Substrate stiffness-mediated YAP activation modulates endothelial function via Dll4-Notch1 signaling

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Abstract

Vascular stiffness becomes progressively altered during vascular development and the formation of vascular lesions. Endothelial cells adapt their functions as a consequence of sensing extracellular substrate stiffness; these alterations allow them to maintain their vascular structure and function. In this study, we investigated the impact of stiffness of cell adhesion substrates on endothelial cell functions. We confirmed that endothelial cells on softer substrates not only elongate cellular aspects in a manner similar to that of angiogenic phenotype, but also attenuate yes-associated protein 1 (YAP) activation compared to cells on stiffer substrates. Endothelial cells on softer substrates also upregulate the vascular endothelial growth factor receptor 1 (VEGFR1) and VEGFR2 mRNA expression that is enhanced by VEGF stimulation. We determined that endothelial cells on softer substrates increased Delta-like ligand 4 (Dll4) expression, but not Notch1 expression, via YAP signaling. Moreover, elevated Dll4-Notch1 signaling in endothelial cells on softer substrates contributed not only to VEGFRs upregulation, but also to suppression of pro-inflammatory IL-6 and PAI-1 mRNA expression and to the facilitation of anti-coagulant thrombomodulin and pro-coagulant tissue factor mRNA expression. Our results suggest that endothelial cells activate the YAP-Dll4-Notch signaling pathway in response to substrate stiffness and dictate cellular function.

Introduction

Endothelial cells constitute the inner layer of blood vessels and embed on an extracellular basement membrane. As the extracellular basement membrane provides both physical and mechanical microenvironments, endothelial cells sense the stiffness of the extracellular matrix (ECM) surrounding a cell, and then adapt numerous cell functions to the physical and mechanical niche for the formation and stabilization of the vessel network [1,2]. It has been reported that not only specific extracellular molecule-mediated signaling, but also vascular stiffness-dependent signaling influences endothelial cell behavior during vascular/lymphatic development and during the pathogenesis of cardiovascular diseases [3,4]. During lymphatic development, soft environments promote the formation of primary lymphatic structures by venous lymphatic endothelial cell progenitors [3]. In addition, vascular stiffening in patients with atherosclerosis and hypertension is a cholesterol-independent risk factor for cardiovascular events closely related to vascular endothelial dysfunctions [5]. Therefore, the mechanisms by which endothelial cells adapt to the physical properties of vascular vessels might be better understood as an onset of vascular dysfunction and/or vascular inflammatory diseases.

The interaction of endothelial cells with components of their surrounding ECM is mediated by ECM receptors and integrins. Dynamic conformational and functional changes in integrins and the association of intracellular proteins in response to ECM stiffness are well-known mechanisms of mechano-sensing [6,7]. Consequently, cells promote focal adhesion formation and cytoskeletal rearrangement that are triggers of focal adhesion kinase-cSrc activation and Rho- MAPK (mitogen-activated protein kinase) activation [7–9]. Recently, the mammalian Hippo pathway tumor suppressor protein, known as yes-

associated protein 1 (YAP), has been found to control cell growth and differentiation by ECM stiffness-mediated mechano-transduction [10,11]. YAP acts as a transcriptional co-activator involved in the regulation of cell proliferation and the suppression of apoptotic genes expression [12]. YAP protein is predominantly located in the cytoplasm in the ordinally Hippo pathway active state. However, upon the onset of the Hippo pathway inactive state from mechanical stress, YAP protein can localize to the nucleus [13]. Thus, as the stiff substrate promotes YAP nuclear localization [10], YAP protein interacts with transcription factors and induces the expression of a number of genes involved in cell growth and differentiation.

Several studies have identified the involvement of the YAP pathway in controlling the vascular system in mice, including angiogenesis [14], atherosclerosis [10,15], inflammation [16], and cardiac development [17,18]. Vascular endothelial (VE)-cadherin–mediated intercellular endothelial cell contact leads to the suppression of YAP activation and thus reduces angiogenesis by decreasing the expression of angiopoietin-2 [19]. Meanwhile, YAP activation has been observed in rigid atherosclerosis-prone regions of arteries in mice [20]. Depletion of YAP in endothelial cells impairs plaque formation in apolipoprotein E-deficient mice [20]. Furthermore, in human umbilical vein endothelial cells (HUVECs), disturbed flow activates YAP and increases the expression of YAP target genes, which is dependent upon integrin α5β1 activation [15,21]. These results suggested the possibility that mechanical stress induces activation of the YAP pathway involved in regulating endothelial cellular function; however, it is unclear how substrate stiffness modulates endothelial cell function via the activation of YAP signaling.

Several studies have shown that the stiffness of diseased arteries (more than 10 kPa) is greater than in healthy vascular vessels (less than about 10 kPa) [22]. Recent studies have shown that endothelial cells sense the altered stiffness of vascular vessels and dictate their differentiation and vascular angiogenesis. This evidence suggests that endothelial cells might regulate their functions in response to the stiffness of the extracellular adhesive substrate. Here, we found that substrate stiffness-mediated YAP activation not only promotes Notch1 signaling by Delta-like ligand 4 (Dll4) expression, but also dictates the capabilities of angiogenesis, inflammation, and blood coagulation, regulated by endothelial cells.

Results

Endothelial cells on soft substrates reduce YAP localization to the nuclei.

In order to establish a cell-culture system that provides a broad range of vascular stiffness, we employed collagen-coated 1, 2, 4, 8, and 25 kPa from hydrogels, and cultured HUVECs on these gels. Under our experimental conditions, the number of dead cells and detached cells did not significantly change across the different gels, whereas the morphology of the cells was remarkedly elongated on softer substrates (Fig. 1A and B). We also confirmed that localization of YAP protein in the nuclei is facilitated in cells on stiffer substrates as has been reported previously (Fig. 1C) [10]. To evaluate YAP transcriptional activity in HUVECs on gels, we evaluated YAP target connective-tissue growth factor (CTGF) and cysteine-rich

angiogenic inducer 61 (CYR61) mRNA expression. Both CTGF and CYR61 mRNA expression in HUVECs on stiffer gels were higher than those in HUVECs on softer gels (Fig. 2A).

It has been known that the Hippo pathways LAT1/2 and MST1/2 phosphorylate YAP protein and promote cytoplasmic localization of YAP protein [23]. We examined whether substrate stiffness influences basal levels of LAT1/2 and MST1/2 mRNA expression in HUVECs. In none of the gels did HUVECs alter the mRNA expression of LAT1 and MST1/2 (Fig. 2B and C). Meanwhile, HUVECs on plastic plates increased CTGF, CYR61, LAT1/2, and MST1/2 mRNA levels to an excessive degree compared to HUVECs on hydrogels. Thus, these results indicated that hydrogels induce YAP activation on stiffer substrates without altering the Hippo pathway, including LAT1/2 and MST1/2 expression. In subsequent experiments, we therefore used 1, 2, 4, 8, and 25 kPa of hydrogels for HUVEC cultures, and analyzed alterations in endothelial cell function vis-à-vis substrate stiffness.

Substrate stiffness-dependent angiogenic-related gene expression

It has been reported that substrate stiffness-mediated YAP activation is involved in angiogenesis or lymphogenesis through the induction of morphological changes and gene expression [19] and that endothelial cells on soft substrates enhance both VEGF internalization and signaling [1]. Next, we evaluated angiogenesis-related mRNA expression with or without VEGF stimulation. The expression of VEGFRs mRNA increased in those HUVECs on softer substrates, and VEGF stimulation increased VEGFR1 and VEGFR3 mRNA expression (Fig. 3A). VRGFR2 mRNA expression also increased in HUVECs on softer gels, while VEGF stimulation decreased VEGFR2 mRNA expression. Notably, although Dll4 mRNA expression was enhanced in softer substrates and under VEGF stimulation, Notch1 expression in HUVECs treated by vehicle was slightly enhanced on softer gels (Fig. 3B).

It has been known that transcription cofactor YAP regulates a number of different gene expression levels; [12] therefore, we examined whether YAP activation regulates Dll4 mRNA expression in a substrate stiffness-dependent manner. The results revealed that YAP activator XMU-MP-1 reduces Dll4 mRNA expression in HUVECs on a 1 kPa gel with VEGF stimulation, and that YAP inhibitor verteporfin enhances it in HUVECs on 25 kPa with VEGF stimulation (Fig. 3C). These data indicated that soft substrates enhance the VEGF-induced pro-angiogenic factors Dll4 and VEGFRs expression in endothelial cells in response to the suppression of YAP activation.

Soft substrates activate the Dll4-Notch1 pathway in endothelial cells

We next examined whether increased Dll4 expression in HUVECs on soft substrates functionally induces Dll4-Notch1 signaling. HUVECs on softer substrates increased Dll4 protein expression and facilitated Dll4 protein induction upon VEGF stimulation (Fig. 4A and B). Of note, although Notch1 protein expression was not altered across all degrees of gel stiffness, NICD (an activated form of Notch1 protein) increased

in HUVECs on softer gels. VEGF stimulation enhanced increase NICD expression on softer gels. Moreover, we evaluated HEY1 and HES1 mRNA expression, which are directly regulated by the Notch1 signaling pathway. HEY1 and HES1 mRNA expression were upregulated in HUVECs on softer substrates without VEGF stimulation (Fig. 4C). Only HEY1 expression was clearly enhanced by VEGF stimulation, similar to that observed with Dll4 and NICD expression. These data indicated that endothelial cells activate the Dll4-Notch1 signaling pathway in response to substrate stiffness and that Dll4-Notch1 signaling activation on softer gels is enhanced by VEGF stimulation.

Soft substrates activate the Dll4 Notch1 pathway in endothelial cells

To investigate whether soft substrate-induced Dll4 expression causes VEGFRs mRNA expression in the presence and absence of VEGF stimulation, we investigated Dll4 siRNA and knockdown Dll4 mRNA expression. We confirmed that Dll4 siRNA remarkedly reduced Dll4 mRNA expression in HUVECs on a 1 kPa gel with and without VEGF stimulation, while Dll4 siRNA slightly reduced Notch1 mRNA expression (Fig. 5A). In addition, only HEY1 expression, which was elevated on soft substrates, decreased as a result of Dll4 siRNA transfection (Fig. 5B). Next, we found that VEGFR1 and VEGFR2 mRNA expression were significantly reduced by Dll4 siRNA treatment, while that of VEGFR3 mRNA remained unchanged (Fig. 5C). These results suggested that soft substrate-induced Dll4 expression generates the emergence of a pro-angiogenic phenotype by inducing VEGFR1 and VEGFR2 mRNA expression.

Soft substrates alter endothelial function-related gene expression

Our results demonstrated that soft substrate-mediated Dll4 expression modulates angiogenesis-related gene expression in response to the extracellular environment. We then examined the expression of ANGPT1 mRNA, which is crucial gene for vascular stabilization. The expression of ANGPT1 mRNA was facilitated by a soft substrate and VEGF stimulation (Fig. 6A). Dll4 siRNA did not alter ANGPT1 mRNA expression in HUVECs without VEGF stimulation, while it abolished ANGPT1 mRNA expression in HUVECs with VEGF stimulation. Moreover, we evaluated the expression levels of a number of genes involved in vascular inflammation and blood coagulation in endothelial cells. Of note, pro-inflammatory IL-6 and PAI-1 mRNA were suppressed by soft substrates, while VEGF stimulation exercised no influence over them (Fig. 6B and C). Although the suppression of IL-6 mRNA expression was abrogated by Dll4 siRNA without VEGF stimulation, the suppression of PAI-1 expression was cancelled by Dll4 siRNA. Furthermore, anti-coagulant THBD (thrombomodulin) and pro-coagulant tissue factor (TF) mRNA were slightly induced on soft substrates and increased remarkedly upon VEGF stimulation on soft substrates (Fig. 6D and E). The increase in mRNA expression on soft substrates via VEGF stimulation was cancelled by Dll4 siRNA. These results suggested that substrate stiffness results in distinct gene expression patterns in endothelial cells via the YAP-Dll4-Notch1 signaling pathway.

Discussion

In this study, we found that endothelial cells on soft substrates not only activate Dll4-Notch signaling by suppressing YAP activation, but also modulate endothelial cellular function. More specifically, we first confirmed that endothelial cells on softer substrates attenuate YAP localization in the nuclei and YAP activation, and that endothelial cells on soft substrates elongate certain cellular aspects in a manner similar to that of the angiogenic phenotype. We then showed that endothelial cells on soft substrates upregulated VEGFR mRNA expression and that VEGF stimulation facilitated soft substrate-induced VEGFR1 and VEGFR3 expression. In addition, we determined that endothelial cells on soft substrates increase Dll4 expression, but not Notch1 expression, via YAP signaling. Elevated Dll4-Notch1 signaling in endothelial cells on soft substrates contribute not only to VEGFR1 upregulation, but also to inflammation and blood coagulation-related gene expression. Thus, endothelial cells might modulate their cellular function as a consequence of adapting to substrate stiffness.

Engler *et al*. have reported that mesenchymal stem cells constitutively differentiate into different type of cells in response to the stiffness of cell adhesion substrates [24]. Numerous studies have subsequently shown that extracellular substrate stiffness is a determinant factor of cell differentiation for several types of cells [24–29]. Furthermore, Segel *et al*. have reported that brain stiffening with age is sufficient to attenuate the proliferation and differentiation rates of oligodendrocyte progenitor cells. For example, the authors showed that isolated aged oligodendrocyte progenitor cells cultured on synthetic scaffolds in order to mimic the stiffness of young brains are molecularly and functionally rejuvenated [30]. In addition to normal healthy cells, tumor cells and other pathogenic cells can also modulate their aggressiveness in response to the stiffness of extracellular environments [31,32]. Taken together, these studies suggest that cell functionality and phenotype are directly linked to the stiffness of the surrounding niche and the extracellular environment. In agreement with these findings, in the current study, we demonstrated that endothelial cells modulate not only the capability of angiogenesis, but also inflammatory and coagulant states in response to substrate stiffness by regulating the activation of Dll4-Notch1 signaling.

Recent studies have reported that the arterial stiffening observed during atherosclerosis and aging is a cholesterol-independent risk factor for cardiovascular events [5,33]. The composition and structure of elastin and collagen, which are predominant components of the vascular wall, determine the passive mechanical properties of the large arteries. Elastic fibers become degraded and fragmented with age and disease, while collagen levels increase [34]. Additional crosslinking between elastin and collagen is a cause of fiber stiffening and prevents enzymatic digestion and degradation, leading to increased vascular stiffness. Endothelial cells are thus exposed to a stiffening environment as aging and arteriosclerosis progress, suggesting that endothelial cells might adapt their function to stiff environments, thereby leading to be onset of endothelial dysfunction.

Endothelial cells play a pivotal role in the regulation and crosstalk of inflammation and blood coagulation in vascular vessels [35,36]. Under healthy conditions, endothelial cells constitutively exhibit anti-inflammatory and anti-coagulant protein on the surface. In contrast, under pro-inflammatory conditions,

they immediately induce the expression of inflammatory cytokines and pro-coagulant factors and reduce anti-inflammatory and anti-coagulant factors [37]. Many earlier studies have indicated that hyperlipidemia [38], high glucose [39], hypertension [40], and disturbed shear stress [41] continuously activate endothelial cells, leading to the endothelial cell dysfunction associated with chronic inflammation. Thus, it is thought that endothelial cell dysfunction-mediated uncontrolled activation of inflammation and blood coagulation is a trigger of vascular inflammatory diseases and cardiovascular events. Our data indicating that endothelial cells on stiff substrates promote continuous pro-inflammatory IL-6 and PAI-1 expression suggests that stiff vascular vessels become an inducible factor of endothelial cell dysfunction.

Stiffness of the extracellular substrate involves in a number of biological processes involving endothelial cells as a result of cell adaptation. Integrin mechano-sensing interaction and YAP mechano-transduction are recognized as an essential mechanism for cell adaptation to extracellular environments [10,15]. Transcription co-factor YAP is known as an essential molecule that regulates various cellular functions by modulating gene expression [3,12]. In particular, YAP activation orchestrates angiogenesis through the regulation of MYC signaling [42], STAT3 (signal transducer- and activator of transcription 3) activation [43], and angiopoietin2 expression [19]. Moreover, it has reported that YAP directly regulates the expression of delta-like ligands and Notch signaling in epidermal progenitors [44]. It is well known that Dll4-Notch1 signaling regulates vascular growth and angiogenesis, including EC sprouting and arterial specification [45,46]. Our findings that endothelial cells on softer substrates induced VEGFRs expression through Dll4 expression and Notch signaling under YAP inactivation have elucidated the mechanism underlying substrate stiffness-mediated angiogenesis.

In addition to angiogenesis, previous studies have implicated both the YAP and Notch signaling pathways in vascular inflammation and coagulation. Lv *et al*. reported that deletion of YAP in endothelial cells markedly augmented the inflammatory response by preventing TRAF6 (tumor necrosis factor receptor-associated factor 6)-mediated NF-κB (nuclear factor-κB) activation [16]. Yi *et al.* reported that knockdown of YAP protein suppresses LPS-induced pro-coagulant TF expression and apoptosis by attenuating the ROCK/YAP/Egr-1 signaling pathway and the YAP/P73/Caspase-3 signaling pathway, respectively [47,48]. In addition, pro-inflammatory stimulation triggers the alteration of Notch family and ligand expression patterns in endothelial cells [49]. Of note, blockade of Dll4-Notch signaling attenuated the development of atherosclerosis in LDL-receptor-deficient mice [50]. These studies, therefore, suggest the possibility that substrate stiffness modulates endothelial cell function through YAP-Dll4-Notch1 signaling. Our data indicate not only that reduced YAP activation in endothelial cells on soft substrates increases Dll4 expression and subsequently Notch1 signaling, but also that Dll4 expression contributes to a part of the alteration of several gene expression patterns involved in endothelial cellular function. As several pathways have been linked to physical stress, more studies are required to further our understanding of how endothelial cells modulate cell function in response to substrate/vascular stiffness. Taken together, our results suggest that endothelial cells might be able to integrate physical signals derived from vascular vessels into biological endothelial cellular functions via activation of YAP-Dll4-Notch signaling. Our work shows that endothelial cells on soft substrates facilitate Dll4-Notch1

signaling by reducing YAP activation and subsequently modulating angiogenesis, inflammation, and blood coagulation-related gene expression. Our study provides novel insights into the fundamental pathway by which endothelial cells modulate endothelial cellular function in response to vascular stiffness.

Methods

Cell culture

Primary HUVECs and their culture media (EGM-2 BulletKit) were obtained from Lonza Japan (Tokyo, Japan). HUVECs were cultured in collagen-coated tissue-culture dishes (BD Biosciences, San Jose, CA) in an atmosphere containing 95% air and 5% CO_2. All experiments were performed with cultured HUVECs during passages 3–5. Confluent HUVECs grown on collagen-coated plastic cell culture dishes were detached with trypsin-EDTA 0.05% and plated on 1, 2, 4, 8, and 25 kPa Softwell™ (Matrigen, St. Louis, MO) coated with collagen for 5 hours in culture media. After adhesion to gels, cells were cultured in FBS-free EGM2 media without VEGF and bFGF for 24 hours on gels. To investigate the effect of VEGF stimulation, HUVECs were stimulated with 50 ng/mL of VEGF for 24 hours.

Quantification of cellular aspect ratio

After culture, brightfield images were captured with an HS all-in-one fluorescence microscope (Keyence, Osaka, Japan). The cellular aspect ratio was measured using built-in functions of NIH ImageJ 1.53a software (US National Institutes of Health, Bethesda, MD). The aspect ratio was defined as the length of the long axis over that of the short axis of the equivalent ellipse. A total 10 single cells were randomly selected and counted for each group.

Fluorescent imaging of YAP protein in HUVECs

HUVECs were fixed by 4% paraformaldehyde, and then permeabilized by 0.05% tween in phosphate-buffered saline. YAP were stained with rabbit anti-human YAP monoclonal antibody (Cell Signaling technology, Beverly, MA) and Alexa488 conjugated anti-mouse IgG antibody (Thermo Fisher Scientific, Waltham, MA). Nuclei were stained using 4’, 6-diamidino-2-phenylindole (DAPI) (Dojindo, Kumamoto, Japan). HUVECs were observed using an HS all-in-one fluorescence microscope.

Quantification of mRNA expression

After stimulation, total RNA was extracted from cells with Trizol reagent (Thermo Fisher Scientific) and mRNA was purified with a PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Reverse transcription of mRNA into cDNA was performed using SuperScript VILO (Thermo Fisher Scientific). A Thunderbird qPCR mix (Toyobo, Osaka, Japan) was used for real-time RT-PCR reactions. The primers and probes used for the Taqman assays were as follows: gapdh Hs99999905_m1, dll4 Hs00184092_m1, notch1 Hs01062014_m1, hey1 Hs01114113_m1, hes1 Hs00172878_m1, vegfr1 Hs01052961_m1, vegfr2 Hs00911700_m1, vegfr3 Hs01047677_m1, Thermo

Fisher Scientific). Other primer sequences for the SYBR green assay are shown in Table 1. Data acquisition and analysis were performed by using the ABI StepOnePlus real-time PCR system (Applied Biosystems). Expression of target mRNA was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and relative mRNA expression was quantified by using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primer sequences for SYBR green real-time RT-PCR

SYBR hGAPDH	Forward	5'-	GAAGGTGAAGGTCGGAGTC	-3'
	Reverse	5'-	GAAGATGGTGATGGGATTTC	-3'
SYBR hCTGF	Forward	5'-	CTTGCGAAGCTGACCTGGAAGA	-3'
	Reverse	5'-	CCGTCGGTACATACTCCACAGA	-3'
SYBR hCYR61	Forward	5'-	GGAAAAGGCAGCTCACTGAAGC	-3'
	Reverse	5'-	GGAGATACCAGTTCCACAGGTC	-3'
SYBR hLATS1	Forward	5'-	CACTGGCTTCAGATGGACACAC	-3'
	Reverse	5'-	GGCTTCAGTCTGTCTCCACATC	-3'
SYBR hLATS2	Forward	5'-	GTTCTTCATGGAGCAGCACGTG	-3'
	Reverse	5'-	CTGGTAGAGGATCTTCCGCATC	-3'
SYBR hMST1	Forward	5'-	TGGTGCTACACGATGGACCCAA	-3'
	Reverse	5'-	GCCACACTTCTCAAACTGCACC	-3'
SYBR hMST2	Forward	5'-	GGCAGATTTTGGAGTGGCTGGT	-3'
	Reverse	5'-	AATGCCAAGGGACCAGATGTCG	-3'
SYBR hANGPT1	Forward	5'-	CAACAGTGTCCTTCAGAAGCAGC	-3'
	Reverse	5'-	CCAGCTTGATATACATCTGCACAG	-3'
SYBR hIL-6	Forward	5'-	AGACAGCCACTCACCTCTTCAG	-3'
	Reverse	5'-	TTCTGCCAGTGCCTCTTTGCTG	-3'
SYBR hPAI-1	Forward	5'-	CTCATCAGCCACTGGAAAGGCA	-3'
	Reverse	5'-	GACTCGTGAAGTCAGCCTGAAAC	-3'
SYBR hTHBD	Forward	5'-	AACGACCTCTGCGAGCACTTCT	-3'
	Reverse	5'-	CCAGTATGCAGTCATCCACGTC	-3'
SYBR hTF	Forward	5'-	CAGAGTTCACACCTTACCTGGAG	-3'
	Reverse	5'-	GTTGTTCCTTCTGACTAAAGTCCG	-3'

Quantification of Dll4 and Notch1 protein expression

After VEGF stimulation, HUVECs were lysed with ice-cold RIPA buffer (Nacarai tesque, Kyoto, Japan). The protein content of the cell lysates was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific), and equal amounts of protein were subjected to sodium dodecylsulfate-polyacrylamide gel

electrophoresis and analyzed by Western blot. Dll4 protein was detected using rabbit anti-human Dll4 mAb (1:1000, Cell signaling technology) and horse radish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:4000, Cell signaling technology) dissolved in CanGetSignal buffer (Toyobo, Osaka, Japan). Notch1 protein and Notch intercellular domain (NICD) was detected using mouse anti-human Notch1 mAb (1:1000, Santa Cruz Biotechnology) and HRP-conjugated anti-mouse IgG secondary antibody (1:2000, Cell signaling technology). GAPDH was detected using mouse anti-human GAPDH mAb (1:4000, Cell signaling technology) and HRP-conjugated anti-mouse IgG secondary antibody (1:2000). Blots were developed using the ECL Prime Western Blotting Detection System (GE Healthcare Life Sciences, Little Chalfont, UK). Membranes were imaged with ImageQuant LAS-4000 analyzer (GE Healthcare Life Sciences). Band intensity was quantified by densitometric analysis using NIH ImageJ 1.53a software.

Dll4 knockdown in endothelial cells by siRNA transfection

After serum starvation, 20 nM siRNA (Silencer Select, Thermo Fisher Scientific) targeting the coding region of Dll4 (assay ID: s534448) or Silencer Select negative control #1 were transfected to HUVECs by using RNAiMax (Thermo Fisher Scientific) for 5 hours. HUVECs were then stimulated with VEGF for 24 hours.

Statistical analysis

The data corresponding to the different stiff gels are expressed as means ± SD and were compared to the 1 kPa group using one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Differences between the two indicated groups were determined by using an unpaired t-test test. Differences between all groups were analyzed by using two-way ANOVA followed by Tukey's test. All of the statistical tests were performed using R software. Statistical significance was set to a P value less than 0.05. The specific and appropriate statistical tests performed are indicated in the relevant figure legends.

Declarations

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Author contributions

E. M. performed most of the experimental work and drafted the manuscript. T. O. conceived of the study, contributed to the data analysis and interpretation, and drafted the manuscript. A. I., E. K., and K. A.

provided technical assistance and contributed to the data interpretation. K. W., M. S., M. T., and A. S. provided study oversight and revised the intellectual content.

Additional Information

All authors have no conflict of interest to declare.

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Figures

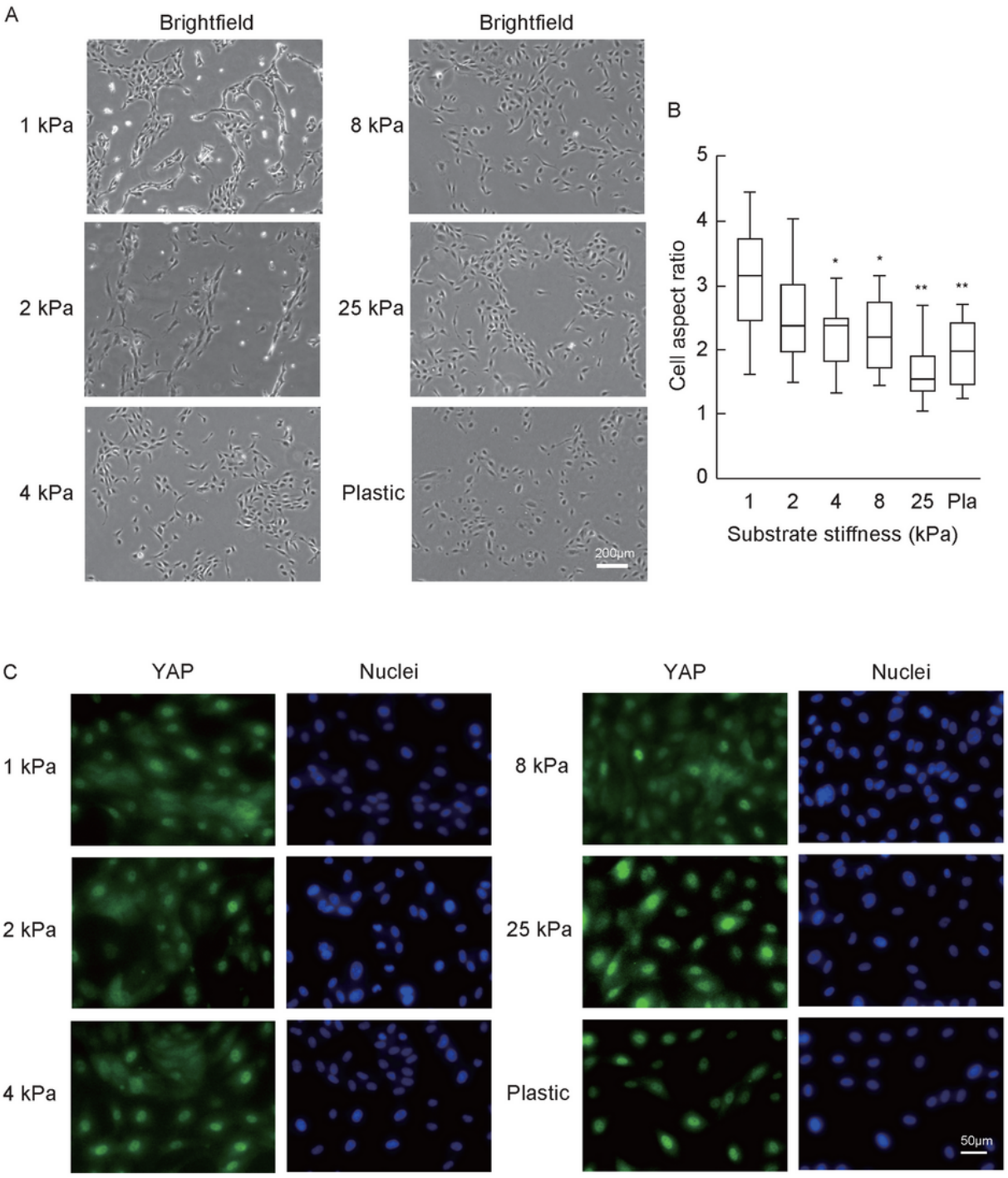

Figure 1

Morphological changes and YAP1 localization of HUVECs on softer substrates. A, Brightfield image of HUVECs cultured on 1, 2, 4, 8, 25 kPa gels, and plastic plate. After plating HUVECs on substrates, cells were cultured in fresh media without FBS for 48 hours. Representative images are shown. Scale bar, 200μm. B, Quantification of the cell aspect ratio in HUVECs on substrates. Aspect ratio is calculated based on the brightfield image as the ratio of cellular major axis to minor axis. The experiments were

repeated three independent times with similar results. Data are expressed as the means ± SD. *P< 0.05; **P< 0.01 vs HUVECs on 1kPa gel, as determined using one-way ANOVA followed by Dunnett's multiple comparison test. C, Immunofluorescent staining of YAP protein in HUVECs on substrates. YAP protein (green) and nuclei (blue) were detected by anti-YAP monoclonal antibody and DAPI, respectively. Representative images from three independent experiments are shown. Scale bar, 50μm.

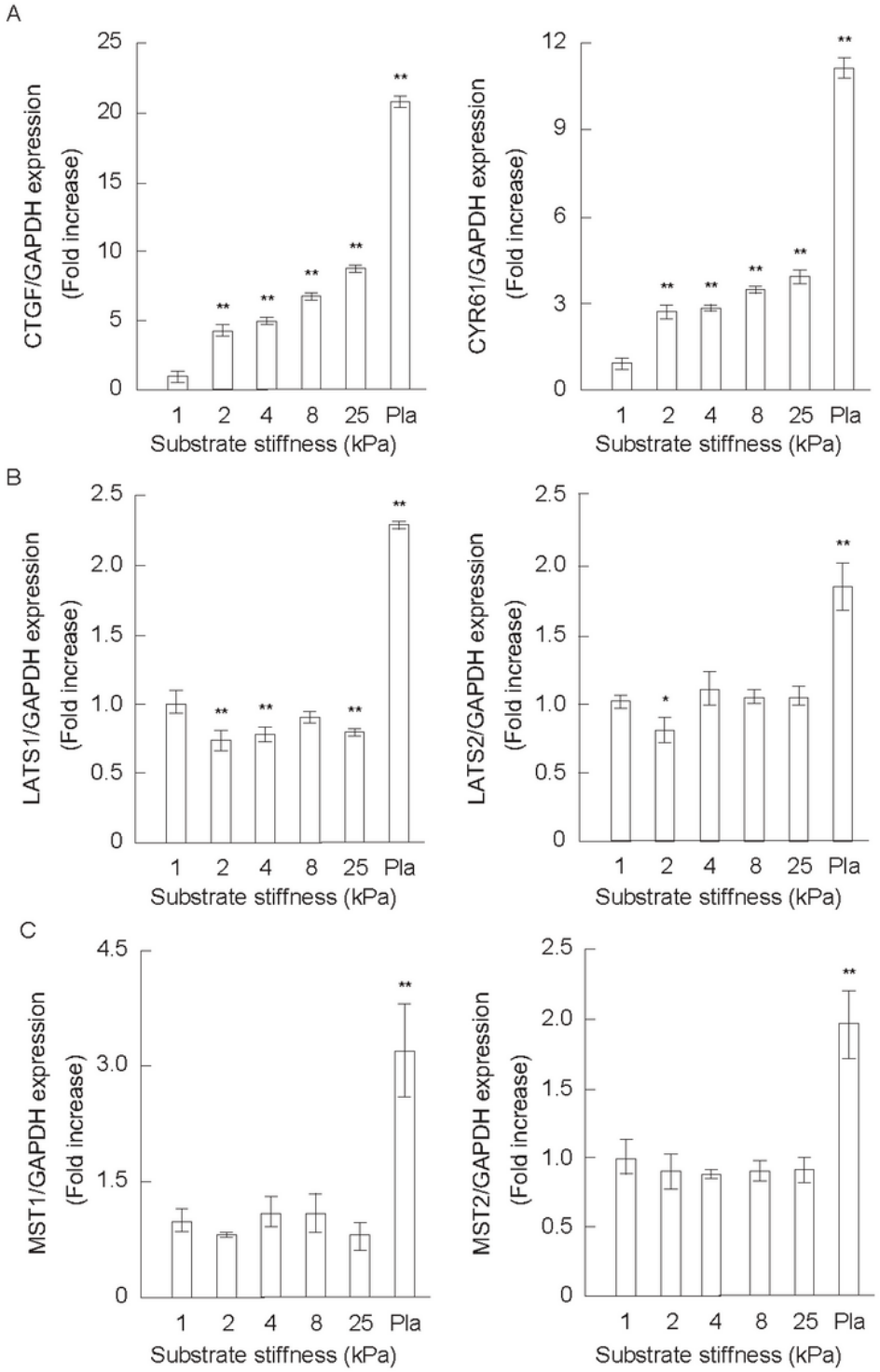

Figure 2

mRNA expression regulated by YAP signaling in HUVECs on softer substrates. A, Real-time PCR analysis of YAP target genes CTGF and CYR61 mRNA expression in HUVECs on substrates. B, Real-time PCR analysis of LATS1 and 2 mRNA expression. C, Real-time PCR analysis of MST1 and 2 mRNA expression. The ΔΔCT values of mRNA were normalized to internal control GAPDH mRNA. Values are reported as fold increase in normalized mRNA expression of HUVECs on 1 kPa hydrogel. The experiments were repeated three independent times with similar results. *P< 0.05; **P< 0.01 vs HUVECs on 1kPa gel, as determined using one-way ANOVA followed by Dunnett's multiple comparison test.

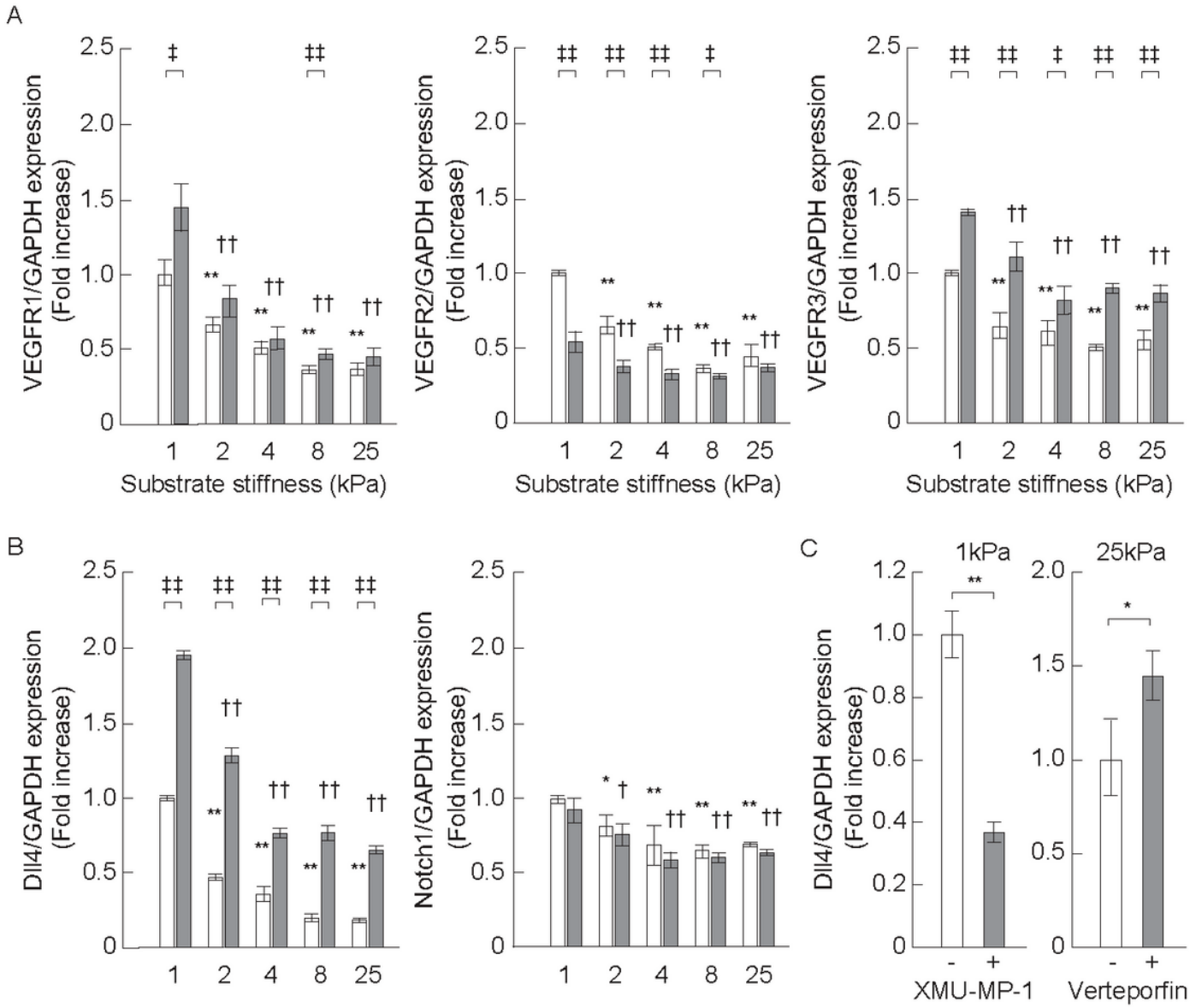

Figure 3

Angiogenesis-related gene expression in HUVECs on softer substrates. HUVECs were cultured on gels for 24 hours and cells were then incubated with VEGF for 24 hours. A, Real-time PCR analysis of VEGFRs mRNA expression in HUVECs on gels. B, Real-time PCR analysis of Dll4 and Notch1 mRNA expression. Values are reported as fold increase in normalized mRNA expression of HUVECs on 1 kPa hydrogel

without VEGF stimulation. Data are expressed as the means ± SD. *, †P< 0.05; **, ††P< 0.01 vs HUVECs on 1kPa gel in the absence or presence of VEGF, as determined using two-way ANOVA followed by Dunnett's multiple comparison test. ‡P< 0.05; ‡‡P< 0.01 between the two groups indicated by the bar, as determined by an unpaired t-test test. C, Effect of YAP activator XMU-MP-1 and inhibitor Verteporfin on VEGF-induced Dll4 mRNA expression. The ΔΔCT values of mRNA were normalized to internal control GAPDH mRNA expression. Values are reported as fold increase in normalized mRNA expression of vehicle-treated HUVECs. The experiments were repeated three independent times with similar results. Data are expressed as the means ± SD. *P< 0.05; **P< 0.01, as determined by an unpaired t-test test.

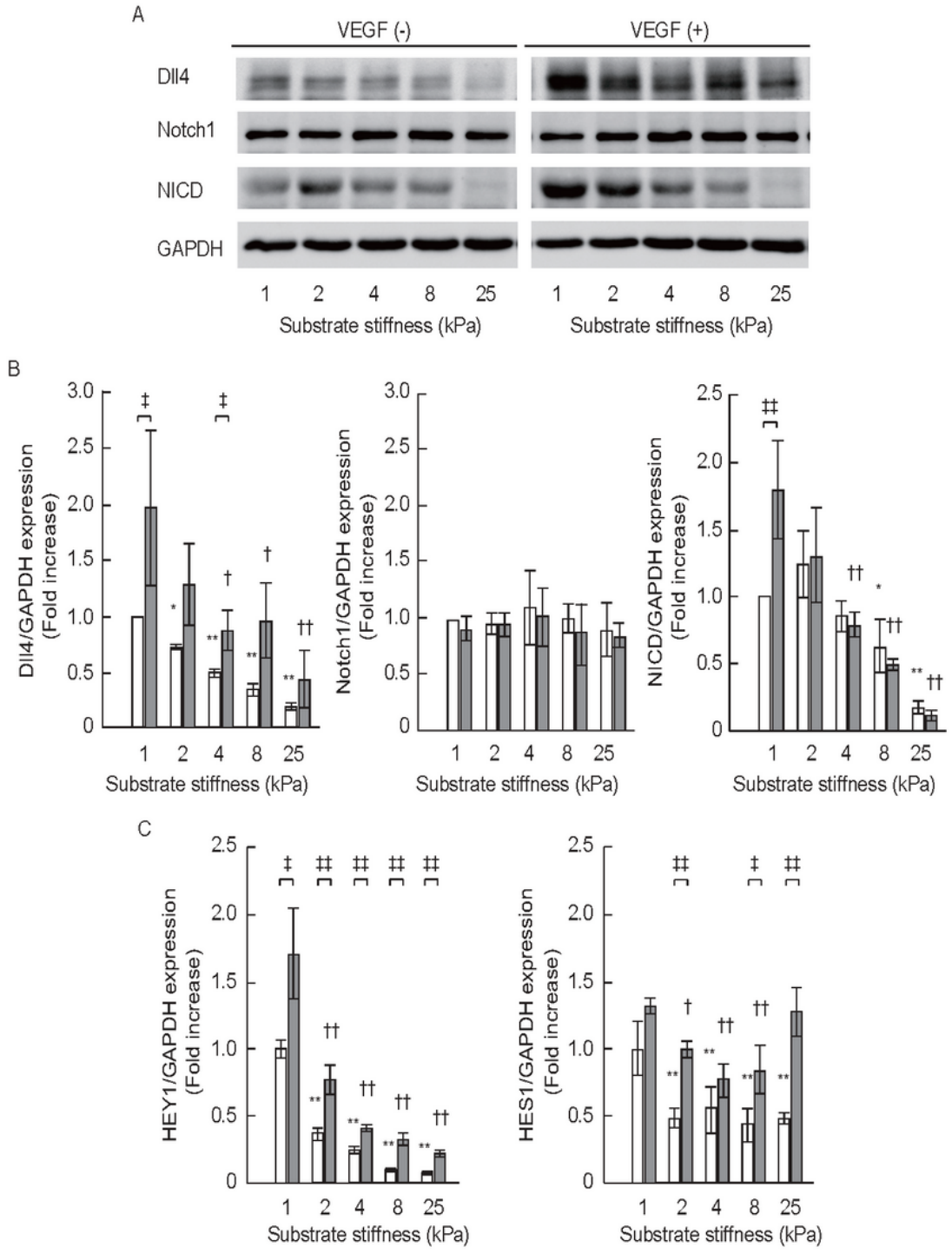

Figure 4

Activation of Dll4-Notch1 signaling in endothelial cells on softer substrates. A, Immunoblot analysis of Dll4, Notch, and NICD protein expression in HUVECs after VEGF stimulation. B, Quantification of protein expression by densitometry analysis using ImageJ software. Intensities of protein were normalized to internal control GAPDH protein. Values are reported as fold increase in normalized protein expression of HUVECs on 1 kPa hydrogel. C, Real-time PCR analysis of NICD target genes HEY1 and HES1 mRNA

expression. Values are reported as fold increase in normalized mRNA expression of HUVECs on 1 kPa hydrogel without VEGF stimulation. Data are expressed as the means ± SD. *, †P< 0.05; **, ††P< 0.01 vs HUVECs on 1kPa gel in absence or presence of VEGF, as determined using two-way ANOVA followed by Dunnett's multiple comparison test. ‡P< 0.05; ‡‡P< 0.01 between the two groups indicated by the bar, as determined by an unpaired t-test test.

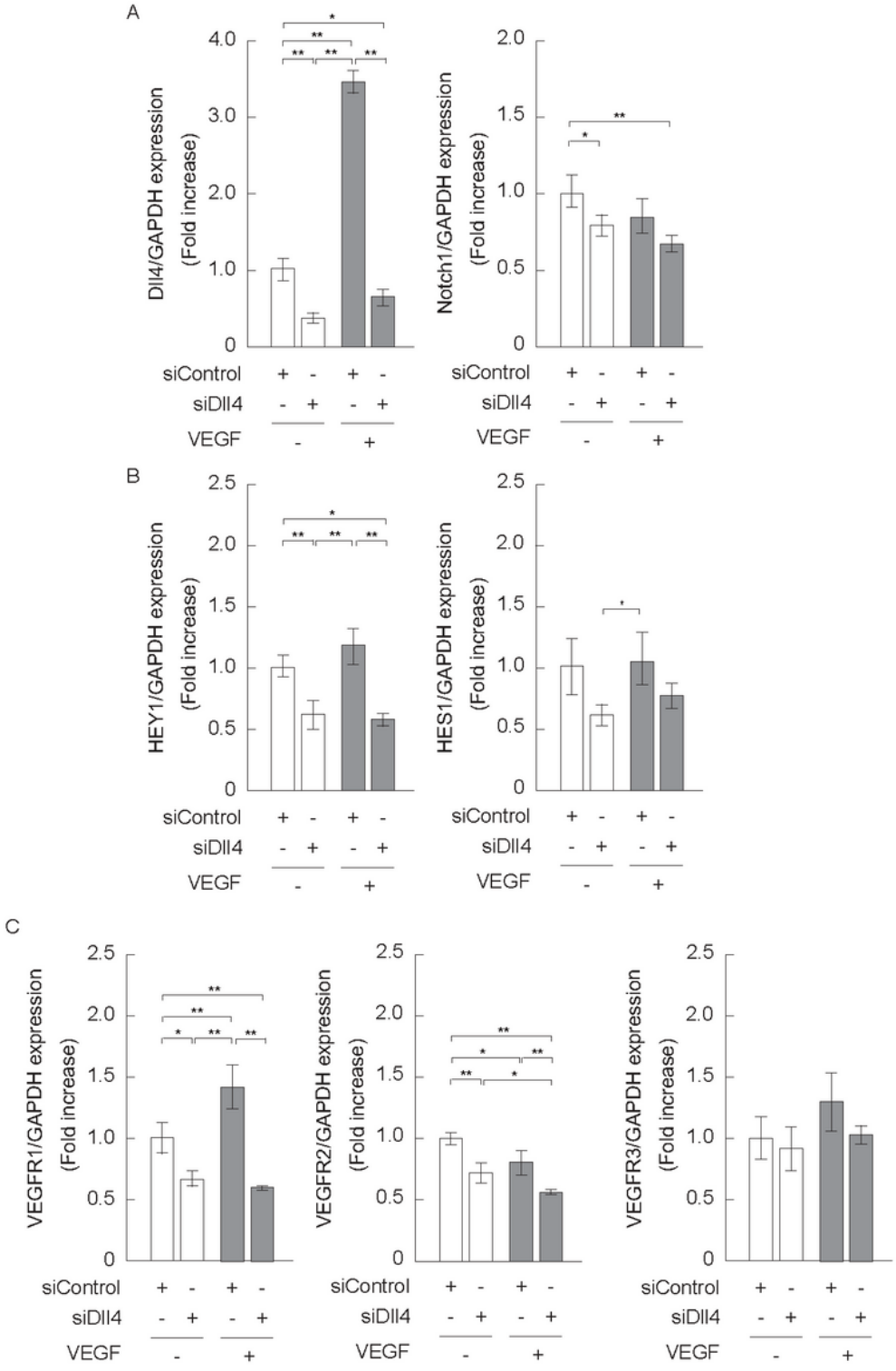

Figure 5

Endothelial cells on softer substrates upregulate VEGFRs expression via the Dll4-Notch1 signaling pathway. Cultured HUVECs on substrate were transfected with Dll4 siRNA (siDll4) and Control siRNA (siControl). At 5 hours post-transfection, HUVECs were stimulated with VEGF for 24 hours. A, Effect of siDll4 on Dll4 and Notch1 mRNA expression in VEGF-stimulated HUVECs on 1kPa gel. B, Effect of siDll4 on HEY1 and HES1 mRNA expression in VEGF-stimulated HUVECs on 1kPa gel. C, Effect of siDll4 on Dll4 and Notch1 mRNA expression in VEGF-stimulated HUVECs on 1kPa gel. Values are reported as fold increase in normalized mRNA expression of siControl-tranfected HUVECs without VEGF stimulation. Data are expressed as the means ± SD. Representative data from three independent experiments are shown. *P< 0.05; **P< 0.01; measured using by two-way ANOVA with Tukey's test.

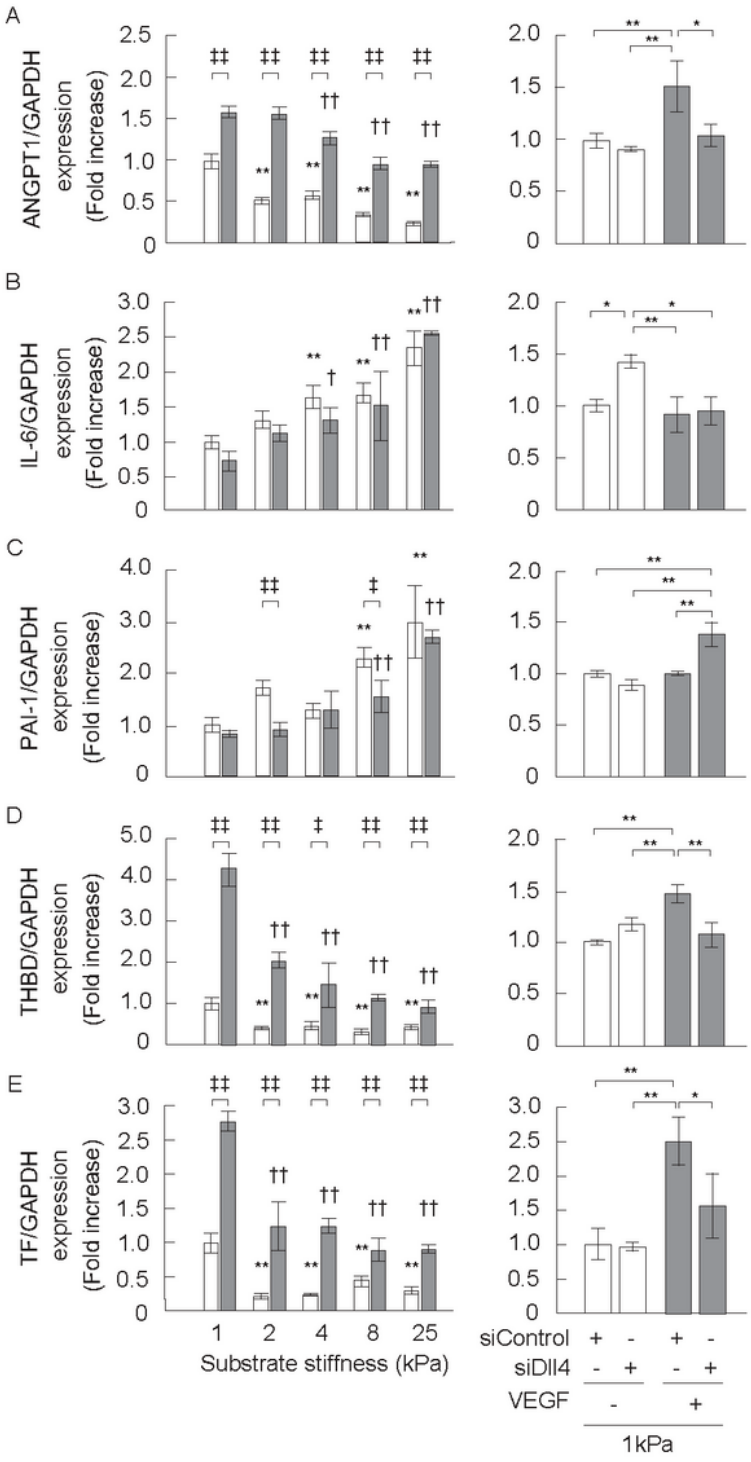

Figure 6

Substrate stiffness dictates endothelial cellular function via the Dll4-Notch1 signaling pathway. Left, Real-time PCR analysis of ANGPT1 (A), IL-6 (B), PAI-1 (C), THBD (D), and TF (E) mRNA expression in cultured HUVECs on substrate were stimulated with VEGF for 24 hours. Values are reported as fold increase in normalized mRNA expression of HUVECs on 1 kPa hydrogel without VEGF stimulation. Data are expressed as the means ± SD. *, †P< 0.05; **, ††P< 0.01 vs HUVECs on 1kPa gel in the absence or

presence of VEGF, as determined using two-way ANOVA followed by Dunnett's multiple comparison test. ‡P< 0.05; ‡‡P< 0.01 between the two groups indicated by the bar, as determined by an unpaired t-test test. Right, Real-time PCR analysis of mRNA expression in siDll4-transfected HUVECs on 1 kPa gels. Values are reported as fold increase in normalized mRNA expression of siControl-tranfected HUVECs without VEGF stimulation. Data are expressed as the means ± SD. Representative data from three independent experiments are shown. *P< 0.05; **P< 0.01; measured using by two-way ANOVA with Tukey's test.