTSeq (5), and differentially expressed genes were identified using DESeq2 R package [32]. Gene ontology (GO) analysis was carried out using DAVID [33,34] to identify enriched biological functional groups and processes.

2.7. Inhibitor assay

Flk-1+ VPC-A3s were plated on single-stiffness surfaces for 24 h then the medium was replaced with medium supplemented with the following inhibitors: 5 µM FAK inhibitor 14 (Tocris Bioscience), 1:200 αvβ3 (Bioss), 1:200 αvβ1 (Bioss), and 10 µg/mL αvβ6 (abcam) for another 24 h. The cells were fixed and stained as previously described.

2.8. Statistical analysis

Statistical analyses were conducted with GraphPad Prism 7 software. Two-way ANOVA with Tukey's multiple comparison for the analysis of three groups was used. Differences at $P \leq 0.05$ were considered statistically significant. P values were calculated by analysis of variance for multiple pairwise comparisons. The data are reported as mean ± SEM.

3. Results

Using Zebraxis hydrogels with alternating stiffer and softer stripes, we were able to verify that Flk-1+ VPCs derived from ESC-A3 cells rapidly migrating to the stiffer stripes only before differentiating into both Tie-2+ GFP and αSMA+ RFP expressing cells (Supplemental Fig. 1). On day 12, cells were fixed and stained with PECAM-1 EC marker, SM22α SMC marker, and DAPI nuclear stain (Supplemental Fig. 2). On both the softer Zebraxis hydrogel (1 versus 10 kPa) and stiffer (10 versus 34 kPa) Zebraxis hydrogel, the cells preferentially migrated to the stiffer stripes over time on the two Zebraxis chips, suggesting that the stiffness or elevation could direct cell placement of the differentiating cells [21,35], but the role of material stiffness in cell fate could not be determined using these Zebraxis hydrogels.

Therefore, to isolate the effects of elastic modulus alone, purified Flk-1+ VPCs derived from both ESC-A3 and ESC-R1 cells were plated onto single-stiffness PA hydrogels tuned to a low stiffness 10 kPa (soft) and 40 kPa (stiff) hydrogels, as well as, a very stiff (>0.1 GPa) tissue culture plastic control [36], and stained with endothelial markers, PECAM-1, and early smooth muscle cell markers, CNN-1, after 3, 7, and 10 days (Fig. 2). Quantification of the PECAM-1+ and CNN1+ cells (Fig. 3) clearly and robustly shows that stiffness-dependence in early (day 3) VPC outgrowths for both mESC-R1 and mESC-A3 lines. Specifically, the lower stiffnesses
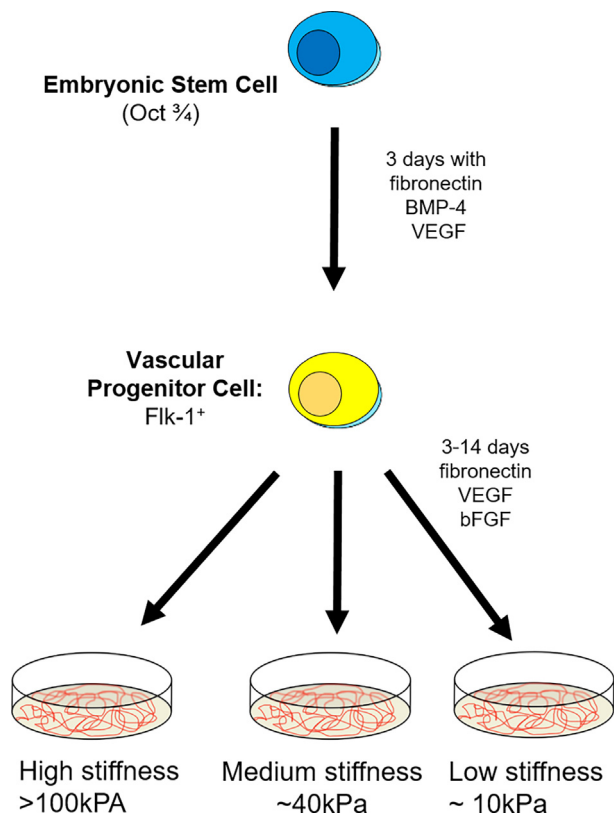

Fig. 1. Overview of vascular induction to examine the role of stiffness from vascular progenitor cells. Murine R1-ESC and A3-ESC were directed to Flk-1+ VPCs, purified, and plated on fibronectin-coated stiffness-controlled surfaces: 10 kPa and 40 kPa, and tissue culture plastic (>0.1 GPa).
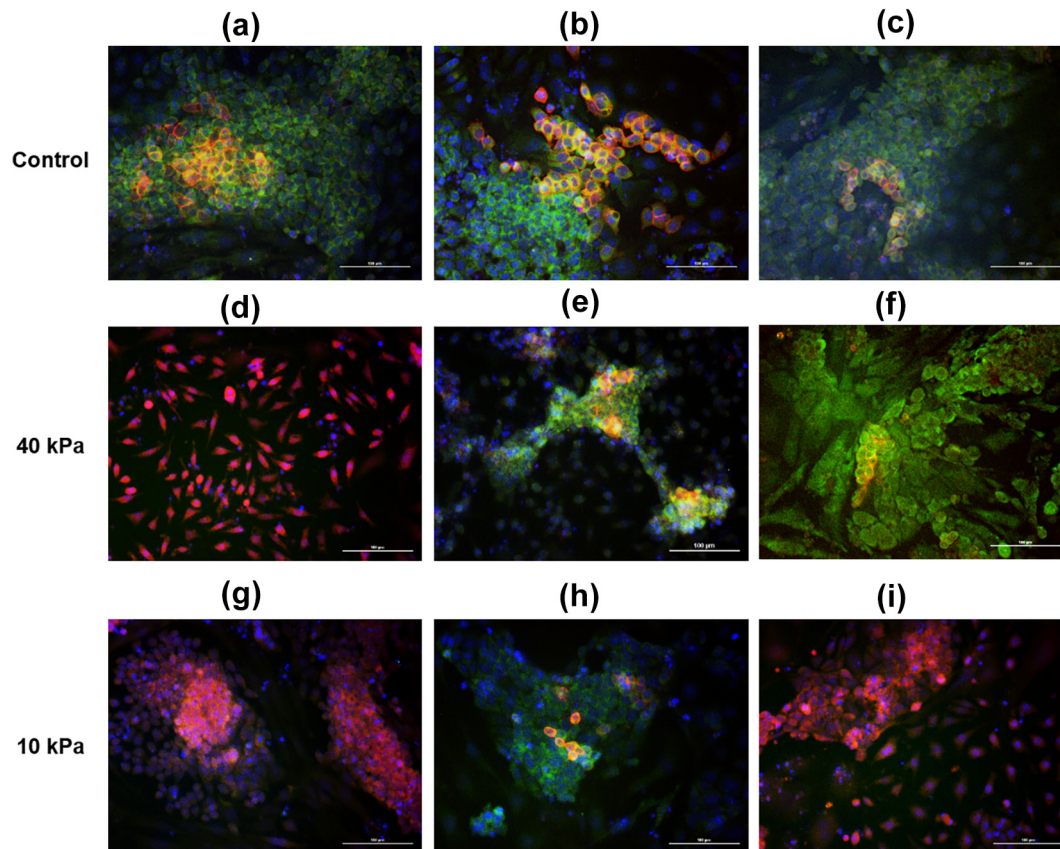
Fig. 2. Fluorescent images of VPC outgrowths on day 7. a-c) Three representative images of Flk-1+ VPC cultured on fibronectin-coated tissue culture plastic (>0.1 GPa). d-f) Three representative images of Flk-1+ VPCs cultured on fibronectin-coated PA hydrogels at 40 kPa. g-i) Three representative images of Flk-1+ cells cultured on fibronectin-coated PA hydrogels at 10 kPa. The cells were fixed and stained for PECAM-1 = red, CNN1 = green, and DAPI = blue. Scale bar = 100um. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
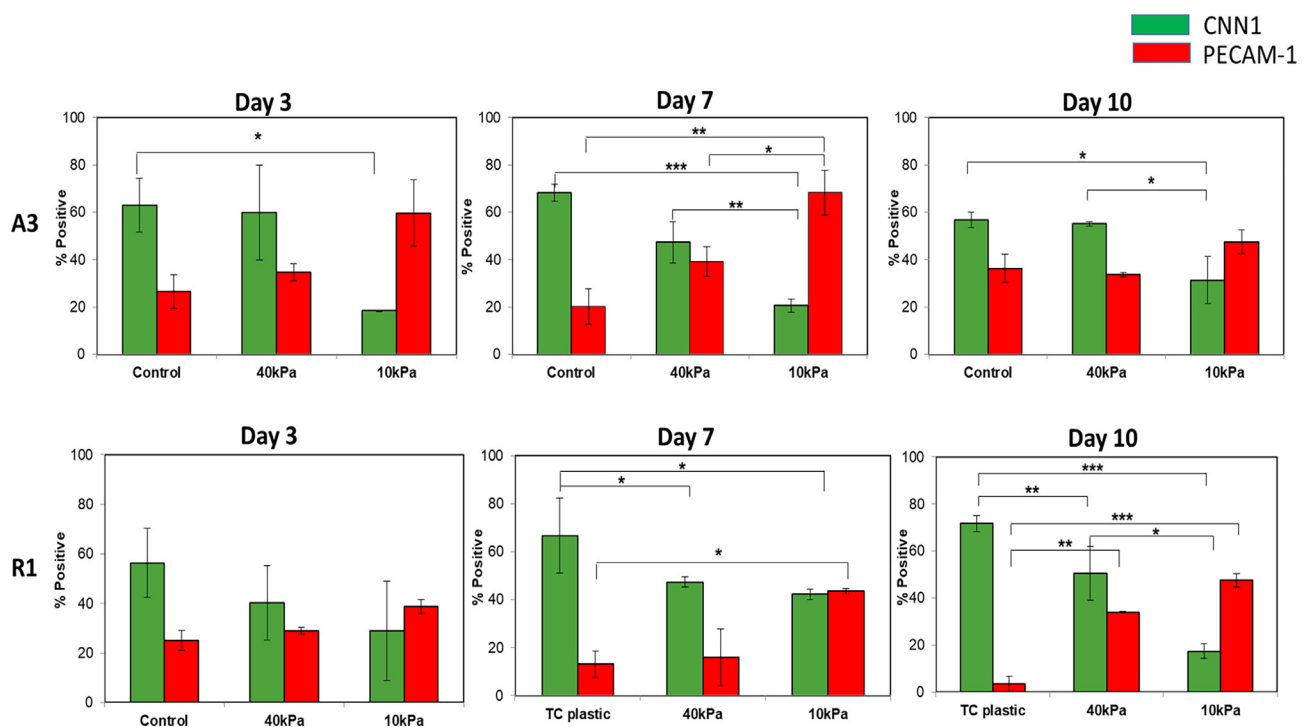

Fig. 3. Flk-1+ cells from ESC-A3 and ESC-R1 on single stiffness substrates. On day 3, more PECAM-1+ ECs are observed on low stiffness materials while more CNN1+ SMCs are on high stiffness materials. Moreover, the role of stiffness-induced maturation of VPCs was consistent across cells from both the ESC-A3 and ESC-R1 lines. * p-value <0.05 ** p-value <0.005 *** p-value <0.0005.

generated more PECAM-1+ ECs while the greater stiffnesses (40 kPa and higher) directed more VPCs towards CNN1+ SMCs. The trend continues through day 3, day 7, and day 10, with CNN1+ cells largely disappearing by day 14. Moreover, the VPC outgrowths were often observed as PECAM-1+ EC islands surrounded by CNN1+ positive SMC, or pure PECAM-1+ populations or pure CNN1+ populations (Fig. 2). Despite their culture in 2D, cells appeared to self-organize according to their native physiology with ECs generating a central colony with SMC loosely associated in the surrounding space.

To more broadly identify gene expression signatures associated with the varying stiffness, we performed RNA-Seq on the VPCs cultured on fibronectin-coated tissue culture plastic (>0.1 GPa), as well as, 40 kPa and 10 kPa hydrogels. The 10 and 40 kPa populations exhibited distinct expression profiles compared to the cells cultured on >0.1GPa with a total of 30 genes exhibiting more than a two-fold expression (P < 0.05) changes from stiffest to softest (Fig. 4). Compared to the cells cultured on the tissue cultures plastic (>0.1 GPa), 11 genes were upregulated, and 22 genes were downregulated on 10 kPa hydrogels, while only 14 genes were downregulated on 40 kPa compared to plastic (Fig. 4). Some of the most highly upregulated genes include: bone morphogenic protein (BMP) binding endothelial regulator (Bmper), receptor tyrosine kinase (TEK), an extracellular matrix-associated heparin-binding protein (CTGF), coagulation factor III or tissue factor (F3), tetraspanin 8 (TSPAN8), and transcription factor Nkx6-2 known to be important in development of the dorsal blood vessel [37]. Overall, hierarchical clustering analysis indicates the distinct genes

are specifically regulated on the stiffest and softest materials (Fig. 5A). VPCs cultured on 10 kPa hydrogel also exhibited higher expression of genes associated with vascular endothelial development including: migration, vasculature development, blood vessel development, blood vessel morphogenesis, and angiogenesis (Fig. 5B and Supplementary Table 1), while genes downregulated on soft material were not as specific to smooth muscle fate, but control signaling and developmental processes.

Mechanosensors, such as integrins (transmembrane proteins) and focal adhesions (link between cytoskeleton and extracellular matrix), translate mechanical signals into cell fate decisions. To further investigate the signaling from vascular-specific integrin and focal adhesion on vascular differentiation, VPC were treated with inhibitors and blocking antibodies to mitigate the signaling pathways that may be activated by stiffness and induce differentiation. FAK 14, which prevents FAK phosphorylation [38], and function blocking antibodies for αvβ3, which binds fibronectin and VEGFR2 leading to mesoderm differentiation [39], and αvβ1, which binds to fibronectin and stimulates angiogenesis [40], were chosen because both αvβ3 and αvβ1 regulate EC function and vascular lumen formation [41]. Blocker for integrin αvβ6, a broader functioning integrin that binds several ECM proteins and activates vascular proliferation, was also examined as a less specific mechanotransducer.

After 48 h of treatment, the FAK inhibitor 14 appeared to mitigate the high stiffness-directed CNN1+ SMC fate, resulting in more robust PECAM-1+ EC proliferation for all stiffnesses (Fig. 6). Integrin αvβ1 blocking mitigated the low-stiffness induced EC fate,
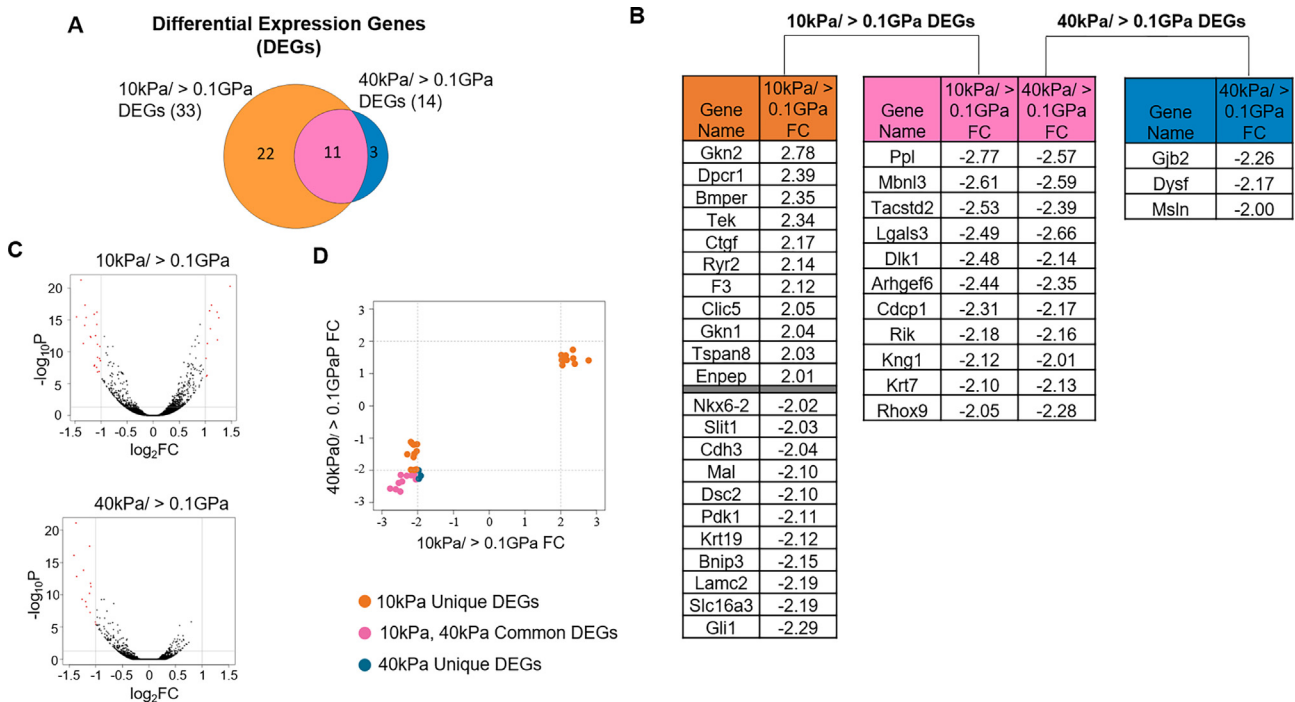

Fig 4. RNAseq gene expression data from VPC cultured on 10 kPa, 40 kPa, and >0.1 GPa. A,B) comparison of differentially expressed genes (DEGs) in 10 kPa vs >0.1 GPa and 40 kPa vs >0.1 GPa and C) their respective volcano plots and D) scatter plot representing genes from A) in terms of fold-change. The genes upregulated on softer 10 kPa materials include: Gastrokine 2 (GKn2), diffuse panbronchiolitis critical region 1 (Dpcr1), BMP Binding Endothelial Regulator (Bmper), TEK receptor tyrosine kinase (Tek), connective tissue growth factor (Ctgf), ryanodine receptor 2 (Ryr2), coagulation factor III (F3), Chloride Intracellular Channel 5 (Clic5), Gastrokine 1 (Gkn1), Tetraspanin 8 (Tspan8), Glutamyl aminopeptidase (Enpep), NK6 Homeobox 2 (Nkx6-2), Slit Guidance Ligand 1 (Slit1), Cadherin 3 (Cdh3), Myelin and lymphocyte protein (Ma)l, desmocollin-2 (Dsc2), Pyruvate Dehydrogenase Kinase 1 (Pdk1), Keratin 19 (Krt19), BCL2 Interacting Protein 3 (Bnip3), Laminin Subunit Gamma 2 (Lamc2), Solute Carrier Family 16 Member 3 (Slc16a3), GLI Family Zinc Finger 1 (Gli1). Genes downregulated on 10 kPa and 40 kPa materials compared with >0.1GPaincluded: Serine/threonine-protein phosphatase PP1 (Pp1), muscleblind like splicing factor 3 (Mbn13), Tumor Associated Calcium Signal Transducer 2 (Tacstd2), Lectin, Galactoside-Binding, Soluble, 3 (Lgals3), Delta Like Non-Canonical Notch Ligand 1 (Dlk1), Rac/Cdc42 Guanine Nucleotide Exchange Factor 6 (Arthgef6), CUB Domain Containing Protein 1 (Cdcp1), Protein RIK (Rik), Kininogen 1 (Kng1), and Keratin 7 (Krt7), Reproductive homeobox 9 (Rhox9). An additional 3 genes were downregulated in 40 KPa material compared with the very stiffest material including: G protein beta subunit (Gib2), dysferlin (Dysf), Mesothelin (Msln).
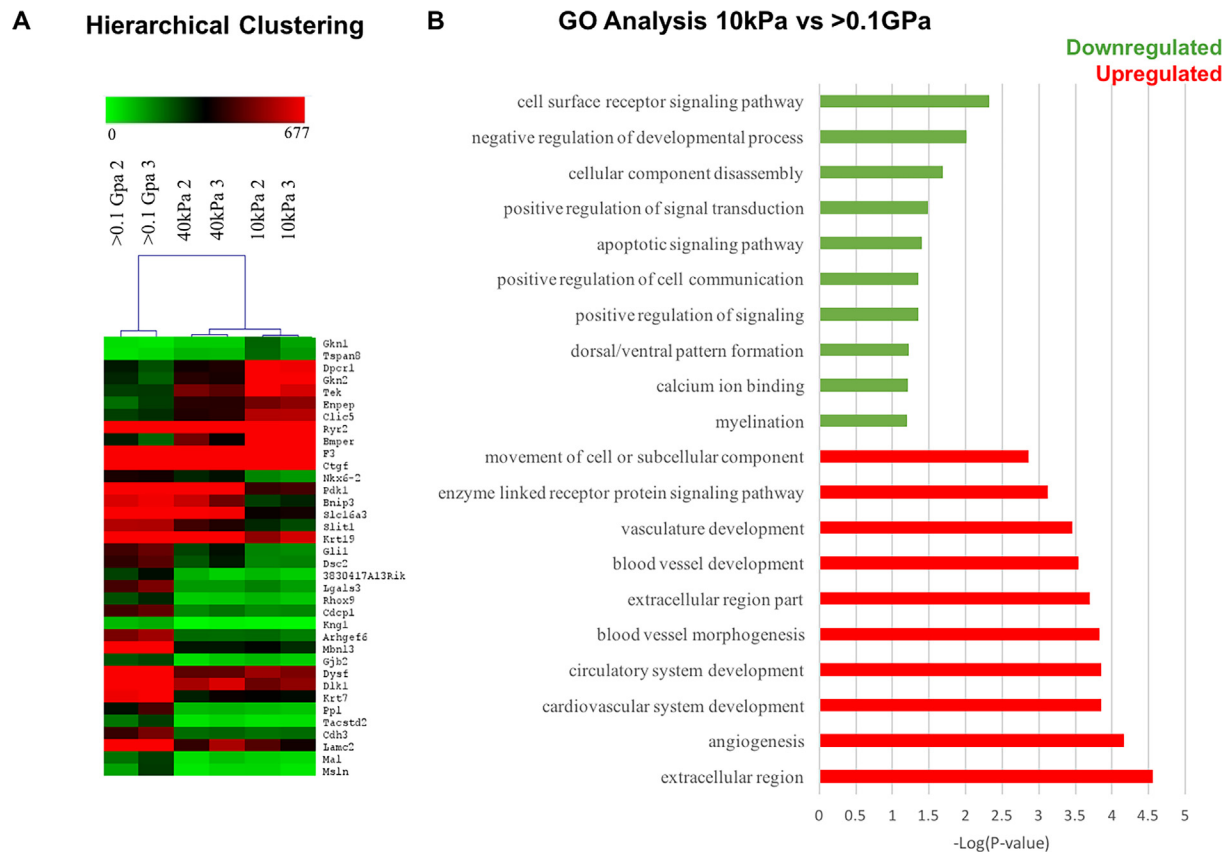
Fig. 5. RNAseq gene expression hierarchical clustering and gene ontology (GO) analysis from VPC cultured on 10 kPa compared and >0.1 GPa. A) Hierarchical clustering analysis of genes upregulated (red) and downregulated (green). Genes with expression differences more than two-fold were selected for B) gene ontology analysis (P < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

whereas αvβ3 blocking appeared to both activate EC fate and mitigate SMC fate. As expected, blocking the more general αvβ6 integrin did not result in significant changes in the stiffness-directed cell fate. Because αvβ1 has been shown to rescue αvβ3 signaling [42], we also added antibodies to both simultaneously. With anti-αvβ3, we observed a slight recovery of the stiffness-directed response suppressed by anti-αvβ1.

4. Discussion

Outgrowths of the VPCs seeded onto softer, 10 kPa, versus stiffer, 40 kPa or greater, materials clearly and robustly show that stiffness plays a significant role in vascular cell fate. Specifically, softer hydrogels generated more PECAM-1+ ECs while the stiffer materials directed VPCs towards CNN1+ SMCs. Stiffness-directed lineage commitment continues up to 10 days, indicating a optimal temporal window for stiffness-directed cell fate at around day 5–6 of total differentiation. Moreover, the outgrowths of Flk-1+ VPCs as PECAM-1+ EC islands surrounded by CNN1+ SMCs suggest that the ECs and SMCs are self-sorting (Fig. 2).

These data, combined with transcriptome analyses, indicate a stiffness-regulated mechanism in vascular cell fate, with overlapping downstream signaling, as detailed in Fig. 7. Specifically, *TEK*, which encodes the angiopoietin-1 receptor called Tie-2, was upregulated on the softer 10 kPa hydrogel. This protein is associated with regulating embryonic vascular development and angiogenesis [39]. Another upregulated gene observed in the VPC outgrowths cultured on 10 kPa was *TSPAN8*, which encodes for tetraspanin proteins that are found on the cell surface and known to

form multiprotein complexes with αvβ3 and α6β4, playing a role in cell development, motility, and angiogenesis [43]. *TSPAN8* can also be found in exosomes and when they are internalized by ECs, the ECs exhibit elevated levels of VWF, VEGF, VEGFR2 and other factors that drive EC proliferation, migration, sprouting and progenitor maturation [44].

F3, coagulation tissue factor (TF) 3, was another gene upregulated in the VPC outgrowths cultured on low stiffness gels, plays a role in blood clotting through thrombin formation with a known association with αvβ3 and β1 integrins in directing cell migration [45], and possibly cell fate as indicted by our integrin studies. *Cdcp1*, a Src kinase family transmembrane protein responsible for cell-matrix adhesion involved in outside-in signal transduction with β1 integrin to induce intracellular phosphorylation of the FAK/PI3/Akt pathway leading to cell survival, proliferation, and migration of tumor cells [46], was downregulated on softer materials substrates.

Regarding SMC fate observed on higher stiffness materials, *Lgals3*, which encodes for beta-galactose binding lectin and is involved in the migration, proliferation, adhesion, and differentiation of vascular SMCs during atherosclerosis development, was downregulated in cells cultured on both 10 kPa and 40 kPa substrates compared to >0.1 GPa. Expression of *Lgals3* was found in models with accelerated plaque formation *in vivo* and *in vitro* SMC. Although *Lgals3* expression is not found in quiescent SMC *in vivo*, *Lgals3* is found in primary cultures of SMC that usually exhibit a synthetic phenotype [47].

Another gene downregulated in cells cultured on softer hydrogels was *Dlk1*. *Dlk1* encodes for a transmembrane protein that belongs to the epidermal growth factor family and plays a role in
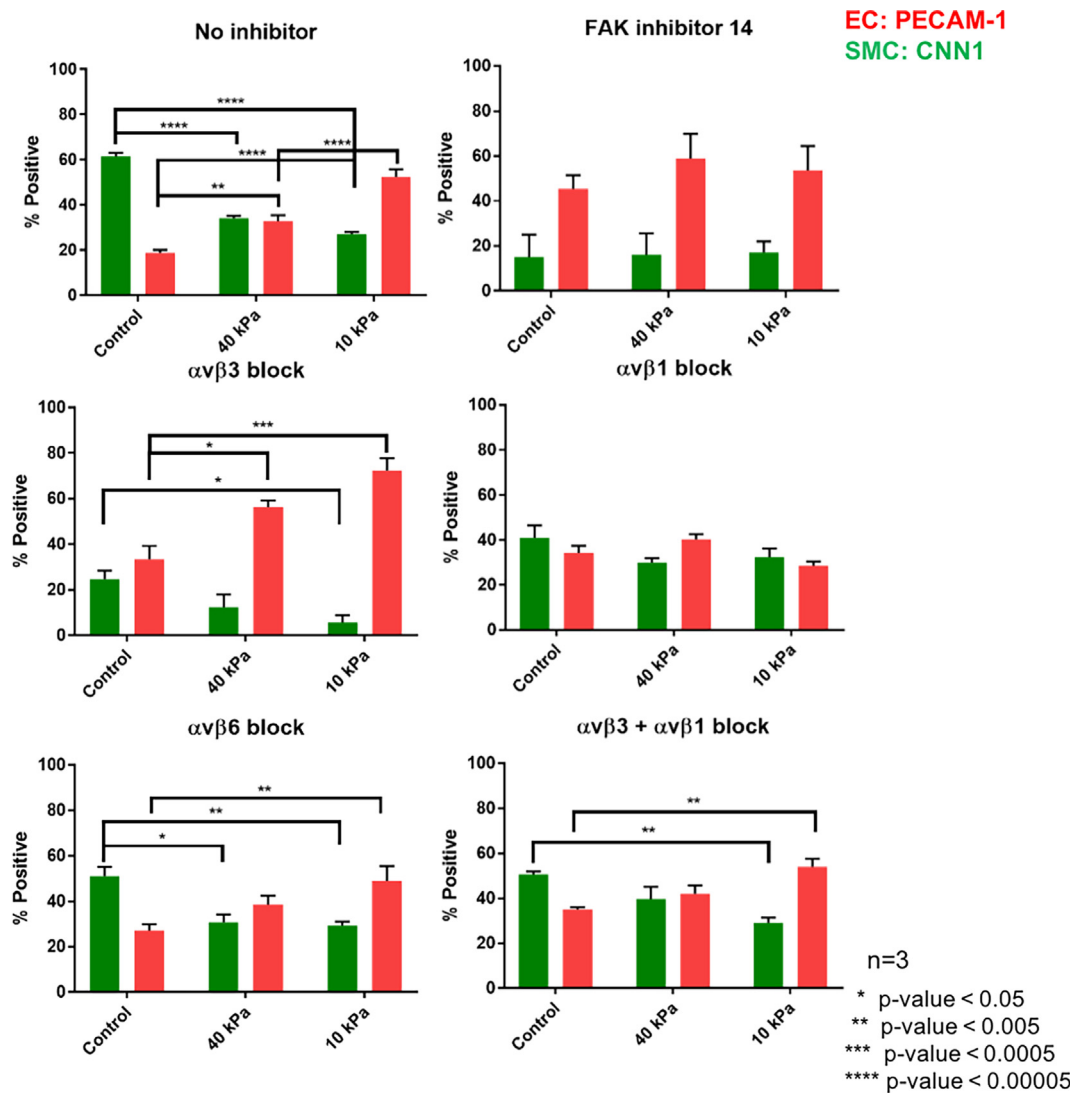
Fig. 6. Inhibitor studies on Flk-1+ VPC-A3 outgrowths on day 5 after 48 hr treatment. Compared to the control (no inhibitor added), stiffness-directed differentiation was mitigated after treatment with inhibitors: FAK14 and $\alpha v \beta 1$. However, $\alpha v \beta 3$ activated endothelial-specific fate leading to an increase in PECAM-1+ cells on lower stiffness hydrogels and a corresponding decrease in CNN-1+ cells. The inhibitor to $\alpha v \beta 6$ did not exhibit a significant effect compared to the controls. * p-value <0.05 ** p-value <0.005 *** p-value <0.0005 **** p-value <0.00005.

several differentiation processes including regulation of Notch-mediated angiogenesis as a non-canonical ligand of the Notch pathway. When knocked down, the lack of *Dlk1* inhibits activation of tip cells, favoring blood vessel stabilization [48] compared with angiogenic activation.

Together with our integrin signaling data and gene expression data further suggest a role for both $\alpha v \beta 1$ and $\alpha v \beta 3$ integrin activation and complex formation with Ang-1/Tie-2 and Ang2/Tie-2, respectively, which can lead to differential cellular outcomes in ECs [49]. Specifically, Ang-1 and Ang-2 are ligands that are agonistic and antagonistic to Tie-2 receptor, respectively. Ang-1 binding to Tie-2 leads to Akt activation and promotes endothelial quiescence and survival, while Ang-2 is an agonist and interferes with Ang-1/Tie-2. Both Ang-1 and Ang-2 recruit $\alpha v \beta 3$ to Tie-2, but only Ang-2 results in the complex formed with Tie-2/$\alpha v \beta 3$/FAK that leads to downstream signaling [49] necessary in EC differentiation [50], rescuing ECs from apoptosis [51], regulation of actin cytoskeleton in ECs, and aiding in endothelial barrier integrity (decreased permeability) [52]. In our study, when anti-$\alpha v \beta 3$ was

added, instead of blocking, we observed an amplification of the stiffness-directed responses - activating EC fate and mitigating SMC fate (Fig. 6). Because integrin blocking antibodies can also result in integrin clustering on the cell surface and lead to signaling [53], it seems that the addition of anti-$\alpha v \beta 3$ in these studies enhanced clustering and complexing with VEGFR2 to further promote endothelial differentiation (Fig. 6) [54]. Therefore, we conclude that the $\alpha v \beta 3$ is not a stiffness-regulating integrin in differentiating vascular cells, while $\alpha v \beta 1$ activation appears to be the dominant stiffness-regulating integrin signaling molecule.

Lastly, we need to acknowledge the literature regarding variable ECM protein composition or surface density when using polyacrylamide hydrogels [55]. While we agree that ECM composition could affect cells while differentiating and responding to their niche, we have shown that covalent chemical attachment in our polyacrylamide hydrogels does not cause large tethers within the cell cultures [25]. Moreover, protein density and pattern presentation for cell attachment have been extensively studied and relatively uniform cell attachments are observed [21]. This does not
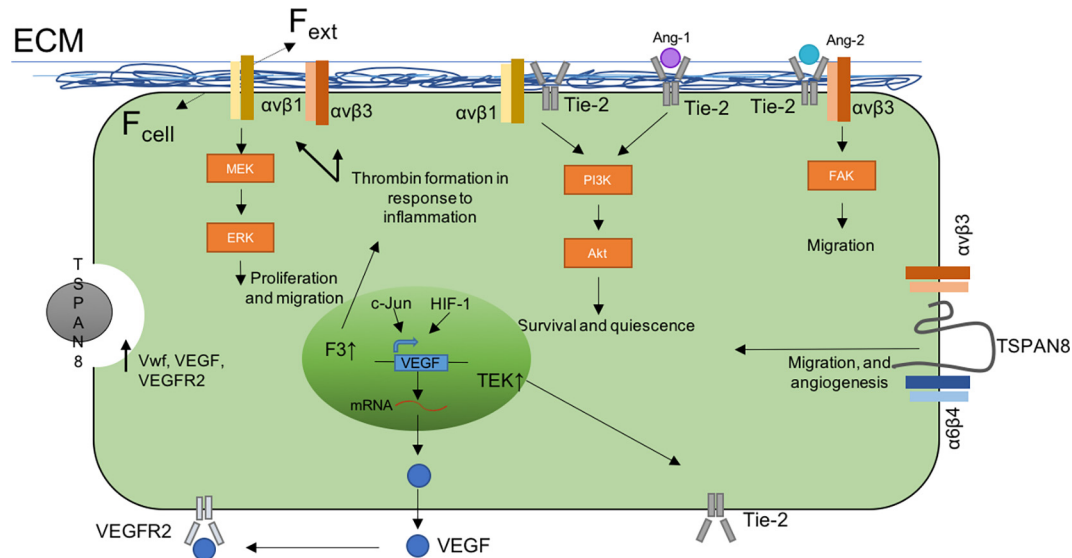
Fig. 7. Genes found to be contributing to stiffness-directed EC differentiation. Specifically, cells cultured on low-stiffness hydrogels upregulated expression of *TEK,* which encodes the angiopoietin-1 receptor called Tie-2 and recruits $\alpha v \beta 3$ leading to downstream activation pathways for EC fate. Also, upregulated TSPAN8, which associates with $\alpha v \beta 3$ and $\alpha v \beta 4$ and drives production of VWF, VEGF, VEGFR2 that lead to EC fate. Additionally, the upregulation of F3 in cells cultured on soft materials is a known regulator of tissue factor in endothelial cell function that can also play a role in upregulation of $\alpha v \beta 1$ and $\alpha v \beta 3$.

negate the possibility of variations of protein density-driven integrin-clustering on the cells, which might be playing an additional role in directing vascular cell fate.

5. Conclusions

Overall, these studies show conclusively that softer hydrogels direct VPCs towards an EC fate compared with greater stiffer hydrogels. The VPCs cultured on the softest material exhibited higher expression of genes known to be associated with migration, vasculature development, blood vessel development, and angiogenesis. Moreover, $\alpha v \beta 1$ activation appears to be the primary stiffness-sensing mechanism with subsequent downstream signaling of *TEK* and *TSPAN8.*

Acknowledgements

Declaration of Competing Interest

The authors do not have any conflicts of interest regarding this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.actbio.2019.07.030.

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