Published in final edited form as:

Biomacromolecules. 2016 September 12; 17(9): 2767–2776. doi:10.1021/acs.biomac.6b00318.

Substrate stiffness combined with hepatocyte growth factor modulates endothelial cell behavior

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Abstract

Endothelial cells (ECs) play a crucial role in regulating various physiological and pathological processes. The behavior of ECs is modulated by physical (e.g., substrate stiffness) and biochemical cues (e.g., growth factors). However, the synergistic influence of these cues on EC behavior has rarely been investigated. In this study, we constructed poly(L-lysine)/hyaluronan (PLL/HA) multilayer films with different stiffness and exposed ECs to these substrates with and without hepatocyte growth factor (HGF)-supplemented culture medium. We demonstrated that EC adhesion, migration, and proliferation were positively correlated with substrate stiffness and that these behaviors were further promoted by HGF. Interestingly, ECs on the lower stiffness substrates showed stronger responses to HGF in terms of migration and proliferation, suggesting that HGF can profoundly influence stiffness-dependent EC behavior correlated with EC growth. After the formation of an EC monolayer, EC behaviors correlated with endothelial function were evaluated by characterizing monolayer integrity, nitric oxide production, and gene expression of endothelial nitric oxide synthase. For the first time, we demonstrated that endothelial function displayed a negative correlation with substrate stiffness. Although HGF improved endothelial function, HGF was not able to change the stiffness-dependent manner of endothelial functions. Taken together, this study provides insights into the synergetic influence of physical and biochemical cues on EC behavior and offers great potential in the development of optimized biomaterials for EC based regenerative medicine.

Keywords

endothelial cell; endothelial cell monolayer; hepatocyte growth factor; substrate stiffness; polyelectrolyte multilayer films

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1 Introduction

Endothelial cells (ECs) which line on the surfaces of blood vessel lumens act as active barriers that dynamically and locally regulate blood vessel homeostasis. Because ECs are positioned at the interface between circulating blood components and the surrounding tissue, ECs play a crucial role in regulating various physiological and pathological processes, such as angiogenesis (blood vessel growth), wound healing, embryonic development, atherosclerosis, tumorigenesis and thrombosis.^{1–4} However, abnormal EC behavior is considered a precursor for various vascular diseases.^{5–8} Therefore, the study and control of EC behavior are of vital significance in the fields of tissue engineering and regenerative medicine.

Substrate stiffness is an important physical cue of cellular extracellular matrices (ECMs) and is known to be a powerful stimulus capable of modulating cellular behavior.^{9, 10} Because ECs are anchorage-dependent cells, substrate stiffness greatly influences EC behavior.^{11, 12} For instance, increasing substrate stiffness promotes EC adhesion and proliferation, whereas reducing substrate stiffness inhibits EC adhesion and proliferation.^{11, 13, 14} Our group recently demonstrated that substrate stiffness significantly affected the EC phenotype and that a reduced substrate stiffness maintained the expressions of EC endothelial markers.¹⁵ Other studies have revealed that high stiffness substrate stimulates ECs to produce functional pro-angiogenic and osteogenic factors.¹⁶ In addition to physical cues, biochemical cues, such as growth factors (GFs), are known to greatly impact cell behavior.^{17, 18} Among them, hepatocyte growth factor (HGF) is a proangiogenic factor. Although initially discovered as a mitogen of hepatocytes,¹⁹ HGF has been demonstrated to promote EC mobility, proliferation and survival.^{20–22} These effects make HGF an important regulator of EC behavior.

The combined influences of physical and biochemical cues on cell behavior are always present in physiological environments. This raises a very interesting and important research issue in the fields of tissue engineering and biomaterials. Recently, publications have studied the combined effects of biochemical cues, such as GFs, and physical cues, such as substrate stiffness, on cell processes.^{23–25} For instance, Tan et al.²³ found that BMP-2 significantly constrained C2C12 myoblast growth on softer matrices, whereas stiffer matrices promoted osteogenesis. Grinnell et al.²⁵ demonstrated that fibroblast spreading was relatively independent of substrate stiffness in an environment rich in platelet-derived growth factor.

The aim of the present study was to investigate the effects of substrate stiffness and HGF on EC behavior. Here, we applied poly(L-lysine)/hyaluronan (PLL/HA) polyelectrolyte multilayer films with different degrees of chemical crosslinking as substrates with different stiffness.^{20, 26, 27} ECs were exposed to PLL/HA films with different stiffness with and without medium supplemented with HGF. EC behaviors, including adhesion, migration and proliferation, were first investigated. After the formation of EC monolayers, the EC behaviors that correlated with endothelial function were investigated by evaluating monolayer integrity, nitric oxide (NO) production and endothelial nitric oxide synthase (eNOS) gene expression.

2 Experiments

2.1 Multilayer film preparation and crosslinking

HA (sodium hyaluronate, MW 360,000 g/mol) was purchased from Lifecore (Chaska, MN, USA) and PLL (P2636) was purchased from Sigma (France). PLL (0.5 mg/mL) and HA (1 mg/mL) were dissolved in a Hepes-NaCl buffer (20 mM Hepes at pH 7.4, 150 mM NaCl). (PLL/HA)₁₂ films (where 12 indicates the number of layer pairs) were prepared as previously described.²⁸ Briefly, 14-mm glass coverslips were dipped in the PLL solution for 8 min. After rinsing three times with NaCl solution (150 mM), the substrates were dipped in the HA solution for 8 min. The coverslips were then rinsed again. This sequence was repeated 12 times. For 6-well plates and 96-well plates, films were fabricated starting with a first layer of poly(ethyleneimine) (branched, 25,000 g/mol, Sigma, China) at 3 mg/mL in a Hepes-NaCl buffer. The (PLL/HA)₁₂ films were crosslinked for 18 h at 4 °C using a crosslinking solution containing 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC, Sigma, France) (30, 70 or 100 mg/mL) and N-hydrosulfosuccinimide (sulfo-NHS, Sigma, France) (11 mg/mL).²⁹ After crosslinking, the films were rinsed with Hepes-NaCl buffer at least 8 times (30 min each time). The crosslinked (PLL/HA)₁₂ films are hereafter named EDC30, EDC70, and EDC100 according to the crosslinking density. The bare underlying materials (14-mm glass coverslips or plastic substrates in multiple-well plates) served as controls.

2.2 Measurement of the amount of diffusing HGF in films

The amount of HGF diffusing into EDC30, EDC70 and EDC100 was monitored by quartz crystal microbalance with dissipation (QCM-D) (Qsense, Sweden) in the dry state. The EDC30, EDC70 and EDC100 films were built crosslinked directly on the gold-coated quartz crystal outside the QCM-D chamber. After drying with nitrogen gas, the film-coated crystals were introduced in the chamber and the baseline resonant frequency f_0 (Hz) was measured. Soluble HGF at 20 ng/mL was then let to adsorb for 4 h and rinsed with PBS for 3 times. After drying with nitrogen gas, the film-coated crystals were introduced in the chamber again to reach a value f_1 (Hz). The amount of HGF was measured according to the Sauerbrey's relation 30:

$$\Delta m = -C\Delta f = -C(f_1 - f_0)$$

where C is the mass sensitivity constant (17.7 ng cm⁻² Hz⁻¹ at 5 MHz).

2.3 Cell culture

Human umbilical vein endothelial cells were purchased from Lonza (C2519A, USA). ECs were cultured in EGM™-2 Medium (CC-3162, Lonza, USA) in Petri dishes at 37 °C and 5 % CO₂. The culture medium was changed every 3 days, and ECs at 80-90 % confluence were used for further experiments. The ECs used for experiments were between 3-8 passages. All samples were sterilized by UV for 30 min before experiments. HGF was purchased from Peprotech (100-39, USA) and reconstituted according to the provider protocol. The concentration of HGF in all culture medium was 20 ng/mL.

2.4 Initial cell adhesion assay

ECs were seeded on EDC30, EDC70, EDC100, and glass coverslip controls at densities of 10000 cells/cm² with and without HGF in the medium. After 6 h culture, cells were fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized in TBS (0.15 M NaCl, 50 mM Tris-HCl, pH 7.4) containing 0.1% Triton X-100 (T8787, Sigma, St. Louis, MO, USA) for 5 min. After rinsing three times with TBS, the slides were blocked with 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) in TBS for 1 h. The cells were then incubated with F-actin (1:500, rhodamine-phalloidin, Sigma, P1951), monoclonal rabbit anti-vinculin (1:400, Sigma V9131), monoclonal mouse anti-focal adhesion kinase (FAK, 1:200, Abcam, ab40794) in TBS with 0.1 % BSA for 60 min. After rinsing three times in TBS, the samples were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:500, Invitrogen, A-11001) or Alexa Fluor 568-conjugated goat anti-rabbit IgG antibody (1:500, Invitrogen, A-11004) in TBS with 0.1 % BSA for 45 min. Finally, the nuclei were stained with DAPI (1:100, Sigma, D8417). All samples were mounted onto glass slides with antifade reagent (ProLong Gold, Invitrogen) and viewed under an Axiovert 200 M microscope or a LSM 700 confocal microscope (both from Carl Zeiss SAS, Le Pecq, France) using 10× or 100× objectives. The fluorescence images were analyzed with ImageJ software (v1.38, NIH, Bethesda). The spot sizes of focal adhesions (FAs) were calculated based on the vinculin immunofluorescence by using the NeuronJ plugin of ImageJ.

2.5 Cell migration assay

EC migration was monitored using time-lapse microscopy. ECs were seeded on EDC30, EDC70, EDC100, and multi-well plate controls at densities of 10000 cells/cm² with and without HGF in the medium. After 6 h culture, cells were imaged every 5 min for 16 h on an LSM 700 confocal microscope (Carl Zeiss Sas, Le Pecq, France) equipped with a controlled environmental chamber (37 °C and 5 % CO₂). Time-lapse images and movies were assembled using ImageJ software (<http://rsb.info.nih.gov/ij/>). Individual cell tracking was performed using the “Manual tracking” plugin, which enables the selection and tracking of a cell and its position in each frame. Each condition was studied in triplicate, and three independent experiments were performed. Approximately 100 cells were tracked for each experimental condition. All data were analyzed using “Chemotaxis tool”, which provides graphical and statistical analyses of the dataset. X/Y Calibration and Time intervals were set up based on the parameters of the time-lapse images.

2.6 Cell proliferation assay

ECs were seeded on EDC30, EDC70, EDC100, and glass slide control substrates at densities of 10000 cells/cm² with and without HGF in the medium. After 1, 2, and 3 days of culture, the cells were imaged using an Axiovert 200 M microscope (10× magnification). Cell populations were counted from at least 10 images for each experimental condition and used to calculate EC proliferation rates and increasing proliferation rates during HGF stimulation.

2.7 HGF receptor analysis

HGF receptor cMet and the phosphorylation of cMet were tested by Western blot. ECs were seeded in 6-well plates coated with EDC30, EDC70, EDC100 and control (6-well plates

only) with and without HGF supplemented in solution. After 6 h adhesion, cells were scraped, rinsed in PBS, and then lysed in T-PER (Tissue Protein Extraction Reagent; Thermo Pierce, 78510) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Pierce, 78440). After boiling, 15 μ L of total protein samples were loaded and run on 12% polyacrylamide gels before being transferred onto PVDF membranes (Millipore, IPVH00010). Membranes were then saturated in 5% BSA in TBS containing 0.1% Tween 20 for 1 h and subsequently, incubated with monoclonal antibodies against cMet (1:1000, Cell Signaling Technology, 3127) and phosphorylated cMet (p-cMet, 1:1000, Cell Signaling Technology, 3126). Membranes were washed and incubated with a peroxidase-conjugated goat anti-rabbit IgG (H+L) secondary antibody (1:5,000, Thermo Pierce, 31210). Peroxidase activity was visualized by ECL (GE, RPN810) using a ChemiDoc MP imaging system (Bio-Rad). Optical density (OD) was analyzed by BandScan 5.0 software.

2.8 Morphology of EC monolayer

ECs were seeded on EDC70, EDC100, and glass slide controls at densities of 30000 cells/cm² with and without HGF supplemented in the medium. The medium was changed every 2 days. After reaching confluence, EC monolayers were fixed, stained with primary antibodies mouse anti-human CD31 (P8590, Sigma, St Louis, MO, USA) and secondary antibodies (Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (A11029, Invitrogen, USA). Finally, samples were stained with DAPI and mounted onto glass slides using ProLong® Gold antifade reagent. EC monolayer morphologies were observed using a Zeiss Axio-vert 200 inverted microscope (10 $\times$ magnification).

2.9 Integrity of EC monolayer

To test the integrity of EC monolayers, 1 U/mL thrombin (T6884, Sigma) was added into cell medium for 15 min after EC monolayers had formed in the EDC70, EDC100, and glass slide control substrates with and without HGF supplemented in medium. Then, cells were fixed and F-actin and nuclei were stained.

2.10 NO release of EC monolayer

Cells were cultured for an additional 2 days after the formation of EC monolayers. The culture medium were collected, and the concentration of NO was measured using an NO assay kit (Boster Bio-engineering, China) according to protocol provided by the manufacturer.²⁰

2.11 eNOS gene expression analysis

eNOS gene expression analysis was carried out by real-time PCR assay. RNA was extracted from ECs after EC monolayer formations 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 eNOS. Human eNOS (NM_000603.4) primers were forward 5'-CCGAGTCCTCACCGCCTTCT-3' and reverse 5'-GGTAACATCGCCGCAGACAAA-3' with an expected product size of 142 base

pairs. PCR products were detected by Real-Time PCR Detection Systems (CFX384, Bio-Rad, USA).

2.12 Statistical analysis

All data were obtained from at least 3 independent experiments with at least three parallel samples per condition in each experiment and are expressed as means $\pm$ standard deviations (SD). Significance was assessed with ANOVA and Student's t-test. A probability value of $p < 0.05$ was considered significant.

3 Results

3.1 EC adhesion

(PLL/HA)_n films are fabricated using layer-by-layer assembly techniques and have been widely applied in the field of biomaterials and tissue engineering. These films can be easily deposited on many types of surfaces to impart new chemical and physical properties.³¹ The stiffness of the (PLL/HA)₁₂ film used in this study can be regulated by tuning the degree of chemical crosslinking.²⁸ Here, we used three differently crosslinked (PLL/HA)₁₂ films, namely EDC30, EDC70 and EDC100, with Young's moduli of approximately 200, 310 and 430 kPa, respectively,¹⁵ as stiffness-controlled substrates. Through transwell migration and proliferation assays, we found that the effects of HGF on EC migration and proliferation were dose-dependent when the HGF concentration was below 10 ng/mL (Figure S1). Above 10 ng/mL, increasing concentrations of HGF did not enhance the effectiveness of HGF on the migration or proliferation of ECs, which was consistent with previous reports.³² In this study, the concentration of HGF was fixed at 20 ng/mL. Indeed, we verified, using quartz crystal microbalance with dissipation measurements (QCM-D) ³³ that, for this HGF concentrations, there was no significant difference between the adsorbed amounts (Table S1). Indeed, a very small amount HGF was able to diffuse into the films, which was independent of the film crosslinking (EDC30, 70, 100).

Cell adhesion is the first cellular event that occurs when a cell comes into contact with a material surface. Cell adhesion has a strong influence on subsequent cellular events, such as migration and proliferation.³⁴ The initial adhesion of ECs on (PLL/HA)₁₂ films with different stiffness with and without HGF was first investigated. The EC morphologies on these different substrates are shown in Figure 1. ECs were almost round and poorly spread on EDC30. They began to spread more evenly on EDC70 and were well spread on EDC100. The EC morphologies showed no apparent differences with or without HGF on EDC30, EDC70 and EDC100. We also quantified the cell density and spreading area for each sample. The EC spreading areas were $451 \pm 259 \mu\text{m}^2$, $1037 \pm 140 \mu\text{m}^2$ and $1795 \pm 534 \mu\text{m}^2$ on EDC30, EDC70, and EDC100, respectively. In medium with HGF, the spreading areas were $566 \pm 186 \mu\text{m}^2$, $1072 \pm 130 \mu\text{m}^2$ and $1716 \pm 452 \mu\text{m}^2$ on EDC30, EDC70, and EDC100, respectively. Regardless of the crosslinking condition, HGF did not strongly influence EC spreading area and density (Figures 1B and C).

FAs were characterized by staining vinculin.³⁵ As shown in Figure 2A, no clear FA plaques appeared when ECs were cultured on EDC30. ECs on EDC70 showed few, punctate and

diffuse FAs. ECs on EDC100 showed numerous, large, elongated FAs. The presence of HGF promoted FA organization in the ECs on EDC30 and increased the FA density of ECs on EDC70. The quantification of FA size is shown in Figure 2B. The FAs were $0.5 \pm 0.2 \mu\text{m}$ long on EDC30, $1.6 \pm 0.3 \mu\text{m}$ on EDC70, and $2.9 \pm 0.7 \mu\text{m}$ on EDC100. The presence of HGF increased FA sizes to $1.1 \pm 0.3 \mu\text{m}$ on EDC30, $2.5 \pm 1.1 \mu\text{m}$ on EDC70, and $5.3 \pm 2.4 \mu\text{m}$ on EDC100. The data suggested that the presence of HGF promoted FA formation in ECs on (PLL/HA)₁₂ films, independent of the film stiffness.

3.2 EC migration

Figure 3A shows EC position tracking over 16 h. We observed that ECs migrated faster on the stiffer (PLL/HA)₁₂ films. The presence of HGF notably increased the distance and speed of EC migration. EC velocities were quantified, as shown in Figure 3B. The velocities were 30.3 ± 8.1 , 64.1 ± 8.2 , and $87.1 \pm 13.2 \mu\text{m/h}$ on EDC30, EDC70, and EDC100, respectively. In medium with HGF, the velocities increased to 93.9 ± 13.8 , 102.7 ± 12.9 , and $122.6 \pm 17.6 \mu\text{m/h}$ on EDC30, EDC70, and EDC100, respectively. Furthermore, the fold increases in EC velocity in presence of HGF on EDC30, EDC70, EDC100 films and control were calculated. The velocities were 3.1 ± 0.5 , 1.6 ± 0.2 , and 1.4 ± 0.2 -folds greater in HGF-supplemented medium on EDC30, EDC70, and EDC100, respectively. Overall, HGF had a stronger influence on migration for the softer (PLL/HA)₁₂ films.

3.3 EC proliferation

EC proliferation on the (PLL/HA)₁₂ films with and without HGF was quantified (Figure 4 and Figures S3 and S4) and appeared to be stiffness-dependent. The proliferation rates were highest on EDC 100 and lowest on EDC30. In fact, ECs on EDC30 did not show any proliferative capacity. Instead, the EC population gradually decreased over the 3 days of culture. With HGF stimulation, although ECs were alive on EDC30, they were not able to proliferate over 3 days. For the ECs on the EDC70 and EDC100 substrates, the proliferation rates were stiffness-dependent and were further significantly enhanced in the presence of HGF. The EC proliferation rate increased 2.4 ± 0.3 -folds on EDC70, 1.5 ± 0.1 -folds on EDC100 and 1.4 ± 0.1 -folds on the control (Figure S4). HGF had the strongest influence on the proliferation rates of softer (PLL/HA)₁₂ films.

3.4 Expression of HGF receptor on ECs

HGF is specific only to its cMet.³⁶ Upon binding, cMet is phosphorylated and downstream intracellular signals are activated, further inducing a series cell processes, such as migration and proliferation.³⁷ cMet and its phosphorylated counterpart (p-cMet) were detected by Western blot, as shown in Figure 5. In the absence of HGF, p-cMet in ECs on all substrates were expressed at very low levels. In the presence of HGF, p-cMet was strongly up-regulated in ECs on all substrates, thereby confirming that the presence of HGF notably activated its receptor.

3.5 Morphology of EC monolayer

The morphologies of the EC monolayers formed on the EDC70, EDC100, and control substrates with and without HGF-supplemented medium was checked by staining with

CD31 (with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody, green) and DAPI (blue). The resultant fluorescent images after merging two color channels are shown in Figure 6. The EC monolayers on the EDC70 and EDC100 substrates exhibited clearer and more continuous distributions of CD31 than the EC monolayers on the control. However, there were no significant differences in cell number among these substrates (data not shown). The EC monolayers on EDC70 and EDC100 with HGF displayed more confluent and flat morphologies than those on EDC70 and EDC100 without HGF. Additionally, the ECs of the monolayers on EDC70 and EDC100 with HGF were regularly aligned and elongated. The ECs of the monolayers without HGF displayed no obvious alignment and orientation. While a higher CD31 expression was observed in the ECs on the control substrate with HGF than those on the control substrate without HGF, the distribution of CD31 at the EC borders were discontinuous.

3.6 Integrity of EC monolayer

The integrity of the EC monolayers was evaluated using thrombin, a special protease produced by injured endothelium. Thrombin disturbs the barrier function of endothelia³⁸ and has been widely used to check the integrity of endothelia.^{39, 40} Before treating with thrombin, EC monolayers on the EDC70, EDC100, and control substrates (with or without HGF) were verified for confluency (Figure 7). After treatment with thrombin, neighboring ECs on EDC70 remained tightly connected and showed few stress fibers; a few small holes in the monolayer were observed. For the EC monolayer on the EDC100 substrate, more stress fibers developed within the cells, and small cell-cell disruptions were observed. ECs on the control substrate showed much more stress fibers, and large holes in the EC monolayer were observed. For all types of substrates, there were no obvious differences in the integrity of the EC monolayers between cultures treated with and without HGF after treatments with thrombin.

3.7 NO release and eNOS gene expression

The production of NO, an important signaling molecule involved in many physiological processes, is one of the most significant functions of the vascular EC monolayer.⁴¹ As shown in Figure 8, the concentration of NO released in the culture medium by a single EC was 0.27 ± 0.06 nM on EDC70, 0.18 ± 0.02 nM on EDC100 and 0.06 ± 0.04 nM on control. In the presence of HGF, the concentration of NO released in culture medium by a single EC was 0.36 ± 0.05 nM on EDC70, 0.28 ± 0.03 nM on EDC100, and 0.14 ± 0.03 nM on control. NO is produced from L-arginine in a reaction catalyzed by eNOS. Here, we detected eNOS gene expression by RT-PCR, as shown in Figure 8B. The relative expressions of the eNOS gene displayed a similar trend as that of the NO release results.

4 Discussion

The aim of this study was to investigate the effects of combined physical (substrate stiffness) and biochemical cues (HGF) on EC behavior. The (PLL/HA)₁₂ film is a mature platform. The physico-chemical properties of this film have been investigated and reported by several groups,^{28, 29, 42, 43} including ours.^{15, 44} The (PLL/HA)₁₂ film was fabricated according to a reported protocol²⁸ and the physico-chemical properties of this film were thus fixed.

This film is an exponentially-growing layer-by-layer film with a thickness of $\sim 1 \mu\text{m}$ for a film made of 12 pairs of layers.^{44, 45} After being crosslinked with EDC/NHS, (PLL/HA) films were stable in culture medium and able to support cell culture for several weeks.³³ More importantly, the stiffness of this film can be regulated by tuning the concentration of the EDC crosslinker.^{15, 28} Here, we thus used these films crosslinked at different levels as stiffness-controlled substrates. Importantly, in the condition of soluble HGF, a negligible adsorption was noted, which was independent on the crosslinking level (Table S1). This is consistent with the fact that both HGF and the films are negatively charged in the cell culture medium (pH ~ 7.4). Indeed, the isoelectric point of HGF is 5.5⁴⁶ and it has previously been shown that the surface of crosslinked (PLL/HA) films is negatively charged.⁴⁷

EC adhesion, migration and proliferation were evaluated on the stiffness-controlled substrates with and without HGF. EC adhesion, migration and proliferation expectedly displayed positive correlations with the substrate stiffness (Figures 1-4), which was consistent with other studies.^{13–15, 48} The presence of HGF promoted EC migration and proliferation on substrates with different stiffness (Figures 1-4), which was attributed to the biochemical effects of HGF.^{20, 49} Moreover, HGF promoted EC adhesion by stimulating the formation of FA and FAK with ECs (Figure 2 and Figure S2), as was observed in a previous report.⁵⁰ However, ECs were unable to normally grow into EC monolayer on EDC30 even in the presence of HGF. One could observe that ECs on EDC30 with HGF were still not well spread (Figure S3). Cell spreading is tightly coupled to proliferation and restricting the spreading area of adherent cells can lead to growth arrest.⁵¹ Thus, we speculated that this inhibited proliferation resulted from the poor adhesion and spreading of ECs on EDC30 films.

Interestingly, the effectiveness of HGF on ECs was stiffness-dependent. ECs on softer films showed stronger migration and proliferation responses to HGF than ECs on stiffer films and the control. This indicates that HGF profoundly influenced the stiffness-dependent growth behaviors of EC. Similar stiffness-dependent effects of GFs have also been reported by Chen and coworkers.²⁴ Importantly, although EC growth was correlated with substrate stiffness in the presence of HGF, the presence of HGF decreased the stiffness-dependent difference in growth. These phenomena could be associated with cMet, the only HGF receptor on ECs, as confirmed by the characterization of p-cMet (Figure 5). The expression level of p-cMet in ECs was up-regulated to a similar level after HGF stimulation independent of the substrate stiffness. This may also be related to the observation that the effectiveness of HGF was stronger on the migration and proliferation of ECs on a softer substrate. This indicates that biochemical cues can weaken the dependency of EC growth behavior on substrate stiffness. Our results agreed with previous findings that biochemical cues can override dependencies on substrate stiffness on cellular processes.^{25, 52}

Effects on EC monolayer morphology were also checked. In our study, the EC monolayer was not able to form on EDC30 because of inhibited proliferation (Figure 4). Therefore, there was no result related to evaluation of EC monolayer on EDC30. The morphologies of the EC monolayers on the EDC70 and EDC100 substrates were similar. There was no significant difference in the CD31 expression levels. This was consistent with our recent findings that the percentages of CD31-positive ECs were similar on the EDC70 and EDC100

substrates.¹⁵ However, the EC morphologies on the EDC70 and EDC100 substrates were quite different from those on the control substrate, regardless of the presence of HGF (Figure 6). The glass control had a GPa stiffness order, i.e., much higher than the stiffness levels of the (PLL/HA) films. However, because the chemical composition of the control was different from those of the (PLL/HA) films, the large difference in stiffness could not necessarily be linked with the observed difference in EC morphology. The ECs in the monolayers on the EDC70 and EDC100 substrates with HGF were well-aligned and elongated. Aligned and elongated ECs have been correlated with resistance to inflammatory reactions, whereas nonaligned or cuboidal ECs seem to promote atherosclerosis.^{53, 54} Therefore, a fully confluent EC monolayer with physiological EC alignment and elongation was obtained on the EDC70 and EDC100 substrates with HGF.

The EC behaviors correlated with the endothelial functions of EC monolayers were investigated. The endothelial functions of EC monolayers are important parameters to consider in evaluating EC behaviors because the complete formation of an EC monolayer is not synonymous with the full restoration of its endothelial functions.^{55–57} Endothelial dysfunction has been associated with severe complications, such as late-thrombosis and neoatherosclerosis.^{58, 59} Endothelial function was generally evaluated in terms of EC monolayer integrity, expression of antithrombotic molecules and NO production.⁵⁹

The physiological endothelium requires a high barrier integrity to be resistant to undesirable external stimuli and is mostly dependent on the regulation of tight intercellular junctions.⁶⁰ Our results show that the EC monolayer integrity was negatively correlated with substrate stiffness, and EC monolayer maintained higher integrity on the substrate with lower stiffness (Figure 7). The presence of HGF did not alleviate the disruption of the EC monolayer after thrombin treatment. Actually, the interactions among ECs increase, and the influence of substrate decreases after formation of EC monolayer. However, cell-cell interaction was influenced by substrate stiffness.⁴⁰ A high stiffness substrate can increase cell-substrate interactions and weaken cell-cell interaction, resulting in the easier separation of neighboring ECs by thrombin stimuli.^{61, 62} Consequently, the endothelium permeability increases and further exacerbates inflammatory responses that could increase the risk of atherothrombosis.⁶³ This result corresponded with fact that high stiffness leads to poor and sensitive integrity of EC monolayer.^{39, 40}

NO is one of the most important vasoregulatory factors and antithrombotic molecules.⁴¹ HGF could promote the NO production ability. However, NO production was also negatively correlated with stiffness. The EC monolayers on lower stiffness substrates displayed higher NO production ability (Figure 8A). These results were consistent with the expression trend of eNOS genes as tested by RT-PCR (Figure 8B). Our results indicated that HGF improved the endothelial function and that endothelial function displayed a negative correlation with substrate stiffness. With respect to endothelial function, HGF did not influence its stiffness-dependent manner. Our study is the first to reveal and highlight the influence of stiffness on the endothelial function of the EC monolayer. A reduced substrate stiffness would favor endothelial function. Because the matrix on which endothelium resides in natural blood vessels is much softer than traditional implant materials, soft substrates should be used to develop healthy endothelium for biomimetic applications. Apart from ECs, the benefits of

lower stiffness substrates on other cells have been reported by other studies. For example, Van Vliet et al. demonstrated that the phenotypic functions of primary hepatocytes were up-regulated on soft substrates.⁶⁴ Taken together, this study revealed that physical (stiffness) and biochemical cues (HGF) exhibited synergistic influences on EC behavior (Scheme 1).

The influence of stiffness together with growth factors (including platelet-derived growth factor (PDGF), TGF- β 1 and BMP-2) on the behavior of specific types of cells (including fibroblasts, chondrocyte and C2C12 myoblast cells) has also been studied by other groups.^{23–25} They found that the presence of growth factors was able to impact the stiffness-dependent manner of cell behaviors. For example, in PDGF-containing medium, fibroblast spreading became relatively independent of substrate stiffness.²⁵ In this study, we firstly investigated the stiffness together with HGF on EC behaviors. In terms of adhesion, migration and proliferation, HGF greatly impacted the stiffness-dependent response, which was consistent with those previous studies. However, we found that, in terms of endothelial functions of EC monolayer, HGF did not change its stiffness-dependent response. Actually, when designing biomaterials for endothelialization, endothelial functions of regenerated EC monolayer should be seriously taken into consideration,⁵⁹ as well as formation process of EC monolayer (EC adhesion, migration and proliferation).⁶⁵ Therefore, our findings provide information with great potential for designing optimized biomaterials in the field of vascular tissue engineering and cancer.

5 Conclusion

In this study, ECs were exposed to stiffness-controlled (PLL/HA)₁₂ films with and without HGF supplemented in the culture medium to investigate the influence of substrate stiffness and HGF on EC behavior. The adhesion, migration and proliferation of ECs displayed positive correlations with substrate stiffness and were further promoted in the presence of HGF. ECs on softer substrates showed stronger migration and proliferation responses to HGF than those on stiffer substrates, suggesting that HGF profoundly impacted the stiffness-dependent behaviors correlated with EC growth. Furthermore, EC behaviors correlated with endothelial function of subsequently formed EC monolayers displayed a negative correlation with substrate stiffness. The presence of HGF improved the endothelial function but did not influence the stiffness-dependent behavior of endothelial function. This study suggested that physical and biochemical cues synergistically influence EC behavior and should be considered when designing effective biomaterials in the field of EC-based regenerative medicine.

Associated Content

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Acknowledgment

This research was supported by Zhejiang Provincial Natural Science Foundation of China under Grant No. LR15E030002, the Key Science Technology Innovation Team of Zhejiang Province (no. 2013TD02), the National Natural Science Foundation of China (51333005, 21374095), the National Basic Research Program of China (2011CB606203), Research Fund for the Doctoral Program of Higher Education of China (20120101130013), International Science & Technology Cooperation Program of China (2014DFG52320), State Key Laboratory of

Molecular Engineering of Polymers (Fudan University) (K2015-10) and PHC CAI YUANPEI (N° 27947ZL). CP acknowledges the European Commission (EC, FP7) for a grant from the European Research Council (GA259370).

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Figure 1. EC adhesion after 6 h culture. Images of actin in ECs on EDC30, EDC70, EDC100 films and control with and without HGF supplemented in medium (A). Cell density (B) and spreading area (C) of ECs quantified after 6 h culture. At least 200 cells were counted for each condition. N = 3 parallel samples per group. The data are representative of three independent experiments, ns: no significant difference. The scale bar is 100 μ m.

Figure 2.

(A) Immunofluorescence images of ECs stained by vinculin (a focal adhesion marker) on EDC30, EDC70, EDC100 films and control with and without HGF supplemented in medium after 6 h adhesion. (B) The spot sizes of focal adhesions (FAs) were quantified from the fluorescent images of FAs. N = 3 parallel samples per group. At least 30 cells were analyzed per sample. The data are representative of three independent experiments, mean $\pm$ SD, * $p < 0.05$. The scale bar is 100 μm .

Figure 3. Influence of HGF on EC migration over 16 h on different stiffness surfaces. (A) Representative migratory trajectories of ECs on EDC30, EDC70, EDC100 films and control with and without HGF supplemented in medium. (B) Migration velocities of ECs on EDC30, EDC70, EDC100 films and control with and without HGF supplemented in medium. N = 3 parallel samples per group. The data are representative of three independent experiments, mean $\pm$ SD, * $p < 0.05$.

Figure 4. Proliferation profile of ECs on EDC30 (A), EDC70 (B), EDC100 (C) films and control (D) with and without HGF supplemented in medium. N = 3 parallel samples per group. At least 8 images were analyzed per sample. The data are representative of three independent experiments.

Figure 5.

Western blot analysis of HGF receptor cMet. (A) The bands of phosphorylated cMet (p-cMet) of ECs on EDC30, EDC70, EDC100 films and control with and without HGF supplemented in medium. (B) Quantification of p-cMet expression using densitometry, normalized by the expression of cMet. N = 3 parallel samples per group. The data are representative of three independent experiments, mean $\pm$ SD, * $p < 0.05$.

Figure 6. Morphology of EC monolayers formed on EDC70, EDC100 films and control with and without HGF supplemented in medium. The immunofluorescence images of EC monolayer were obtained by staining CD31 (green) and DAPI (blue). The scale bar is 200 μm .

Figure 7.

Integrity of EC monolayers on EDC70, EDC100 films and control with and without HGF supplemented in medium. The fluorescent images of EC monolayers were obtained by staining for F-actin (red) and DAPI (blue). 1 U/mL thrombin was added for 15 min. The white arrows refer to the holes in EC monolayers after treatment with thrombin. The scale bar is 50 μm.

Figure 8.

(A) Concentration of NO released in culture medium per cell and (B) relative endothelial nitric oxide synthase (eNOS) expression of EC monolayers on EDC70, EDC100 films and control with and without HGF supplemented in medium. N = 3 parallel samples per group. The data are representative of three independent experiments, mean $\pm$ SD, * p < 0.05.

Scheme 1.

Illustration of influence of substrate stiffness combined with HGF on EC behaviors, including EC growth (A and B) and endothelial function of EC monolayers (C and D).