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Substrate stiffness differentially impacts autophagy of endothelial cells and smooth muscle cells

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ABSTRACT

Stiffening of blood vessels is one of the most important characteristics in the process of many cardiovascular pathologies such as atherosclerosis, angiosteosis, and vascular aging. Increased stiffness of the vascular extracellular matrix drives artery pathology and alters phenotypes of vascular cell. Understanding how substrate stiffness impacts vascular cell behaviors is of great importance to the biomaterial design in tissue engineering, regenerative medicine, and medical devices. Here we report that changing substrate stiffness has a significant impact on the autophagy of vascular endothelial cells (VECs) and smooth muscle cells (VSMCs). Interestingly, our findings demonstrate that, with the increase of substrate stiffness, the autophagy level of VECs and VSMCs showed differential changes: endothelial autophagy levels reduced, leading to the reductions in a range of gene expression associated with endothelial function; while, autophagy levels of VSMCs increased, showing a transition from contractile to the synthetic phenotype. We further demonstrate that, by inhibiting cell autophagy, the expressions of endothelial functional gene were further reduced and the expression of VSMC calponin increased, suggesting an important role of autophagy in response of the cells to the challenge of microenvironment stiffness changing. Although the underlying mechanism requires further study, this work highlights the relationship of substrate stiffness, autophagy, and vascular cell behaviors, and enlightening the design principles of surface stiffness of biomaterials in cardiovascular practical applications.

1. Introduction

Cardiovascular diseases (CVDs) are one kind of top threatens to public health and causing huge economic losses each year. It is reported by the World Health Organization (WHO) that more than 18 million people died from CVDs in 2018, representing nearly one-third of global deaths [1]. During these pathological processes, lipids and cells accumulate in the conduit arteries. Due to the progression of these phenomena, the diseased vessels become stiff, while healthy blood vessels are soft and elastic [2]. Accordingly, vascular endothelial cells (VECs) show the attenuated generation of endothelial autacoids and losing the function to regulate vascular tone and homeostasis [3]; And vascular smooth muscle cells (VSMCs) perform uncontrolled proliferation and migration, shifting to the inflammatory/synthetic phenotype and correspondingly increasing extracellular matrix (ECM) synthesis [4]. Although the influence of extracellular matrix stiffness on vascular cell dysfunctions and disease progression has been largely researched, the relationship between autophagy and mechanotransduction is often overlooked in such vascular cell based experiments.

Autophagy is a catabolic mechanism for cells to remodel cytoplasmic constituents, to control organelle quality, and to manage the quantity of intracellular biomass for maintaining cellular homeostasis [5]. It has been attracting a great deal of interests in many research fields in cell biology, physiology and pathology [6–8]. Growing evidence suggest that autophagy has a close correlation with various cellular processes, including but not limited to cell motility and differentiation [9,10],

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chemosensitization [11], cell reprogramming and oncogenicity [12]. Recently, considerable indications link autophagic activities to vascular abnormalities. For instance, defective autophagy of VSMCs was found to accelerate cellular senescence, promoting neointima formation and atherogenesis [13,14]. Deletion of autophagy genes ATG 5 and ATG 7 would lead to impaired endothelial secretion of von willebrand factor (vWF), an essential factor in the thrombotic events [15]. Impaired autophagic activities were also shown to cause endothelial dysfunction directly by regulating intracellular level of Beclin 1 [16].

Several researches suggested that autophagic activities can be regulated by matrix stiffness and play an important part in mechanotransduction. In fact, a recent study fabricated fibronectin-coated polyacrylamide hydrogels whose stiffness can be modulated between 0.23 kPa (soft) to 38 kPa (stiff) [17]. Breast cancer cells autophagy were inhibited on soft substrates, accompanied with the improved ability of maintaining breast cancer stem cells and promoting chemotherapeutic resistance. Human liver cells were found to have a similar intracellular autophagic level on polydimethylsiloxane (PDMS) gels with stiffness varying from 11 to 1220 kPa, but the location and distribution of the autophagosomes exhibited stiffness-dependency [18]. Various investigations have been conducted to study how vascular cells behave in response to changing in substrate stiffness [19–22]. Previously, we have demonstrated that adhesion, migration and proliferation of VECs and VSMCs were reduced on the substrate with lower stiffness [20,23–26]. VECs would lose their phenotype markers, such as vWF and CD31, in a stiffness-dependent manner, and the softer substrate favored the maintaining of VEC phenotype and resisting the endothelial-to-mesenchymal transition (EndMT) that was induced by transforming growth factor β1 (TGF-β1) *in vitro* [23]. Improved endothelial function was achieved on substrates with low stiffness as shown with the tighter integrity, higher production of nitric oxide, and expression level of endothelial function related genes [21]. These studies indicate a very profound influence of substrate stiffness on vascular cells. However, rare evidence shows the impact of extracellular matrix stiffness on VECs and VSMCs autophagy, which makes huge contributions to many developmental and physiological processes.

Given that both substrate stiffness and autophagy are closely correlated with vascular cell behaviors, we would like to question whether substrate stiffness impacts cell autophagy, which could strengthen our understanding of how substrate stiffness affects vascular cells. Here, by culturing VECs and VSMCs on the polyelectrolyte films with different stiffness, we present that cellular autophagy was significantly influenced by substrate stiffness. We measured autophagic levels of cells by assessing monodansylcadaverine (MDC), microtubule-associated light chain 3 (LC3) protein, and mammalian target of rapamycin (mTOR). The gene of endothelial function-related proteins and calponin/vinculin were further measured for testing the status of VECs and VSMCs, respectively. The particularly interesting result is that substrate stiffness has opposite impacts on VECs and VSMCs, which shed light on designing criteria of the biomaterial surface in the field of cardiovascular practical applications.

2. Materials and methods

2.1. Materials

Poly (L-lysine) (PLL, Mw 30,000–70,000), Monodan-sylcadaverine (MDC), Hanks' balanced salt solution (HBSS), Spautin-1, Triton X-100, FITC-phalloidin, mouse antihuman calponin monoclonal antibody rabbit antihuman vinculin and DAPI were purchased from Sigma-Aldrich (U.S.A.). Hyaluronic acid (HA, Mw 351–600 kDa) was purchased from Lifecore Biomedical (U.S.A.). *N*-[2-Hydroxyethyl] piperazine-N'- [2-ethanesulfonic acid] (HEPES) and phosphate buffered saline (PBS) were obtained from Sangon Biotech (Shanghai, China). 1-Ethyl-3-(3-(dimethylamino) propyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS) were purchased from Aladdin (Shanghai, China). Endothelial Cell Medium and Smooth Muscle Cell Medium were purchased from ScienCell Research Laboratories (U.S.A.). All solutions were prepared using deionized water (18 MΩ, Milli-Q Ultrapure Water System, Millipore). Human umbilical vein smooth muscle cells (VSMCs) and human umbilical vein endothelial cells (VECs) were isolated from newborn umbilical cords, following the rules of the local ethical committee. Legal signature of donors for informed consent was obtained according to the regulation.

2.2. Fabrication of PLL/HA polyelectrolyte multilayer films

The PLL/HA polyelectrolyte multilayers with different stiffness were constructed according the methods described previously [23]. Glass coverslips (diameter is 14 mm) were pretreated by immersion in piranha solution (30% H_2O_2/98% H_2SO_4 = 3/7, v/v) for 30 min, and then cleaned by deionized water thoroughly. PLL (0.5 mg mL^{-1}) and HA (1 mg mL^{-1}) were dissolved in HEPES/NaCl buffer solution (20 mM HEPES, 0.15 M NaCl, pH = 7.4). The PLL/HA multilayers were fabricated by alternate deposition of glass coverslips into PLL and HA solution for 8 min each. Between each step, NaCl solution (150 mM, pH = 6.4) was used as wash liquor to flush the remanent molecules on substrates. This sequence was repeated until $(PLL/HA)_{12}$ films were finished. The films were crosslinked for 18 h at 4 °C with a solution containing EDC (70 mg mL^{-1}, 100 mg mL^{-1}, 150 mg mL^{-1}) and sulfo-NHS (11 mg mL^{-1}) in NaCl (150 mM, pH = 5.5). Finally, films were rinsed with HEPES/NaCl buffer solution for at least 8 times.

2.3. Measurement of films stiffness

The young's modulus of films was measured by nanoindenter (Piuma, probe elastic contant 0.50 N m^{-1}) together with PIUMA controller/drive (Optics11, Amsterdam, The Netherlands) in HEPES/NaCl buffer solution. The indenter sensed samples placed on top of a z-piezoelectric translator. The deflection of the cantilever during indentation was measured via Fabry–Pérot interferometry. Each sample was measured at nine different sites, and results were obtained by dataviewer software using Hertzian contact model with a fitting range of 10%.

2.4. VECs and VSMCs culture

VECs were cultured in Endothelial Cell Medium (ECM, ScienCell Research Laboratories, Cat No. 1001, U.S.A.) supplemented with fetal bovine serum, endothelial cell growth factor supplements (ECGS) and streptomycin/penicillin at 37 °C and 5% CO_2. VSMCs were cultured in Smooth Muscle Cell Medium (SMCM, ScienCell Research Laboratories, Cat No. 1001, U.S.A.) supplemented with fetal bovine serum, smooth muscle cell growth supplement and streptomycin/penicillin at 37 °C and 5% CO_2. The culture medium was changed every 2–3 days and cells with 90% confluence were passaged or seeded for further experiments. VECs and VSMCs were used for experiments between 3 and 8 passages.

2.5. Monodansylcadaverine staining

MDC was used to determine the formation of autophagic vacuoles. After washed by PBS, VECs and VSMCs were treated with 50 μM MDC (in HBSS) at 37 °C for 30 min in the dark. Then, the cells were washed with PBS for three times to remove the unreacted fluorescent dye. The visualization of MDC stained vacuoles was determined by inverted microscope (Axiovert 200 M, Zeiss, Germany). Optical density, cell numbers and area of cells were analyzed by software ImageJ. Average optical density analysis was calculated by the formula:

Average optical density = MDC fluorescence intensity per cell/Cell area.

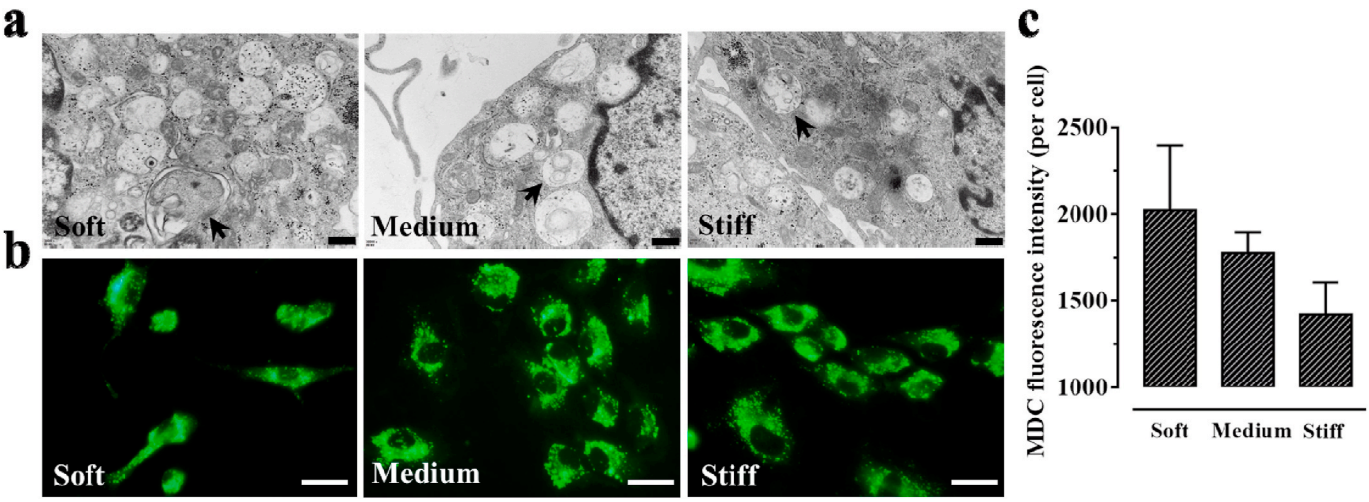

Fig. 1. Autophagic features in VECs induced by stiffness. (a) TEM images of autophagosomes. Black arrow, autophagosomes. Cell density, 2×10^4 cm^{-2}, Scale bar, 500 nm. (b) MDC stained fluorescent microscopy images of cells cultured on substrates with different stiffness. Cell density, 1×10^4 cm^{-2}, Scale bar, 200 nm. (c) Average optical density analysis of the fluorescent images. The data were representative of three independent experiments (mean ± SD) and were calculated by the formula: Average optical density = MDC fluorescence intensity per cell/Cell area.

2.6. Transmission electron microscopic (TEM) observation

VECs and VSMCs were collected and fixed overnight in 2.5% glutaraldehyde in phosphate buffer (PB, pH = 7.0). After washed by PB for three times, the specimen was postfixed with 1% osmic acid for 1.5 h. Dehydration process was performed by rinsing the specimen in alcohol with a graded series of concentration (30%, 50%, 70%, 80%, 90%, 95% and 100%) for 15 min each step and transferred to acetone (100%) for 20 min. Then, the specimen was infiltrated by Spurr resin mixture and embedded by specific medium for about 9 h. Finally, after stained by uranyl acetate and alkaline lead citrate, samples were observed in TEM of Model H-7650.

2.7. Western blot analysis

For western blotting, samples were isolated by radio immunoprecipitation assay (RIPA) lysis buffer with protease inhibitors. The lysates were centrifuged at 12,000 rpm at 4 °C for 15 min, and separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels. After transferred to a poly (vinylidene difluoride) (PVDF) membrane (Millipore, MA, USA), the proteins were incubated overnight with antibodies against LC3, *t*-mTOR, *p*-mTOR, β-actin. Membranes were then incubated with secondary antibody (Thermo Pierce). Finally, signals were detected using the ECL (ECL Western Blotting Substrate, Pierce, USA) system and quantitated by densitometry using BandScan 5.0 software.

2.8. Real-time quantitative PCR analysis

Expression of endothelial function relative genes were analyzed by real-time quantitative PCR (RT-qPCR). Briefly, cells were seeded in 6-well plates. RNA was extracted using a TRIzol Reagent kit (Haogene Biotech, China). The extracted and purified RNA samples (500 ng) were reverse transcribed into cDNA using a 1st-Strand cDNA Synthesis Kit (Haogene Biotech, China). Generated cDNA samples were used as templates to perform a standard PCR analysis using Power SYBR Master Mix (Invitrogen). PCR primers were designed to amplify human genes. And PCR products were detected by Real-Time PCR Detection Systems (CFX384, Bio-Rad, USA).

2.9. Immunofluorescence assay

To visualize the differentiation marker of VSMCs, cells were fixed with paraformaldehyde (4% PBS) after proliferation for 15 min at room temperature. After treatment with 0.1%Triton X-100 for 15 min, samples were stained with FITC-phalloidin (1:200), mouse antihuman calponin monoclonal antibody (1:200), rabbit antihuman vinculin (1:150) and DAPI (1:100). All samples were mounted onto clean coverslips with antifade reagent and observed by fluorescence microscopy (Axiovert 200 M, Zeiss, Germany).

Optical density and area of cells were analyzed by software ImageJ. Average optical density analysis was calculated by dividing optical density with cell area. Fold change of cells were calculated as following:

$$\text{Folds} = \text{Nn} \div \text{N1} \quad (1)$$

where Nn was the absolute cell number on certain day of culture and N1 was the absolute cell number on the first day of culture.

2.10. Statistical analysis

Data were expressed as mean ± SD ($n \geq 3$). The statistical significance was determined using one-way ANOVA analysis. ($^*P < 0.05$).

3. Results

3.1. Preparation of substrates with different stiffness

The (PLL/HA) polyelectrolyte films based on the layer-by-layer assembly technique were employed as substrates because this kind of films have been well characterized and widely applied as a model platform for the study of stiffness roles in various cell behaviours [27–30]. Films with various stiffness can be conveniently fabricated by tuning the concentrations of chemical cross-linking reagents (EDC and sulfo-NHS), and no extracellular matrix protein precoating of these films is required [29]. In this study, we controlled the film with different stiffness through three EDC concentrations of 70, 100, and 150 mg mL^{-1}. The Young's modulus of films was tested by nanoindenter (probe elastic constant0.50 N m^{-1}) together with PIUMA controller/drive (Optics11, Amsterdam, The Netherlands). The indenter sensed film samples placed on top of a z-piezoelectric translator. The deflection of the cantilever during indentation was measured via Fabry–Pérot interferometry. Based on the load-displacement curves the reduced Young's modulus (RedYM) was calculated, similar to an Atomic Force Microscope (AFM) [31,32]. The Young's modulus of EDC70, EDC100, EDC150 crosslinked (PLL/HA) multilayers was measured as 279.8 ± 29.8, 340.5 ± 70.7, and 478.3 ±

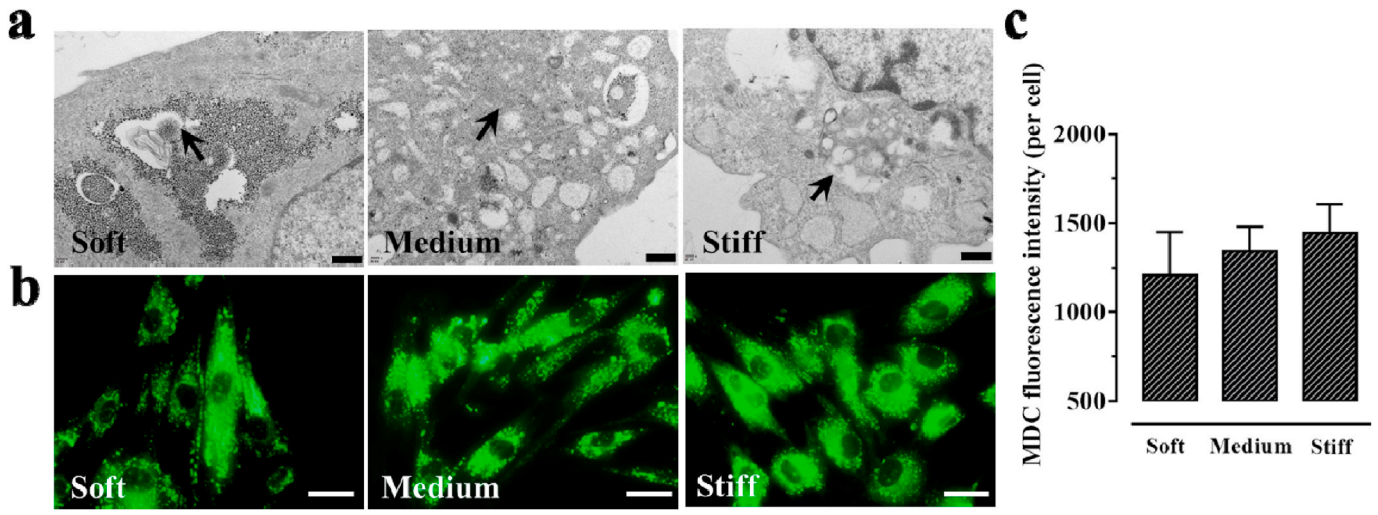

Fig. 2. Autophagic features in VSMCs induced by stiffness. (a) TEM images of autophagosomes. Black arrow, autophagosomes. Cell density, 2×10^4 cm^{-2}, Scale bar, 500 nm. (b) MDC stained fluorescent microscopy images of cells cultured on substrates with different stiffness. Cell density, 1×10^4 cm^{-2}, Scale bar, 200 nm. (c) Average optical density analysis of the fluorescent images. The data were representative of three independent experiments (mean ± SD) and were calculated by the formula: Average optical density = MDC fluorescence intensity per cell/Cell area.

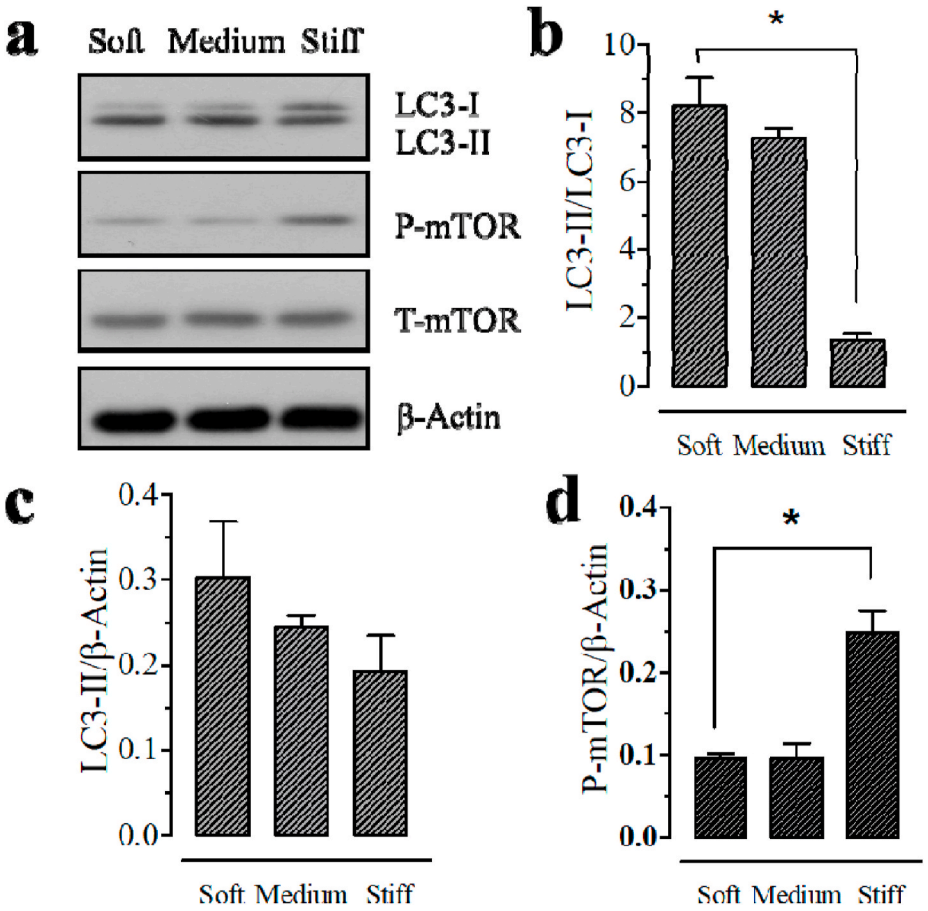

Fig. 3. LC3 activities and phosphorylated mTOR activities of VECs cultured on substrates with different stiffness were analyzed by Western blot. Cell density, 2×10^4 per well. The data were representative of three independent experiments (mean ± SD), $^*P < 0.05$.

47.3 kPa, respectively. The films were hereafter denoted as soft, medium, and stiff.

3.2. Substrate stiffness differentially impacts autophagy of VECs and VSMCs

Cellular autophagy was typically assessed by TEM and MDC staining for observing autophagosomes [9]. TEM technique is the only tool that

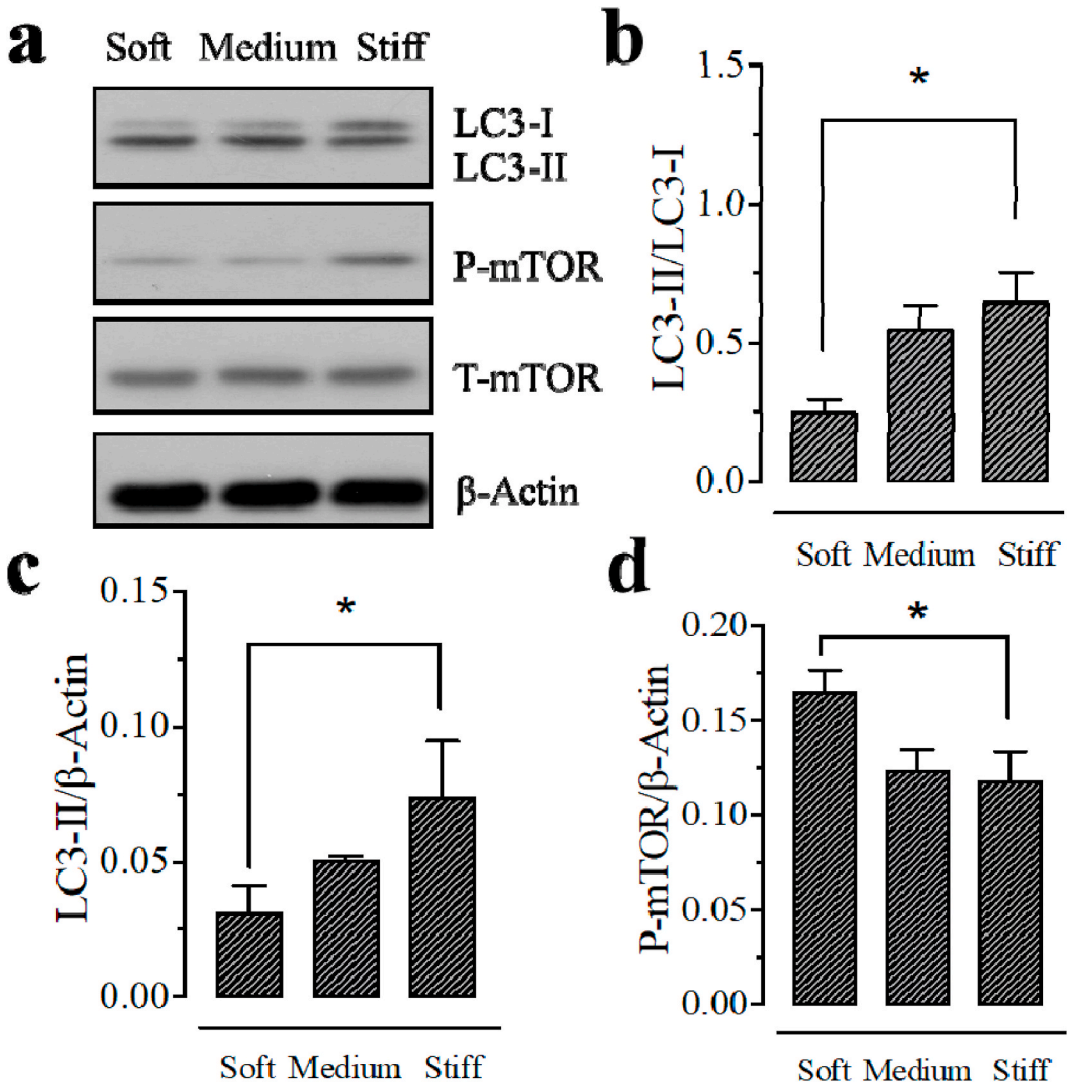

Fig. 4. LC3 activities and phosphorylated mTOR activities of VSMCs cultured on substrates with different stiffness were analyzed by Western blot. Cell density, 2×10^4 per well. The data were representative of three independent experiments (mean ± SD), $^*P < 0.05$.

can reveal all of the dynamic stages of a forming autophagosome in the nanometer range and in its natural environment. Cytoplasmic materials including membrane structure, engulfed cellular organelles like mitochondria or endoplasmic reticulum usually can be observed in autophagic vacuoles. VECs and VSMCs were cultured on the soft, medium and stiff (PLL/HA) films for 72 h and then submitted to TEM experiments. As shown in Figs. 1a and 2a, both VECs and VSMCs in all three substrates stiffness displayed autophagosome containing cytoplasmic materials, which had similar structure with autophagic vacuoles in other reports [9,33]. The MDC staining was further conducted (Figs. 1b and 2b). Numbers of intracellular vacuoles were observed in VECs and VSMCs as shown in green dots. The average MDC fluorescence intensity of cells was then measured. Although the differences of the fluorescence intensity were not significant ($P = 0.0685$, $P = 0.2318$), we found that, for VECs, autophagic vacuoles had a tendency to decrease with the increase of substrate stiffness; while, an opposite trend was observed for VSMCs (Figs. 1c and 2c). These results demonstrate that VECs and VSMCs in all three substrates stiffness displayed autophagosome, suggesting autophagic activities.

To explore this differential impact further, we tested protein LC3 that associated with microtubule and activation pathway related mammalian target of rapamycin (mTOR) in VECs and VSMCs. LC3-II derives from the lipidation of LC3-I in the cytoplasm, and biochemical analysis of the conversion of LC3-I to LC3-II is widely used to monitor autophagosome formation [34]. During the process of autophagy, LC3-II is recruited to the membrane of autophagosomes and it is a convincing indicator of autophagosome number [33,35]. As shown in Fig. 3a, for VECs, densities of western-blotting bands of LC3-I and *P*-mTOR showed increase with the substrate stiffness, and the ratio of LC3-II/LC3-I for the soft film exhibited 11.7% and 83.7% higher than that of the medium and stiff ones, respectively (Fig. 3b). In addition, as seen in Fig. 3c and d for the ratio of LC-II/β-actin and 36.5% higher and 61.6% lower than that of cells on the stiff film, respectively. These data clearly indicate that a stiffer surface weakens autophagy of VECs. In contrast, when VSMCs were cultured on these films, the autophagy changes showed an oppositely trend (Fig. 4). The ratios of LC3-II/LC3-I, LC-II/β-actin, and *P*-mTOR/β-actin of the cells on the soft film were increased by 163.3% and 137.6%, and decreased by 28.6%, respectively, compared with cells on the stiff film. mTOR is a conserved serine/threonine protein kinase that senses and integrates various physiological and environmental signals to regulate cell behaviours; The phosphorylated active form of mTOR, *P*-mTOR, is involved in activation of protein transcription and inhibition of the autophagic activity [36]. There were significant differences in *p*-mTOR expression between soft and stiff films, indicating that the stiffness-induced autophagy was mTOR-dependent.

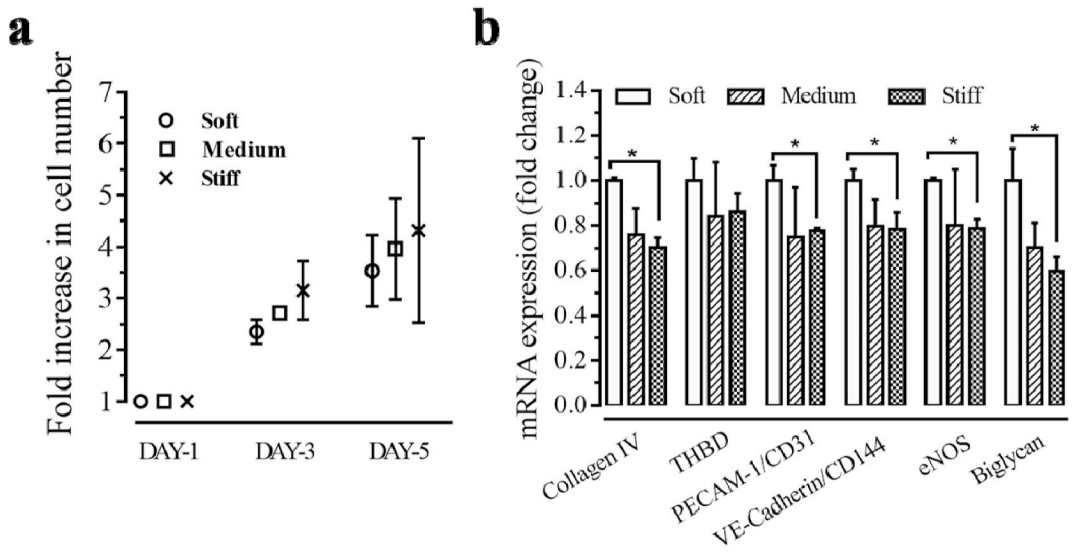

Fig. 5. Increasing of substrate stiffness reduced expression of endothelial functional genes. (a) VECs proliferation on substrates with different stiffness. (b) Endothelial function RT-PCR assay of VECs cultured on substrates with different stiffness. Cell density, 1×10^4 cm^{-2}. The data were representative of three independent experiments (mean ± SD), $^*P < 0.05$.

3.3. Increasing of substrate stiffness impairs VECs

We considered that the substrate stiffness would impact VECs, in which autophagy would be involved. To explore this, we investigated VEC proliferation and gene expressions related to endothelial function. As seen in cell numbers (Fig. 5a), VECs were proliferated well on all three films. Although not significant, stiffer film improved VEC proliferation a little; nevertheless, the VECs were grown to confluence in 5 days. We continuously cultured cells for another 2 days and collecting cells for mRNA measurement. Endothelial function correlated genes including collagen IV, THBD, PECAM-1/CD31, VE-cadherin/CD144, eNOS, and biglycan were analyzed. All these genes were higher when cells cultured on the soft film compared with cells on the medium or stiff films (Fig. 5b). For instance, the expression of eNOS and VE cadherin/CD144 of cells on soft film was 21.5% and 21.7% higher than that of cells on stiff film. These data suggest that, although the proliferative capacity and shapes of VECs were similar, the substrate stiffness do have an influence on functional molecules release, and increasing of substrate stiffness would impair VEC functionality.

3.4. Increasing of substrate stiffness induces phenotype transition of VSMCs

We further explore the influence of substrate stiffness on VSMCs. In healthy vessels, differentiated VSMCs are contractile and unresponsive to growth signals [4]. However, if the vascular microenvironment pathologically changed, VSMCs would transit from contractile (differentiated) phenotype to synthetic (proliferative) phenotype [37]. We test whether the substrate stiffness would influence phenotypic plasticity of VSMCs. The cells were cultured on the films for 3 days to achieve confluence. Like the case of VECs, the proliferation of VSMCs on three films showed almost same levels (Fig. 6c). Then, we tested cell phenotype when they cultured on the films with different stiffness. We chose calponin and vinculin as contractile and synthetic phenotype markers, respectively [38]. As seen in calponin and vinculin immunostaining (Fig. 6a, b, d, e), cells on the soft film showed much stronger calponin fluorescence than the cells on the medium or stiff films. On the contrary, cells on the stiff film showed the strongest vinculin staining than other two ones, which may suggest more robust focal adhesion of cells on the stiff substrate and favoring phenotype transition [38].

3.5. Autophagy inhibition attenuates endothelial functionality

To explore the relationship between autophagy and endothelial functionality, we treated VECs with 10 μM spautin-1 to down-regulate cell autophagic level. The cells were cultured on the soft film to achieve confluence and then switched to the spautin-1 containing medium for another 2 days. We chose the soft film as cell culture substrate because VECs on this film showed the highest autophagic level in three substrate stiffness. The confluent VEC sheet was collected for mRNA measurement. Beclin 1 and ATG5 are typical autophagy related genes that have been extensively researched in the studies for autophagic activity and cardiovascular dysfunction [15,16], the functional consequence of their mRNA modulation by inducers in vascular cells is typically investigated. As seen in Fig. 7a, spautin-1 effectively down-regulated cellular expression of Beclin-1 and ATG-5, indicating the reduced autophagic level. Accordingly, we found the mRNA expressions of Biglycan, eNOS, THBD, Collagen IV, PECAM-1/CD31, VE-cadherin/CD144 were decreased by 23.1%, 14.8%, 22.4%, 32.3%, 24.3%, and 12.8%, respectively (Fig. 7b). The data suggested that, in the case of VECs, autophagy may play a role to protect cells and in favor of endothelial functionality.

3.6. Autophagy inhibition attenuates the influence of substrate stiffness on VSMCs

Since the increasing substrate stiffness leads to reducing of VSMC autophagy, we were wondering whether autophagy inhibition would attenuate this influence. To test this, we chose VSMCs cultured on the stiff film for the 10 μM spautin-1 treatment. As shown in Fig. 8a, in 2 days treatment, mRNA expressions of Beclin-1 and ATG-5 in VSMCs were decreased by 28.0% and 15.6%, respectively, compared with control. In contrast, the expression of calponin in cells was increased by 75.2% as measured by western-blot assay (Fig. 8b and c). The increase of calponin was further demonstrated by immunostaining (Fig. 8d). Taken together, we verified that the autophagy inhibition could attenuate the influence of substrate stiffness on VSMCs and reducing phenotype transition of VSMCs.

4. Discussion

In this study, we provide evidence for the important role of substrate

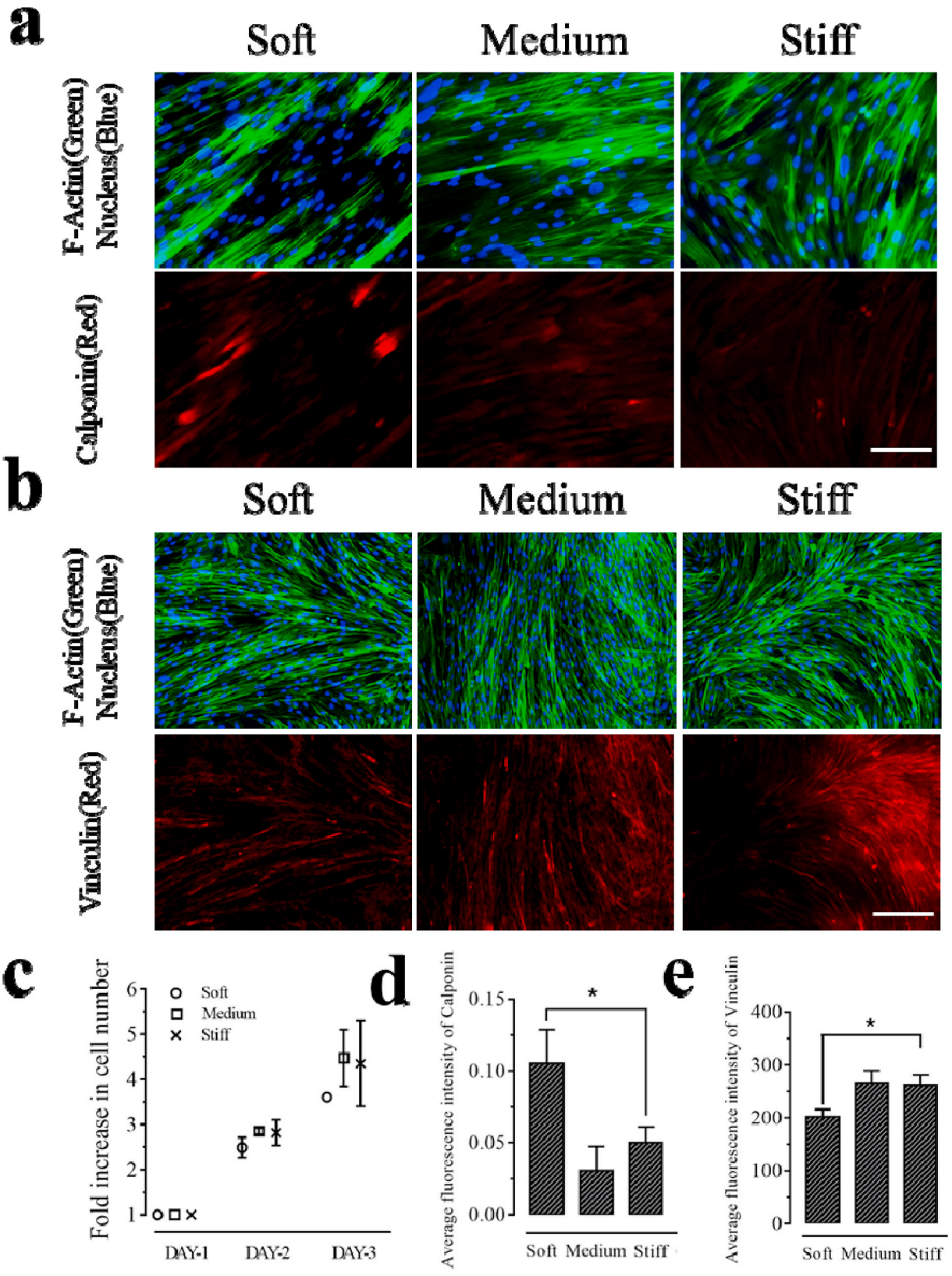

Fig. 6. Increasing of substrate stiffness reduces calponin expression in VSMCs. Immunofluorescence staining of (a) calponin (scale bar, 100 nm) and (b) vinculin (scale bar, 200 nm) in VSMCs. Cell density, 1×10^4 cm^{-2}. (c)VSMCs proliferation on substrates with different stiffness during 3-Days culture. (*d*–e) Average optical density analysis of calponin and vinculin fluorescent images. The data were representative of three independent experiments (mean ± SD), $^*P < 0.05$.

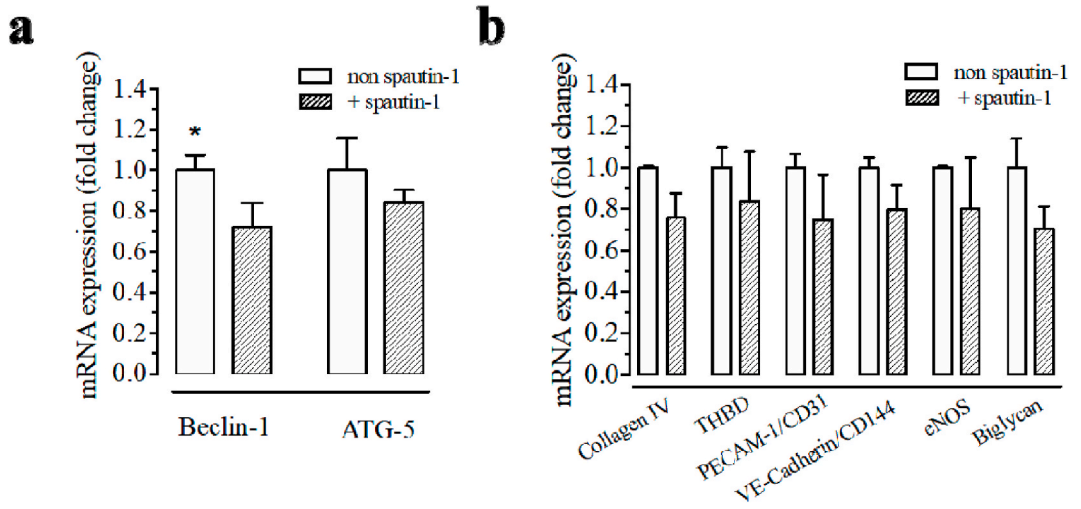

Fig. 7. Autophagy inhibition attenuates endothelial functionality. (a) Autophagy level and (b) endothelial function RT-PCR assay of VECs on soft films after addition of autophagy inhibitor spautin-1. Cell density, 2×10^4 cm^{-2}. The data were representative of three independent experiments (mean ± SD), $^*P < 0.05$.

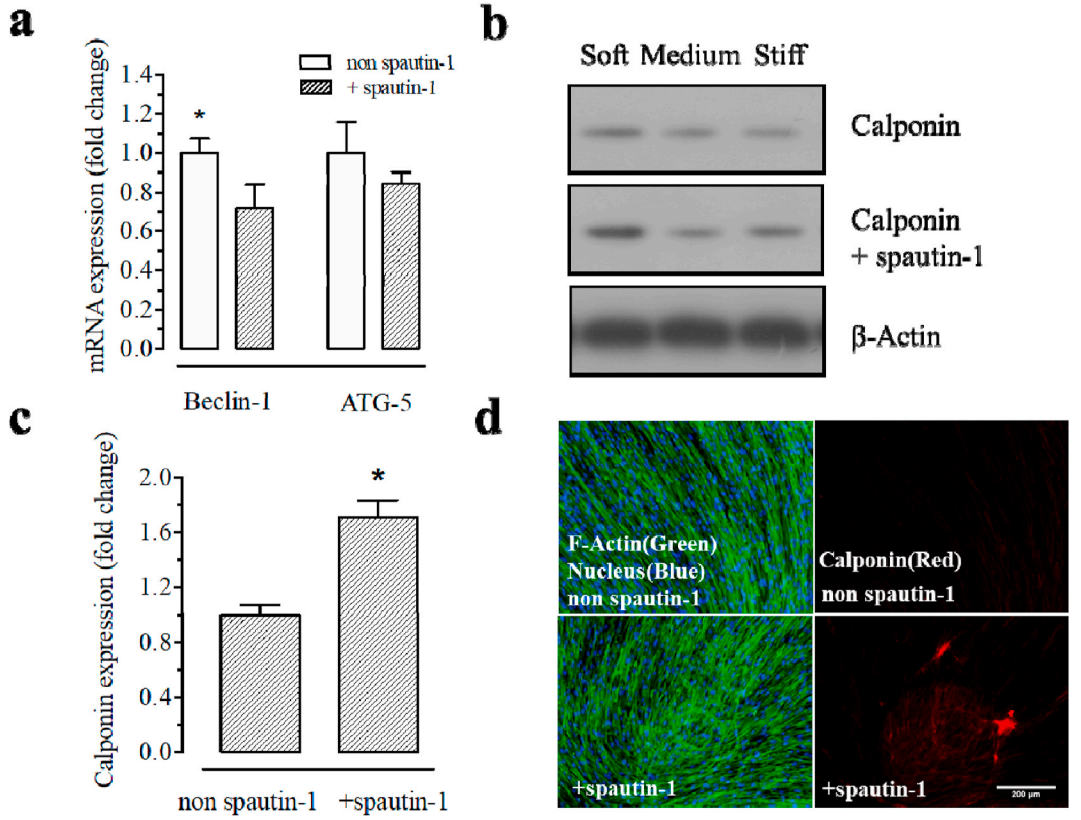

Fig. 8. Autophagy inhibition attenuates the influence of substrate stiffness on VSMCs. (a) Autophagy level of VSMCs on stiff films before and after addition of autophagy inhibitor spautin-1. (b) Calponin expression of VSMCs assayed by western blotting. (c) Quantification on western blotting results of stiff samples. (d) Immunofluorescence staining of VSMCs on stiff films before and after addition of autophagy inhibitor spautin-1. Scale bar, 200 nm. Cell density, 2×10^4 cm^{-2}. The data were representative of three independent experiments (mean ± SD), $^*P < 0.05$.

stiffness in the influence of the autophagic behavior of VECs and VSMCs. Interestingly, the changes in autophagy induced by substrate stiffness were different in VECs and VSMCs. VECs showed a higher level of autophagy and potential endothelial function when they cultured on soft substrate, suggesting a protective role of autophagy in VECs. On the contrary, VSMCs showed a higher level of autophagy on stiff substrate, which induced a transition of cells from contractile to synthetic phenotype. Our findings suggest that substrate stiffness plays an important role in vascular cells abnormalities that may contribute to the development of various CVDs such as angiosclerosis and atherosclerosis.

Cells continuously detect their surrounding microenvironment and respond accordingly [39,40]. Autophagy which is a stress responsive catabolic process can be activated by various cellular stresses such as nutrient deprivation, organelle damage, hypoxia, cytokines, and pathogen infection [5,41,42]. In recent years, materials with physicochemical properties especially nanomaterial-based agents have emerged as

inducers of autophagy. Nanomaterials, such as gold nanoparticles [43], carbon nanotube [44], silver nanoparticles [7], lanthanide-based nanocrystals [33], cerium dioxide nanoparticles [45], and graphene quantum dots [46], all showed the influence on autophagy in some certain extent. Till now, the biomechanical environment of extracellular matrix is identified to play a considerable role in various cellular processes and tissue development, however, few evidence shows the impact of stiffness on cellular autophagy. It is highly desirable to exploit whether substrate stiffness would induce the change of autophagy, since substrate stiffness has shown playing a crucial role in many cellular events and physiological/pathological processes [47,48].

As a result, both VECs and VSMCs responded to the substrate stiffness. Our data including visualization of autophagic vacuoles, and LC3 and mTOR expression have demonstrated that changes in cellular autophagy elicited by the substrate stiffness are distinct and have differential impacts on VECs and VSMCs. We showed that, with the increasing of substrate stiffness, the autophagy of VECs was downregulated (Fig. 3); while, the autophagy of VSMCs was upregulated (Fig. 4). Simultaneously, alteration expression of endothelial functional-related gene (Fig. 5) and shift of VSMCs phenotype (Fig. 6) were observed. It was previously reported that soft substrate was in favor of VECs to generate autacoids and VSMCs to maintain contractile/differentiated phenotype [21,26,38]. We observed that VECs on soft substrate showed a higher level of autophagy and endothelial function compared with cells on stiff substrate. Inhibiting VEC autophagy using spautin-1 further downregulated endothelial autacoids expression (Fig. 7). Our findings suggest that loss of autophagy may be involved in the process of which the higher substrate stiffness induces endothelial dysfunction. Similar findings were recently reported, and the difference in cell autophagic levels can be caused by other inducers such as shear stress, angiotensin-II, and glucose [49–52]. In contrast, autophagy was upregulated and contractile protein calponin was downregulated when VSMCs cultured on the stiff substrate (Fig. 4); meanwhile, cells shifted to a synthetic phenotype (Fig. 6b). Inhibiting VSMC autophagy using spautin-1 compensated the expression of calponin (Fig. 7). Previously reported data demonstrated that high intracellular tension triggered by high substrate stiffness can increase VSMC autophagosomes [53], and the autophagy suppression prevented the hyperproliferation of VSMC [54].

Autophagy is a fundamental self-digested process, and more and more latest studies confirm that it has a very important role in control cell behaviors [5,10,55]. Autophagy has been widely recognized to play a major cytoprotective role, defending the cell from damaged organelles/mitochondria, misfolded proteins/DNA, pathogens, improving the quantity of intracellular biomass, and maintaining cellular homeostasis [5]. It was previously reported that cellular autophagy could be influenced through modulating cytoskeleton [10,56]. We thus speculate that the autophagy changes of VECs and VSMCs on different substrate stiffness may also be partially ascribed to the changes in the cytoskeleton. Although the underlying mechanism needs further investigation, our findings suggest that the stiffening of blood vessels may cause vascular cells dysfunction through impairing autophagy of VECs and activating phenotype shift of VSMCs, which could speed up the development of the CVDs such as angiosclerosis and atherosclerosis. Induction of proper autophagy levels may represent a novel mechanism of the vascular protective actions. Consequently, cellular autophagy may be a potential target for cell manipulation in tissue engineering and could be used to derive new therapies in CVDs.

5. Conclusion

In conclusion, we investigated the influence of different substrate stiffness on the autophagy of VECs and VSMCs. With the increase of substrate stiffness, autophagy levels of VECs reduced, leading to the decreased endothelial function related gene release; whereas autophagy levels of VSMCs increased, showing reduced VSMC calponin expression. Through inhibiting cell autophagy, we further showed that the expressions of endothelial functional gene were further reduced and the expression of VSMC calponin increased, suggesting a vital role of autophagy in response of the cells to the challenge of microenvironment stiffness changing.

CRediT authorship contribution statement

M Hu and KF Ren designed the research. M Hu performed the experiments. M Hu, KF Ren, GS Fu, J Ji, and YB Wang analyzed the data. M Hu wrote the manuscript. KF Ren and GS Fu supervised the project and revised the manuscript. All authors substantially contributed to the research and reviewed the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2020.10.013.

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