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Co-culture of endothelial cells and patterned smooth muscle cells on titanium: Construction with high density of endothelial cells and low density of smooth muscle cells

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ABSTRACT

Endothelialization has been considered a promising method to improve the biocompatibility of vascular implanted biomaterials. However, little is known about the anti-coagulation, anti-inflammatory, anti-atherosclerosis and anti-shedding property of the attached endothelial cells (ECs) and the relationship with their bio-environment and material-environment, which are both important evaluations to the cardiovascular biomaterials designed for tissue engineering applications and *in vivo* implantation. In this *in vitro* study, a novel co-culture model was built, where vascular smooth muscle cells (SMCs) were cultured on the hyaluronic acid (HA) micro-strip patterned titanium (Ti) surface on a low density to biomimetic the EC pericyte environment. Subsequently, the EC number and its functional factor, including nitric oxide (NO), prostacyclin (PGI_2), tissue factor pathway inhibitor (TFPI), thrombomodulin (TM), and the inflammatory induced factor, endothelial leukocyte adhesion molecule-1 (E-selectin) were quantified, respectively. The anti-shedding property was also assessed by the blood flow shear stress (BFSS) acting. The results showed that the novel co-culture model possessed better EC coverage, functional factor release and anti-shedding functions than the control.

1. Introduction

Cardiovascular diseases (CVDs) are not only the first cause of death worldwide, but also the leading cause of death in modern society [1]. According to World Health Organization (WHO) statistical data, there are about 17 millions of people who die from CVDs each year on a global scale [2]. After more than 30 years of development, percutaneous coronary intervention (PCI) has gradually matured and become a main treatment of CVDs. A lot of cardiovascular implants have been developed for the therapeutic of CVDs, including vascular grafts, stents, and artificial heart valves, et al. Nevertheless, a remarkable number of patients (up to 20%-30%) develop restenosis at the 3rd to 6th month after the PCI operation [3]. Most research attribute neointima formation and in stent restenosis to mechanical injury caused during the operation and the subsequent endothelial cell dysfunction, as well as thrombosis at the site of injury and smooth muscle cell (SMC) proliferation and migration [3]. In fact, the problem should be attributed to the inadequacy of the cardiovascular implant function on long-term antithrombotic and anti-hyperplasia which is relevant to their own biocompatibility [4]. Recent research generally consider that a confluent layer of endothelial cells (ECs) is the best surface to prevent adverse cardiac events [5]. Therefore, surface endothelialization has become the research hot tip of biomedical device surface modification and attracts more and more attention.

As mentioned above, surface endothelialization is an effective methodology for improving the biocompatibility of cardiovascular implants. Thus, biomaterials for improving their endothelial cell coverage are receiving increasing attention [6,7]. However, it is well known that endothelial cell function is influenced by complex vascular microenvironments, including extracellular matrix [8], pericytes [9], blood flow shear stress [10], etc. Smooth muscle cells (SMCs) as an important pericyte of endothelial cell, exist in two main types of cell forms: one type distributes around the vessel wall in contractile phenotype, constructing the middle membrane of the vascular [11]; the other type marginally distributes in the extracellular matrix between ECs and SMCs, and orients along the blood flow shear stress [12]. For decades, Several co-culture models have been developed to mimic the pericyte environment of ECs [12]. However, SMCs of these co-culture models have not been cultured in a bionic environment. So, building a biomimetic

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pericyte environment of ECs on the vascular implants' surface in the purpose of enhancing their physiological functions may be a better method.

In the present study, the ECs were co-cultured with low density micro-patterned SMCs on titanium (Ti). The anti-coagulation and anti-hyperplasia factor (NO, PGI_2, TM and TFPI) and inflammation relevant factor (E-selectin) of the attached endothelial cells were determined and evaluated, respectively. Additionally, the cell anti-shedding ability, as an important function, after exposed to the blood flow shear stress (BFSS) was also investigated.

2. Materials and methods

2.1. Micro-patterned surfaces

MP silicon templates with 25 μm wide lanes and 25 μm spacing were fabricated previously. Poly-dimethylsiloxane (PDMS) stamps were made from silicon templates by mixing at a 10:1 wt/wt ratio the 184-elastomer base to curing agent Sylgard, Dow Corning), and polymerizing at 120 °C. PDMS stamps were inverted on Ti substrates which were treated by 1 M NaOH solution at 80 °C for 24 h (samples labeled as TiOH), thereby creating micro-strips (samples labeled as TiOH/HAP), which were incubated for 24 h with 5 mg/ml hyaluronan (1×10^6 Da, Sigma). For non-patterned surfaces, TiOH substrates were used as control. The whole fabrication process is displayed in Fig. 1. The surface morphology of the TiOH and TiOH/HAP samples was characterized by Alcian-Blue-staining method and observed under a light microscope [13].

2.2. Smooth muscle cell and endothelial cell isolation

Primary culture of human umbilical artery smooth muscle cells (HUASMCs) and Human umbilical vein endothelial cells (HUVECs) were created by a conventional method which was described in the previous work [14,15], and the 3rd generation of HUASMCs and HUVECs were used in the experiment.

2.3. HUASMC density optimization for HUASMCs/HUVEC co-culture

HUASMCs in 5 different densities, 1×10^5 cells/ml, 5×10^4 cells/ml, 2.5×10^4 cells/ml, 1.25×10^4 cells/ml and 0.625×10^4 cells/ml, were seeded on the TiOH/HAP samples respectively, and cultured for 4 h, 1 day and 3 days each. A cck-8 assay was used to investigate the HUASMC proliferation. The medium was removed and the samples were washed three times with PBS. Then, fresh medium (have no phenol red) containing CCK-8 reagent was added to each sample and incubated at 37 °C for 4 h in standard culture conditions. Afterward, 170 μl of the blue solutions were transferred to a 96-well plate. The absorbance was measured at 570 nm by a micro-plate reader (BIO-TEK Instruments, USA). All proliferation experiments were performed in triplicate, and the seeded density of HUASMCs which caused a stable proliferation was chosen for HUASMC/HUVEC co-culture.

2.4. Co-culture of patterned HUASMCs and HUVECs

Cell Trackers with two different colors (red and green) were used in the whole co-culture process to distinguish the co-cultured HUASMCs and HUVECs clearly. The Tracker with red color was used for HUASMCs while the Green one was used for HUVECs, and the cells were firstly marked by the trackers before seeded. Briefly, HUASMCs were seeded on the patterned substrates at the density chosen in Section 2.3 and incubated at 37 °C for 4 h. After removing the culture medium and a tightly rinsed step with warm PBS (Sterile, pH 7.2), the micro-patterned HUASMCs were incubated with 50 μg/ml collagen IV (Sigma) for 1 h. Thereafter, HUVECs were plated onto the collagen IV layer at the density of 1×10^5 cells/ml and incubated at 37 °C for 1 day and 3 days, respectively. After rinsing three times with PBS in 37 °C, the cells were observed by fluorescence microscope. HUVEC number from 25 random optical microscope fields was counted and calculated statistically [16].

2.5. NO, PGI_2, thrombomodulin and tissue factor pathway inhibitor release of HUVECs

The amount of NO, PGI_2, thrombomodulin (TM) and tissue factor pathway inhibitor (TFPI) released from HUVECs on each sample were detected to evaluate the anticoagulation and inhibitory atherosclerosis properties of the HUVECs preliminarily. The NO, PGI_2, TM and TFPI release was examined by the methods as described in the previous work [17]. The amount of NO, PGI_2, TM and TFPI were finally normalized to cell number.

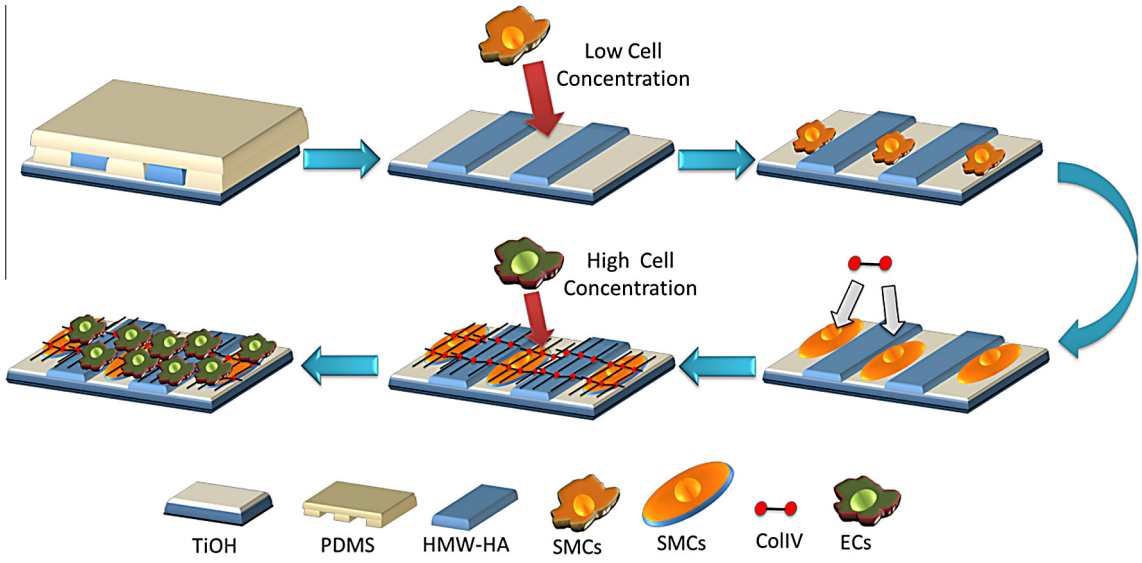

Fig. 1. The scheme of co-culturing endothelial cells and smooth muscle cells on TiOH/HAP substrate.

2.6. Investigation of HUVEC lesion

The endothelial cell activation, damage and dysfunction are related to the inflammation, thrombosis and further atherosclerosis [18]. E-selectin, an endothelial cell-specific surface molecule, has been proven to be involved in the endothelial cell activation, damage and dysfunction through its continuous upon expression [19]. Thus, detection of E-selectin was performed to evaluate the HUVEC activation and damage in this work. E-selectin secreted by HUVECs on the co-culture model and TiOH samples were examined by the related kits according to the manual after 1 and 3 days of incubation, respectively. The specific factor E-selectin was finally normalized to cell number.

2.7. Retention of HUVECs

Retention of HUVECs in this co-culture model was detected using the previous method [16], and retention of HUVECs cultured on the TiOH samples was used as control.

2.8. Statistical analysis

Mean values ± SD are given with representative images. Statistical significance required a p value <0.05.

3. Results and discussions

3.1. Characterization of micro-patterned cell template

Light microscopy photographs of the samples after Alcian-Blue-staining were obtained to show the morphology of the TiOH/HAP and TiOH (Fig. 2A). It could be seen from Fig. 2A-a that there was a uniformly porous surface on the TiOH samples which were fabricated by 1 M NaOH of 80 °C for 24 h. This structure might help for the immobilization of the HA micro-strips and the maintenance of the HUASMC contractile phenotype. The breadths of the TiOH strip (yellow strip) and HA strip (green strip) on the TiOH/HAP samples were also depicted. In this work, the breadth of the strips was still both 25 μm (Fig. 2A-b), consistent with the previous parameter [12,16]. The reason was that the micrometers ranging from 20 to 30 μm would be closer to the physiological state for HUVECs [12,17]. The light microscopy results might be the evidence for confirming integrity of the micro-pattern.

3.2. HUASMC density optimization for HUASMCs/HUVEC co-culture

In our previous work, The co-culture model of micro-patterned HUASMCs and HUVECs was successfully built on the Ti surface and the physiological functions of the HUVECs were evaluated [12,16]. However, the HUVECs could not completely cover the HUASMCs, and the reason suggested was the cell densities. It was reported by Tu et al. that HUVECs with high density would completely cover HUASMCs with low density [20], but HUASMCs with extremely low density could not survive on the TiOH/HAP surface, and the density should be suitable for the micro-pattern surface. Therefore, the HUASMC density optimization experiment was designed to choose a suitable HUASMC density for building the co-culture model on the TiOH/HAP surface.

The effect of TiOH/HAP on the attachment and proliferation of HUASMCs with 5 different densities (1×10^5 cells/ml, 5×10^4 cells/ml, 2.5×10^4 cells/ml, 1.25×10^4 cells/ml, 0.625×10^4 cells/ml) was investigated by a cck-8 assay after incubation for 4 h, 1 day and 3 days, respectively. Fig. 2B shows the amount and proliferation of HUASMCs with 5 different densities on the TiOH/HAP surface. The absorbance was proportional to the amount of the cells, the more the cells on the surface, the larger the absorbance. It could be seen from the cck-8 results that the HUASMCs with the density of 1×10^5 cells/ml and 5×10^4 cells/ml displayed excessive growth tendency after 1 day and 3 day culture, while the HUASMCs with the density of 1.25×10^4 cells/ml and 0.625×10^4 cells/ml showed no better proliferation activity, especially for the HUASMCs with the density of 0.625×10^4 cells/ml who presented worse growth appearance. Thus, HUASMCs with the density of 2.5×10^4 cells/ml which displayed a stable growth tendency during 4 h, 1 day and 3 day culture were chosen for the co-culture with the HUVECs.

3.3. Co-culture of patterned HUASMCs and HUVECs

It is well known that cardiovascular wall contains both endothelial cells (ECs) and vascular smooth muscle cells (SMCs) [12]. ECs, which provide an interface between the blood and the vessel wall, are constantly subjected to the influences of SMCs beneath. It has been reported by Wang et al that SMCs can promote EC adhesion via microtubule dynamics and activation of paxillin and the extracellular signal-regulated kinase pathway [9]. Therefore, pericyte environment of SMCs plays an important role for the implanted device surface endothelialization. In the present study, a novel co-culture model of ECs (high density, 1×10^5 cells/ml) and SMCs (low density, 2.5×10^4 cells/ml) which followed our previous work [12,16] was built successively. Fig. 3A shows the morphology and behavior of both HUVECs and HUASMCs on each sample after culture of 1 day and 3 days, respectively. The HUASMCs (red) in the co-culture model still exhibited long spindle-shaped morphology and formed several long stripes, and the co-cultured HUVECs (green) still distributed in the HUASMCs-blank area at the 1st day. However, after 3 days of culture, the co-cultured HUVECs completely covered the micro-patterned HUASMCs as expected previously, despite not showing a spreading or elongated morphology. Fig. 3B shows the HUVEC amount on each sample after culture of 1 day and 3 days, respectively. Significantly different from the co-culture models in the previous work [12,16], the novel one possessed a remarkable advantage of HUVEC amounts compared with the TiOH sample, indicating an effective approach to enhance the HUVEC coverage. Nevertheless, the anti-coagulation function of the HUVECs in the co-culture model should be further evaluated.

3.4. Anti-coagulation factor release

It is widely accepted that nitric oxide (NO) plays a significant role in cardiovascular regulation through the modulation of vascular tone and endothelial function [21], while the latter mainly reflected in the anticoagulation and inhibiting smooth muscle proliferation and migration. Thus, the NO release was detected as a metric of the HUVEC function in the novel co-culture model. Fig. 4A shows the NO release amount of HUVECs on the micro-patterned HUASMCs and TiOH surface, respectively. Obviously, HUVECs on the micro-patterned HUASMCs released more NO compared with HUVECs on the TiOH surface. NO is produced from L-arginine by NOS [22]. There are three isoforms of NOS, namely eNOS, neural constitutive nitride oxide synthase (nNOS), and iNOS responsible for NO biosynthesis [22], while the eNOS are the master NO synthases in vascular ECs [22]. PI3 kinase/Akt kinase pathway has been considered the main regulation which can affect the eNOS activation [23]. The presence of SMC exposed fluid flow shear stress or elongated by micro-pattern can stimulate the PI3 kinase/Akt kinase of ECs up-regulated via TGF-β1 secretion [24], and further eNOS activation, then more NO released.

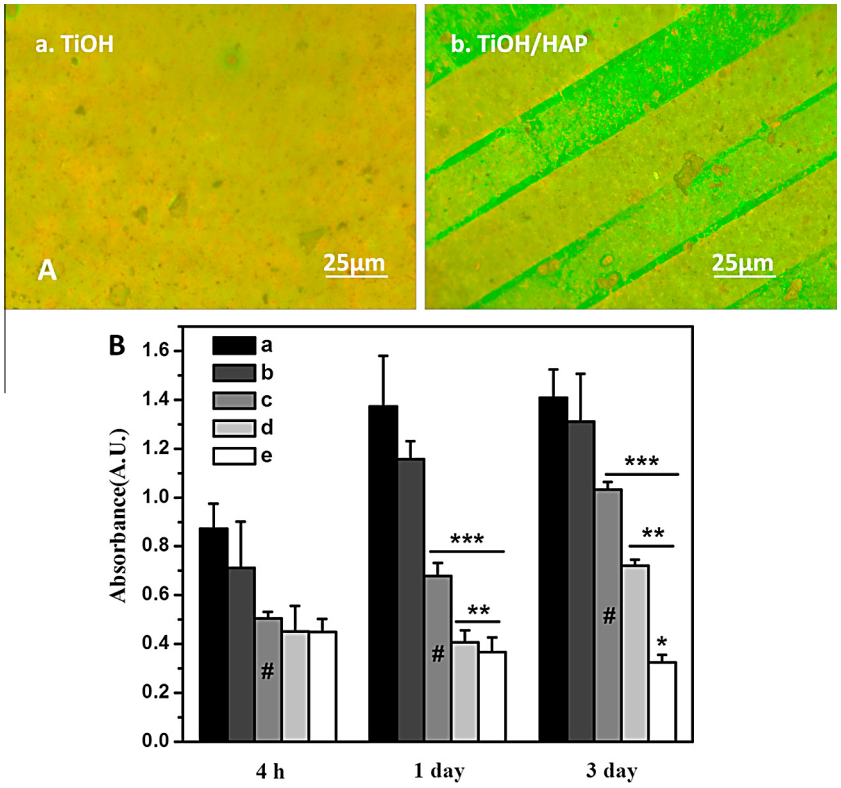

Fig. 2. (A) Light microscopic images of Alcian-Blue-stained HA micro-patterns: (a) TiOH and (b) TiOH/HAP (Yellow section: alkali activated titanium surface; green section: HA micro-patterns); (B) Investigation of SMC proliferations on TiOH/HAP samples after incubation for 4 h, 1 day and 3 days, respectively. The seed densities of the SMC were different: (a) 1×10^5 cells/ml; (b) 5×10^4 cells/ml; (c) 2.5×10^4 cells/ml; (d) 1.25×10^4 cells/ml; (e) 0.625×10^4 cells/ml. The # marked group was the SMC density applied in the EC/SMC co-culture. ($N = 3$, mean ± SD, $^{*}p < 0.05$ compared with d, $^{**}p < 0.05$ compared with c, $^{***}p < 0.05$ compared to (a) and (b)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The prostacyclin (PGI_2) detection shows a similar trend with the NO release in Fig. 4B: HUVECs on the micro-patterned HUASMCs released more PGI_2 compared with HUVECs on the TiOH surface. PGI_2 is a potent inhibitor of platelet aggregation and SMC proliferation [25], and is synthesized by ECs in response to a variety of vasoactive stimuli such as bradykinin, histamine, thrombin, and trypsin [25]. Intracellular calcium concentration ($[Ca^{2+}]_i$) is also an influence factor of PGI_2 synthesis [25], the increase of ECs $[Ca^{2+}]_i$, which is regulated by the TGF-β1 secreted from SMCs [12], will lead to more PGI_2 synthesis.

Fig. 4C shows the thrombomodulin (TM) expression of HUVECs on micro-patterned HUASMCs and TiOH, respectively. From the statical results, HUVECs on the micro-patterned HUASMCs possessed an advantage of TM expression compared with HUVECs on the TiOH surface, which was consistent with the NO and PGI_2 release. TM is a transmembrane glycoprotein and endothelial cell receptor of thrombin, converts thrombin from a pro-coagulant enzyme to an anti-coagulant [26]. *In vivo*, the protein-C-TM mechanism culminates in the generation of activated protein-C, a potent anti-coagulant on endothelial surfaces by surface-bound thrombin, which is sequestered on endothelial surfaces by TM [27]. It has been reported by Ma et al. that TM mRNA of ECs can be increased threefold to sixfold by either basic fibroblast growth factor or platelet-derived growth factor, which can be synthesized by the SMCs [28].

Tissue factor pathway inhibitor (TFPI) is the master inhibitor of tissue factor (TF)-mediated coagulation [29]. In atherosclerotic plaques TFPI co-localizes with TF, where it is believed to play an important role in attenuating TF activity [29]. Fig. 4D shows the TFPI detection results of HUVECs on micro-patterned HUASMCs and TiOH, respectively. TFPI detection presented a consistent trend with the NO, PGI_2 release and TM expression: HUVECs on the micro-patterned HUASMCs secreted more TFPI compared with HUVECs on the TiOH surface. The explanation may be the fact that TFPI is constitutively synthesized by both the vascular ECs and vascular SMCs [30,31].

3.5. E-selectin detection

Inflammatory mediators like tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), increase the expression of E-selectin on endothelial cells [32]. The E-selectin, on inflammatory stimulation breaks the endothelial barrier and its release regulates the attachment and trans-endothelial migration of leukocytes which damages the vascular tissues [32]. Thus, the E-selectin quantitative characterization was performed as an evaluation of the potential HUVEC lesion. Fig. 4E shows the E-selectin detection of the HUVECs on micro-patterned HUASMCs and TiOH each. It could be seen from the result, the HUVECs on micro-patterned HUASMCs exhibited significantly higher E-selection expression than the HUVECs on TiOH after 1 day culture, suggesting more probability of HUVEC lesion. Whereas the E-selection expression of the HUVECs on micro-patterned HUASMCs gradually decreased after 3 day culture, significantly lower compared to the value of the HUVECs on TiOH, which indicated that the existence of micro-patterned HUASMCs could affect HUVEC E-selection expression and protect the HUVEC from cell lesion.

3.6. Anti blood flow shear stress function

In native vascular, the monolayer of endothelial cells coated on the interior surfaces of blood vessels is constantly exposed to blood

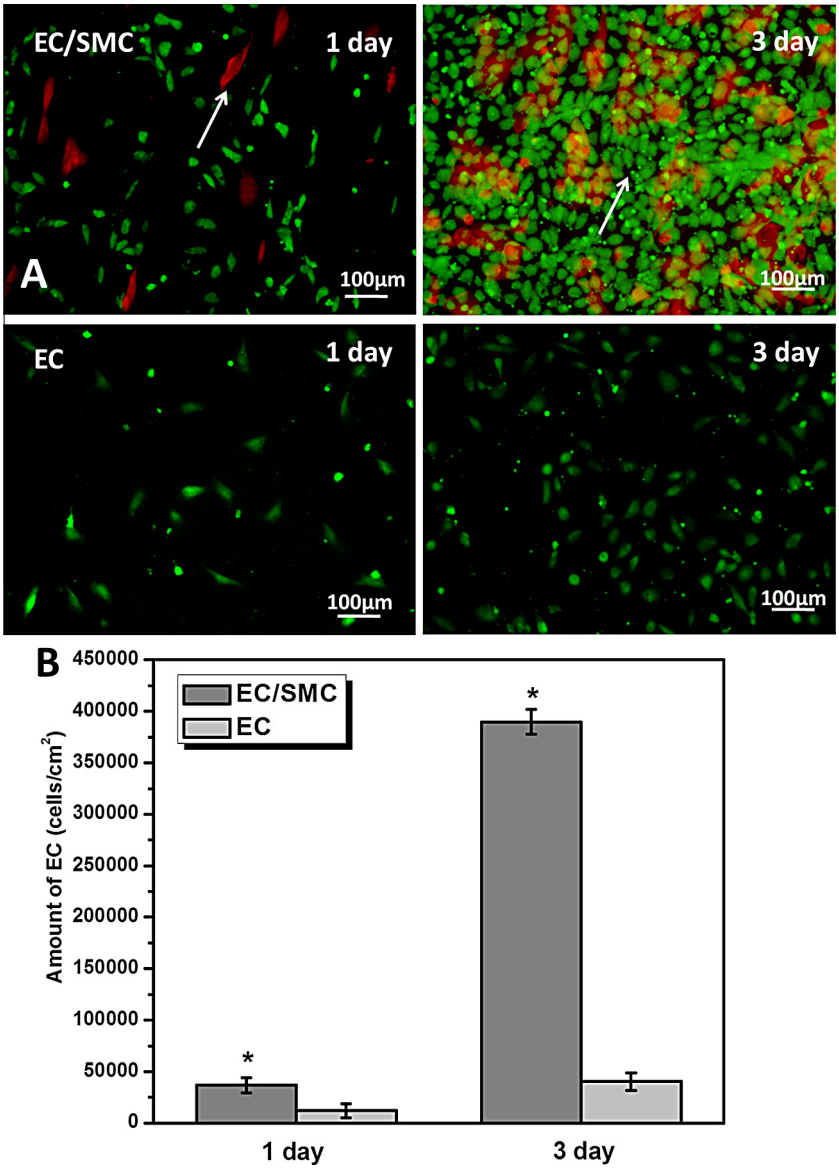

Fig. 3. (A) Fluorescence graphics of SMC (red) and EC (green) in the groups of EC/SMC co-culture and EC alone, respectively; (B) Amount of EC in the groups of EC/SMC co-culture and EC alone, respectively ($N = 5$, mean ± SD, $^{*}p < 0.05$ compared with the group of EC alone). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

flow [33]. BFSS regulates endothelial physiology and pathophysiology in a magnitude- and flow pattern-dependent manner [33]. The injury and shedding of ECs caused by BFSS will expose the underlying extracellular matrix, stimulate macrophage cells and leukocyte adhesion and aggregation, and lead to the pathological phenotype transformation of SMCs, further involving in the development of atherosclerosis [34]. Thus, the retentions of HUVECs on micro-patterned HUASMCs and TiOH were investigated as the evaluation of their anti-BFSS function, and the results were displayed in Fig. 4F. It could be seen that co-culture model exhibited a remarkable advantage of HUVEC retention compared with single HUVEC model, especially at the 3rd day, there was almost no HUVEC shedding in the co-culture model (retention), suggesting an excellent anti-BFSS function of the complete EC monolayer with the micro-patterned pericytes (SMCs).

In summary, we successfully built the co-culture model of vascular endothelial cells (high density) and micro-patterned vascular smooth muscle cells (low density) on blood contact material titanium (Ti) in this study, and the endothelial cells covered on the micro-patterned smooth muscle cells completely. The endothelial cells in the co-culture model take significant advantages in NO, PGI_2, TM, TFPI release and/or expression, inhibiting E-selectin expression, and cell retention under the BFSS compared with the endothelial cells on the control samples. In conclusion, the micro-patterned smooth muscle cells (low density) improve the endothelialization of the Ti surface, and also enhance the anti-coagulation, anti-atherosclerosis, anti-inflammatory and anti-shedding functions of the co-cultured endothelial cells, which would be potential for the development of a new generation of cardiovascular grafts. The promising initial study only gives a short term information about the co-cultured cell state and cytokines release *in vitro*. Additional studies are needed to investigate the detailed relationship between the micro-patterned smooth muscle cells and endothelial cytokines release, and to detect the long term endothelial cell function in the co-culture model.

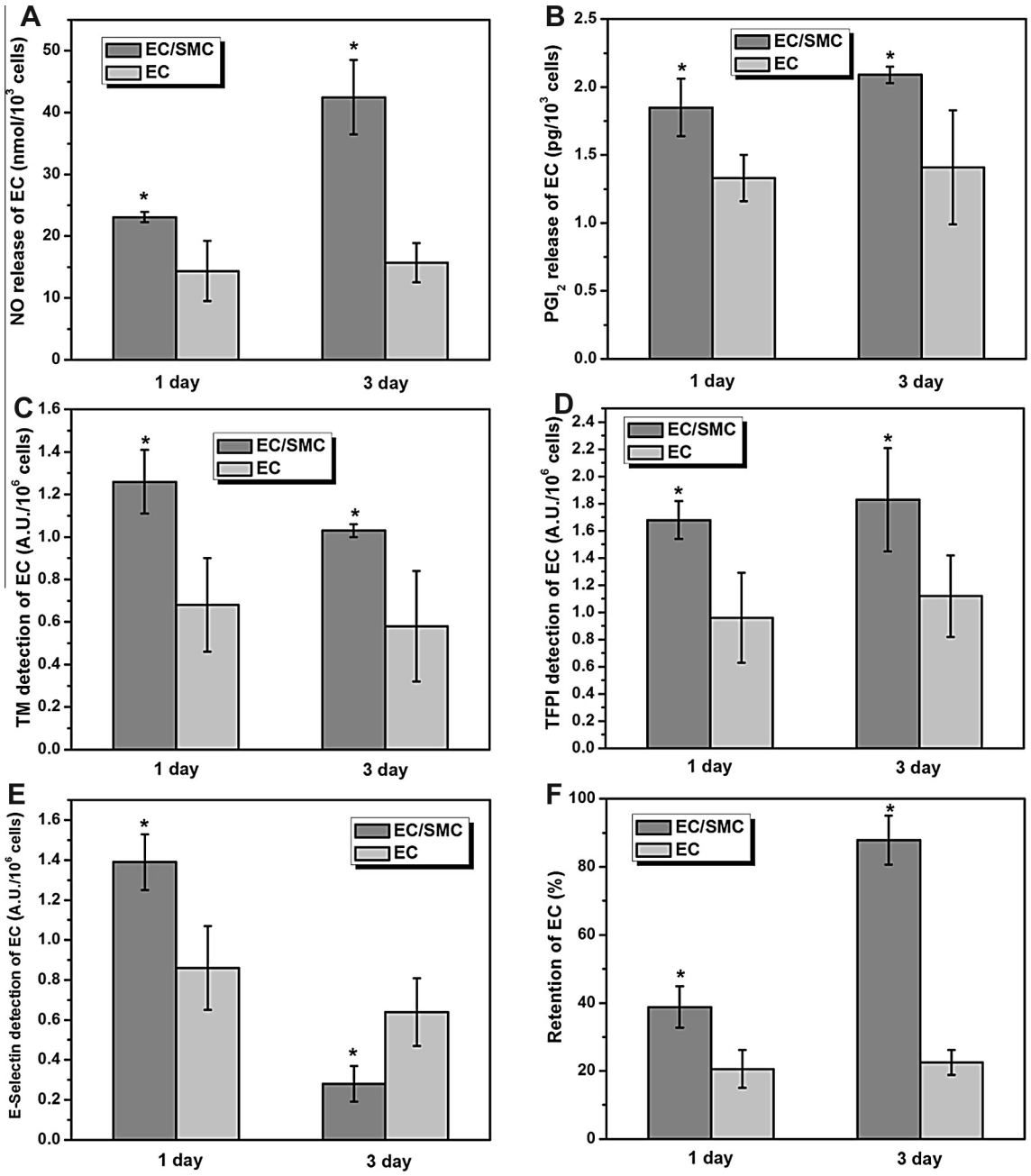

Fig. 4. Detection of (A) NO, (B) PGI_2, (C) TM, (D) TFPI, (E) E-selectin and (F) retention of EC in the group of EC/SMC and EC alone after incubation for 1 day and 3 days, respectively ($N = 5$, mean ± SD, $^*p < 0.05$ compared with the group of EC alone). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

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