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Tailoring of the titanium surface by preparing cardiovascular endothelial extracellular matrix layer on the hyaluronic acid micro-pattern for improving biocompatibility

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ABSTRACT

It has been proved that high molecular weight hyaluronic acid (HMW-HA, 1×10^6 Da) micro-strips on titanium (Ti) surface can elongate the human vascular endothelial cell (EC) morphology, subsequently enhance endothelial extracellular matrix (ECM) deposition in our previous work. The HMW-HA micro-strips were anticipated to possess good hemocompatibility and EC compatibility simultaneously. However, the single HMW-HA micro-strips on Ti substrate showed bad hemocompatibility. To solve this problem, a method combining HA micro-pattern and EC decellularization was developed, and the endothelial extracellular matrix layer on the HA micro-pattern (ECM/HAP) showed excellent hemocompatibility and endothelial progenitor cells (EPCs) compatibility (cell number: $14.3 \pm 0.5 \times 10^5$ cells/cm^2 > $2.2 \pm 0.7 \times 10^5$ cells/cm^2 on ECM/TiOH, $7.5 \pm 1.3 \times 10^5$ cells/cm^2 on TiOH, $3.4 \pm 0.9 \times 10^5$ cells/cm^2 on TiOH/HAP and $3.6 \pm 1.2 \times 10^5$ cells/cm^2 on Ti). We also found that the ECM/HAP coating could significantly inhibit the excessive proliferation of smooth muscle cells (SMCs) (cck-8 absorption: $0.25 \pm 0.06 < 1.18 \pm 0.09$ A.U. on ECM/TiOH, 0.87 ± 0.15 A.U. on TiOH and 1.55 ± 0.11 A.U. on Ti) and the attachment of macrophages (cell number: $1.3 \pm 0.1 \times 10^3 < 9.2 \pm 1.5 \times 10^3$ cells/cm^2 on ECM/TiOH, $8.8 \pm 0.3 \times 10^3$ cells/cm^2 on TiOH, $7.3 \pm 0.7 \times 10^3$ cells/cm^2 on TiOH/HAP and $9.6 \pm 0.9 \times 10^3$ cells/cm^2 on Ti in 12 h). These data suggest that the multifunctional ECM/HAP coating can be used to build the bionic human endothelial ECM on the biomaterials surface, which might provide a potential and effective method for surface modification of cardiovascular devices.

1. Introduction

Cardiovascular disease is the number one cause of death in the world currently [1,2]. Stents implantation, which is the main method to treat coronary artery diseases, may provoke a series of cellular and biochemical events that induce pathological processes [3,4]. The sequence of events is: inflammation and smooth muscle hyperplasia that may produce thrombosis due to further inflammation or rupture of the plaque. The cycle does repeat itself if the thrombotic event does not occlude the vessel [3,5–8]. This thickening of the vessel wall may due to mechanical mismatch at the anastomosis between the stents and natural blood vessel [9,10] and poor re-endothelialization on the stents [3]. A monolayer of endothelial cells (ECs) cannot only prevent thrombosis but also mediate the phenotype and proliferation of the SMCs [3]. Thus, the available and effective techniques of re-endothelialization on the vascular implant are very important for clinical treatment to prevent the thrombosis and ISR.

Immobilizing biomolecules on biomaterials surface is an hot spot which has attracted a lot of attention and research in recent years [11,12]. Most biomolecules are components of the ECs extracellular matrix [1,3], and immobilizing specific extracellular matrix components can promote endothelial adhesion to biomaterials. Nevertheless, these extracellular matrix components also promote SMCs hyperplasia [3,13]. Meanwhile, the inflammatory response is another aspect that is often neglected [14]. In the stents

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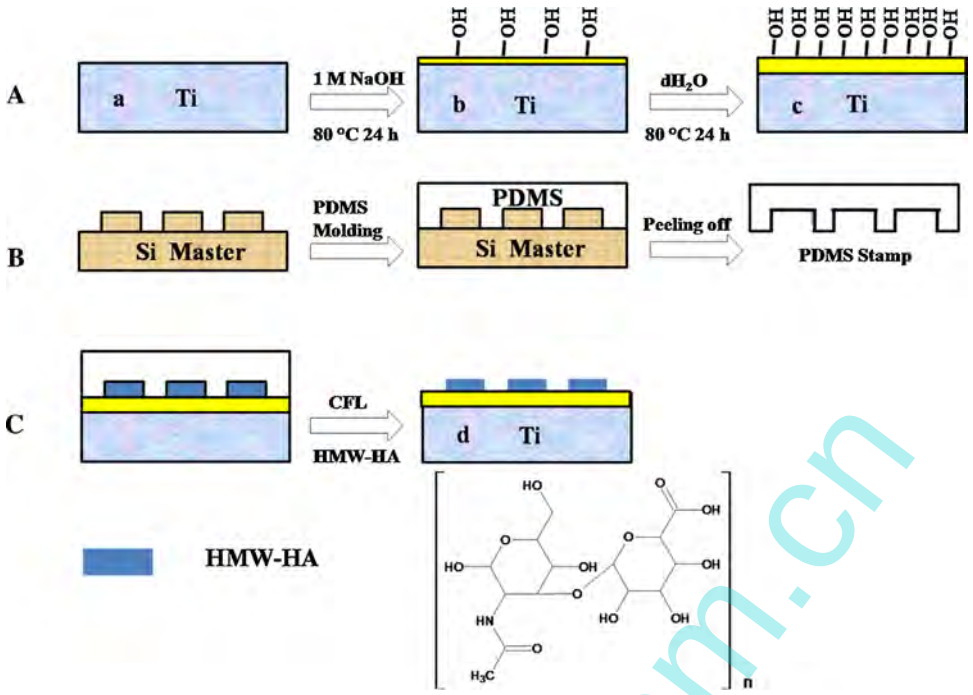

Fig. 1. The scheme of preparing HA micro-pattern on Ti surface. (A) Preparation of the functional layers of hydroxyl groups: (a) polished Ti substrate; (b) preparation of the functional layers of hydroxyl groups by 1 M NaOH at 80 °C for 24 h; (c) further enriching the hydroxyl groups by immersing b in the dH_2O at 80 °C for 24 h; (B) fabrication of the PDMS stamp from a silicon mold; (C) fabrication of the HA micro-pattern on the hydroxyl groups enriched Ti substrate by the PDMS stamp: (d) HA micro-patterned TiOH substrate.

implantation, response of SMCs and macrophages to vascular intimal damage and endothelial cell death play a great part in the EC dyfunctions, thrombosis and inflammation [14,15]. Therefore, it is also an urgent need to develop a multifunctional surface which possesses the functions of anticoagulation, promoting endothelialization, inhibiting SMCs hyperplasia and anti-inflammation simultaneously.

It is well known that the monolayer of endothelial cells from the body's own blood vessels is the best natural barrier and functional organization [16], and the autologous ECs are elongated by the blood flow shear stress (BFSS) and grow along the blood flow *in vivo* [17]. It has been generally accepted that simulating the biological microenvironment *in vivo* of the ECs on the biomaterials surfaces *in vitro* may be a effective method to build the autologous EC monolayer [18–21]. The micro-strips of biomolecules fabricated on biomaterials by soft lithography using a polydimethylsiloxane (PDMS) stamp can elongate the cells without the shear stress [20]. Several micro-patterns of biomolecules have been prepared to be applied for the biomaterials, including hyaluronic acid (HA) [20], phosphatidyl choline [22], collagen I [23] and the mixtures of fibronectin and heparin [24], and all these micro-strips can elongate the EC morphology. However, the extracellular matrix covered surface fabricated by the method of the elongated EC detachment and its biocompatibility have not been reported.

HA is a linear polysaccharide composed of repeating disaccharide units of D-glucuronic acid and N-acetyl glucosamine linked by β(1, 4) and β(1, 3) glucosidic bonds [25]. This biomolecules have high viscosity and absorbent [25], thus can be easily fixed on a hydrophilic and rough surface by physical adsorption [20]. The presence of —COOH group leads to the weak acidity of HA, which can cause an acid–base reaction between the HA and metal surfaces, and the both can be easily combined with hydrogen bonds. In addition, HA is a component of the ECM, owning good cell compatibility and no cytotoxicity [26]. High molecular weight HA (HMW-HA, $\geq 1 \times 10^6$ Da, 5 mg/ml in PBS) has been widely used for the regulation of cell behavior [20,27]. Therefore, HA is chosen as an excellent material of biomolecules for preparing micro-patterns on biomaterials surface for the purpose of mimicking BFSS effect.

In this work, HMW-HA micro-patterns were fabricated on the titanium (Ti) surface for the excellent biocompatibility [11] and mechanical performance [28]. The ECM of elongated ECs was prepared on the HMW-HA micro-patterned titanium (Ti) surface by the method of cell detachment. This bionic surface was demonstrated not only having good blood compatibility and EC compatibility, but also obtaining excellent ability in inhibiting the proliferation of SMCs and adhesion of macrophages. We expect this bionic ECM surface will provide potential application for blood contact implanted devices.

2. Materials and methods

2.1. Materials and reagents

Ti substrates (diameter = 10 mm) were prepared using 99.5% pure Ti foils (Baoji, China). Ti substrates were polished and ultrasonically cleaned four times successively in acetone, ethanol and deionized water (dH_2O) for 5 min each and then dried at room temperature. HMW-HA (Sangon Biological Engineering Co. Ltd., China) was diluted to a concentration of 5 mg/ml in dH_2O. Polydimethylsiloxane (PDMS) prepolymer and catalysts (Sylgard 184) were obtained from Dow Corning (Midland, MI, USA). Medium 199 (M199), medium DMEM-F12 (F12), fetal calf serum (FBS), trypsin and type II collagenase were supplied by Gibco BRL. Cell counting kit-8 (CCK-8), mouse anti human SM-myosin antibody (bs-2178R), FITC conjugated rabbit anti-human type IV collagen antibody (bs-0806R-FITC) and TMB (3,3′,5,5′-etetramethylbenzidene) for the immunochemistry tests were provided by BD Biosciences (China). The actin staining reagent kit (SABC-FITC, SA2004), 4,6-diamino-2-phenyl indole (DAPI), horseradish peroxidase (HRP) conjugated goat antimouse IgG antibody (BA1050) and mouse anti human α-SMA antibody (BM0002) were stocked by Boshide (China). Mouse monoclonal antihuman Collagen IV antibody (ab6311),

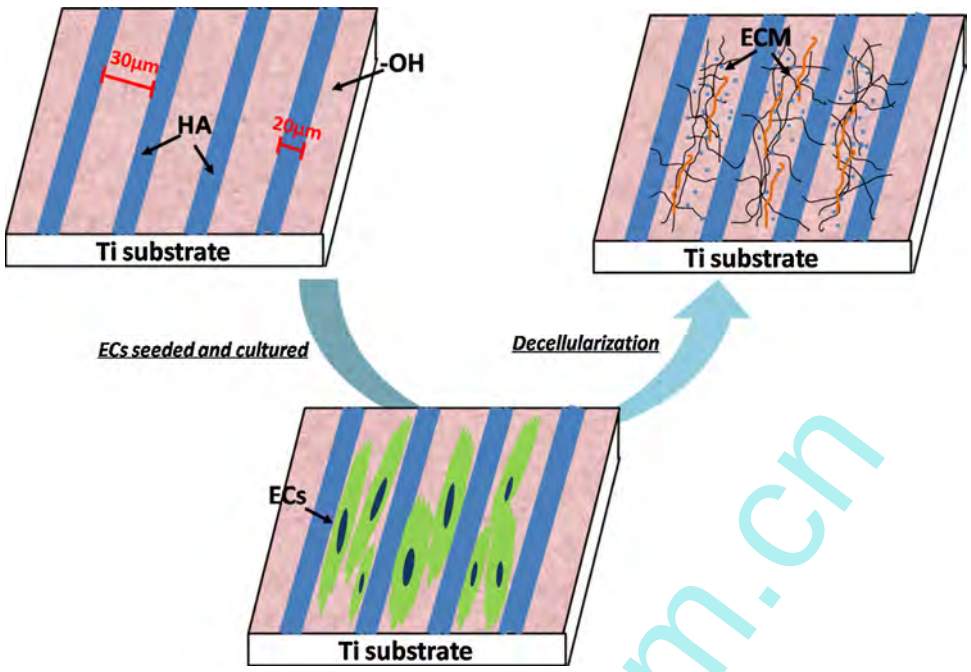

Fig. 2. The scheme of culturing ECs on TiOH/HAP surface and subsequent decellularization.

mouse monoclonal antihuman fibrinogen (FGN) γ chain antibody (ab119948) and HRP conjugated goat antihuman fibrinogen antibody (ab64664) were purchased from abcam (UK). Prostacyclin (PGI_2) ELISA kit was provided by Wuhan ColorfulGene Biological Technology Co. Ltd., China. All the other reagents were analytical of grade.

2.2. Preparing HMW-HA micro-pattern on Ti substrate

Fig. 1 shows the fabrication process of HA micro-pattern on Ti substrate. The cleaned Ti plates were immersed in 1 M NaOH solution at 80 °C for 24 h, and then rinsed thoroughly with dH_2O and then immersed in the dH_2O at 80 °C for 24 h to obtain more —OH groups. The NaOH-activated Ti substrate is labeled as TiOH. Next, a PDMS mold is fabricated by casting a liquid prepolymer against a master whose surface has been patterned with complementary structures by photolithography. After curing at 120 °C in a vacuum oven for 1 h, the PDMS stamps were peeled off the silicon master. After 2 μl of 5 mg/ml HA solution added onto the TiOH surface (labeled as TiOH/HA), the PDMS stamp was pressed down on the TiOH/HA surface by the force of 8 Newton for 4 h at a ventilated environment. Through this process, the micro-pattern on the silicon master was moved to the TiOH/HA surface (labeled as TiOH/HAP) by the method of capillary force lithography (CFL).

2.3. ECs culture

The ECs obtained from newborn umbilical vein were isolated and cultured using the method that we have reported [20]. The 3rd passage of ECs with the concentration of 5×10^4 cells/ml were seeded on the TiOH/HAP and TiOH samples surface and cultured for 3 days. ECs obtained from newborn umbilical vein (Kun Zhang et al., 2014; Maternal and Child Health Hospital in Chengdu, China) were approved by the Maternal and Child Health Hospital Institutional Review Board, and all the work complied with Helsinki Declaration.

2.4. The fabrication of EC-ECM by decellularization

To obtain the ECM of ECs on the TiOH/HAP sample, a decellularization operation was carried on as follows. Briefly, after a standard culture of ECs for 3 days, the used culture medium was removed and the samples were rinsed twice with sterile normal saline of 37 °C. Then, 1 ml decellularization reagent was added and incubated at 37 °C for 20 min. Finally, after removing the decellularization reagent, the rinse step was duplicated, and the ECM of ECs on the TiOH/HAP sample (labeled as ECM/HAP) was obtained. The ECM of ECs on the TiOH sample (labeled as ECM/TiOH) was used as control. All the processes were operated in sterile environment and displayed in Fig. 2.

The decellularization reagent was prepared as follows. In brief, the triton (Triton X-100, Sigma) was dissolved to a concentration of 0.5% with PBS at 60 °C. Subsequently, the aqueous ammonia was diluted to a concentration of 0.034% with the 0.5% triton solution at 18 °C. Finally, a filtration procedure was performed using filter membrane to ensure that the reagent was sterile. The decellularization reagent was stored at 4 °C and sterile environment.

2.5. Surface characterization

2.5.1. SEM

The surface morphology and roughness of the Ti substrate, TiOH sample and the TiOH/HAP samples were characterized using a scanning electron microscopy (SEM, JSM-7001F, Japan). Before observed by SEM, all the samples were gold deposited.

2.5.2. AFM

The surface topography and roughness of ECM/HAP and ECM surfaces were characterized using a Nanowizard II AFM (JPK Instruments, Berlin, Germany) in tapping mode with spring constants of 0.06 N/m. AFM was performed at room temperature in air at a rate of one line scan per second. Image analysis was performed using the CSPM Imager software.

2.5.3. Immunofluorescence staining of type IV collagen in the ECM

Type IV collagen (CoIV) is the main component of the endothelial cell ECM [29,30]. Thus, we stained the CoIV on the ECM/HAP and ECM/TiOH using FITC conjugated rabbit anti-human type IV collagen kit (bs-0806R-FITC, BD Biosciences, China, diluted 1:100 in NS) and got the immunofluorescence images to investigate the ECM distribution, the TiOH samples, TiOH/HAP samples, and ECs on the both samples were also stained as control. The relative abundance of the CoIV on the ECM/TiOH and ECM/HAP samples were also detected by ELISA assay (first antibody: mouse monoclonal anti-human Collagen IV antibody, ab6311, abcam, UK, diluted 1:250 in NS; second antibody: HRP conjugated goat antimouse IgG antibody, BA1050, BD Biosciences, China, diluted 1:100 in NS).

2.6. Fibrinogen attachment and conformational change of fibrinogen

Fibrinogen (FGN) attachment test is a conventional evaluation of the surface and/or interface blood compatibility. The FGN attachment on the ECM/HAP, ECM/TiOH, TiOH/HAP, TiOH and Ti samples were measured by an ELISA method. Briefly, platelet-poor plasma (PPP) obtained from Fresh ACD blood of a healthy volunteer by centrifuging at 3000 rpm for 15 min was used. The samples were put into a 24-well culture plate and 50 μl/well PPP was dropped onto the sample surface and incubated for 1 h at 37 °C; then the samples were thoroughly rinsed three times with normal saline (NS). Subsequently, the samples were blocked with 1 wt% bovine serum albumin (BSA) in NS at 37 °C for 30 min. Thereafter, 20 μl HRP conjugated goat antihuman fibrinogen antibody (ab64664, abcam, UK, diluted 1:250 in NS) was added and incubated at 37 °C for 1 h. Further, 100 μl TMB solution was added onto the sample surfaces and reacted in dark for 10 min (blue color), and then 50 μl 1 M H_2SO_4 was used to stop the peroxidase catalyzed reaction (yellow color). Finally, 140 μl supernatant was transferred to a 96-well plate and the absorbance at 450 nm was measured on a micro-plate reader.

The conformational change of FGN could cause the platelet further adhesion and aggregation. The conformational change of FGN on the ECM/HAP, ECM/TiOH, TiOH/HAP, TiOH and Ti samples were measured the same method as FGN attachment [11]. The first anti body was 20 μl mouse monoclonal antihuman fibrinogen γ-chain antibody (diluted 1:250 in NS, the first antibody), and the second anti body was 20 μl HRP conjugated goat anti mouse IgG antibody (diluted 1:100 in NS, second antibody).

2.7. Cell compatibility

The ability of the micro-patterned ECM in rapid endothelialization, inhibiting SMCs proliferation, and anti-inflammatory was determined *in vitro* by EPCs compatibility, SMCs culture and macrophage adhesion. The Ti, TiOH, TiOH/HAP and ECM/TiOH samples were used as control.

2.7.1. Endothelial progenitor cells

Bone marrow mesenchymal EPCs were obtained from the mononuclear cells isolated using bone marrow obtained from the long leg bones of SD rat (Dashuo Co. Ltd., Chengdu, China) by density gradient centrifugation [31]. Mononuclear cells were re-suspended in DMEM-medium containing vascular endothelial growth factors (VEGF) and 20% fetal calf serum and seeded into a culture flask. The medium was changed every 48 h, and finally EPCs were isolated after 7 days of culture. The trypsinization was performed when cells approached confluence. The 8th generation of the EPCs was seeded on the samples with the density of 2×10^4 cells/ml and cultured for 1 day. All the manipulations above were operated in sterile and ventilated environment.

To study the morphology of the EPCs on the ECM/HAP surface, immunofluorescence staining of actin using SABC-FITC kit was performed: The samples were fixed with 4% paraformaldehyde for 2 h at room temperature, and washed with NS for three times; then blocked with 1% BSA for 30 min at 37 °C, and washed step again; after the rabbit anti-human actin antibody (SA2004, Boshide, China, 1:100 diluted in NS) was added and incubated at 37 °C for 30 min, the washed step was repeated again; then goat anti-rabbit IgG antibody (SA2004, Boshide, China, 1:100 diluted in NS) was added and incubated at 37 °C for 30 min, and rinsed step again; Finally, FITC conjugated antibody (SA2004, Boshide, China) was introduced and incubated at 37 °C for 30 min and rinsed with NS for five times [11]. EPCs proliferation was investigated by CCK-8 kit after 1 day of incubation. The medium was removed and the samples were washed twice with normal saline. Subsequently, fresh medium without phenol red containing CCK-8 reagent was added to each sample and incubated at 37 °C for 4 h in a humidified atmosphere containing 5% CO_2. Afterwards, 180 μl of the blue solutions were transferred to a 96-well plate. The absorbance was detected by a microplate reader (BIO-TEK instruments, USA). The number of EPCs on each sample was determined by a prepared standard curve.

The amount of NO and PGI_2 released from EPCs on each sample were detected to evaluate the anticoagulation and inhibitory hyperplasia properties of the EPCs. The NO release was examined by a typical Griess Reagent method as described in the previous work [20], and the PGI_2 detection was performed by ELISA kit according to the manual. The amount of NO and PGI_2 were finally normalized to cell number.

2.7.2. Smooth muscle cells

SMCs derived from human umbilical artery were isolated and cultured as described method in the previous work [20]. In brief, the human umbilical cord was washed thoroughly with normal saline to remove the blood outside, and subsequently the artery was excised from the umbilical cord and opened at its length. Then, Connective tissue outside and fibroblast layer outside were peeled off, and the intima inside were gently scraped by a sharp tweezer. Afterwards the muscle tissue was washed thoroughly with normal saline and cut into small fragments. The fragments were then seeded in a culture flask filled with 4 ml medium F12 and 1 ml fetal calf serum (FCS, Gibco BRL) mixture, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Finally, the fragments were removed after SMCs migrated to the wall of the culture flask. Cells were fed with freshly prepared growth medium every 24 h. The 3rd passage of SMCs with the concentration of 2×10^4 cells/ml were seeded on the samples surface and cultured for 1 day. All the manipulations above were operated in sterile and ventilated environment.

To study the SMCs morphology on the samples, immunofluorescence staining of actin using SABC-FITC kit was performed [11]. SMCs proliferation was also tested by CCK-8 kit after 1 day of incubation. For the purpose of investigating the phenotype of the SMCs, the amounts of α-SMA and SM-myosin of the SMCs were tested using an ELISA method according to we described in the previous work [32].

2.7.3. Macrophages

Peritoneal macrophages were obtained from SD rats (Dashuo Co. Ltd., Chengdu, China) by flushing the peritoneum with a 10 ml cold Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Canada). The harvested cells were centrifuged at 1200 rpm for 5 min and resuspended in DMEM containing 10% fetal bovine serum, 1%-L-glutamine, 1% penicillin/streptomycin, counted and viability determined by 0.2% trypan blue exclusion [14]. The suspended macrophages with the concentration of 5×10^4 cells/ml were seeded on the samples surface and cultured for 3 h, 6 h, 12 h,

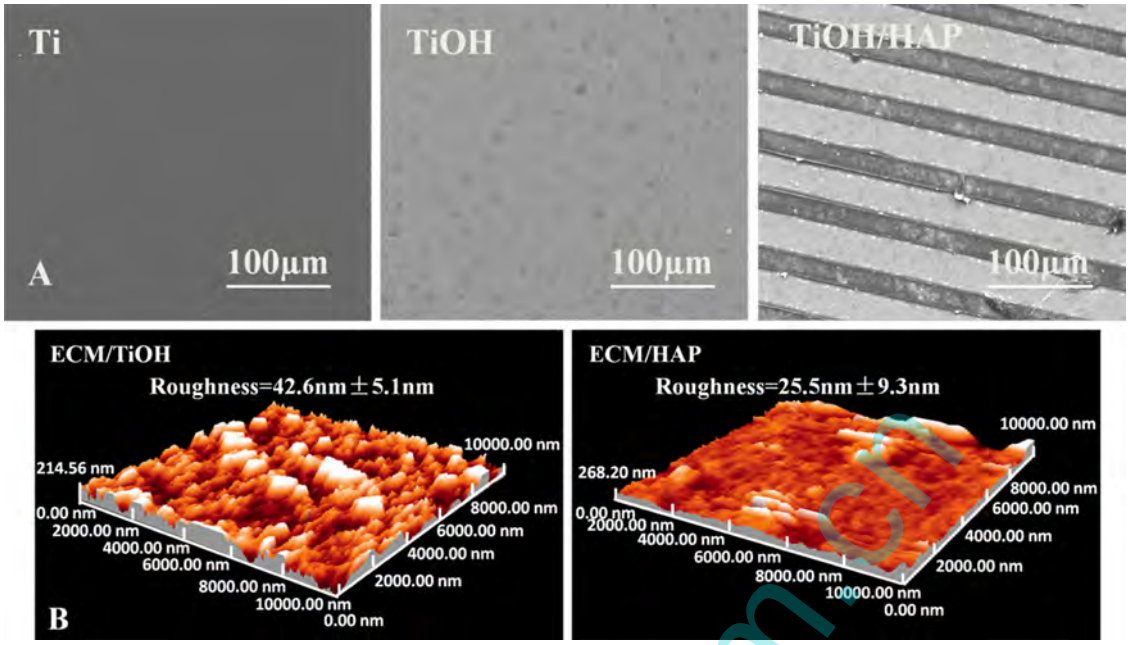

Fig. 3. (A) SEM images of Ti, TiOH and TiOH/HAP; (B) AFM of ECM/TiOH and ECM/HAP.

respectively. All the manipulations above were operated in sterile and ventilated environment.

To study morphology of the macrophages on samples, immunofluorescence staining of actin of macrophages using SABC-FITC kit was performed. Cell nucleus staining was also carried out to calculate the amount of the adhering macrophages on the surfaces. In brief, the cells on the samples stained with SABC-FITC kit were stained with 4,6-diamidino-2-phenylindole (DAPI, 50 μg/ml diluted in normal saline) at room temperature for 5 min and rinsed five times with NS. The cells were photographed using fluorescence microscope, and amounts of macrophages attached onto samples were calculated from at least 15 images.

2.8. Data analysis

The data were statistically evaluated using ANOVA by homogeneity test of variances firstly, and *post hoc* test was prepared subsequently in LSD method for comparison. They were expressed as mean ± standard deviation (SD). The probability value $p < 0.05$ was considered as a significant difference. The data analysis was performed using the software SPSS 11.5 (Chicago, IL).

3. Results

3.1. Surface characterization

3.1.1. SEM

The SEM images in Fig. 3A depict the surface morphology of Ti, TiOH and TiOH/HAP. The images exhibited that the Ti sample got a rougher surface after activation in NaOH solution. The width of the TiOH strip and that of the HA strip were 30 μm and 20 μm, respectively. The rationale for the specific micro-pattern is that the minor diameters of endothelial cells range from 20 μm to 30 μm [20,33]. The reason why 30 μm for TiOH strip and 20 μm for HA strip can be attributed to that the cell for endothelialization may be not only endothelial cells but also endothelial progenitor cells, and the minor diameters of endothelial progenitor cells are bigger than endothelial cells, this pattern geometry may be help to the attachment and differentiation of the endothelial progenitor cells.

3.1.2. AFM of ECM

The topography difference between ECM and ECM/HAP was depicted from AFM images (Fig. 3B). The surface of ECM seemed to be regularly lined by a great number of large round granules, whereas ECM/HAP seemed to be lined with fewer little round granules. Moreover, the ECM/HAP surface showed smoother than the ECM surface (roughness decreased from 42.6 to 25.5 nm) according to the AFM data.

3.1.3. Immunofluorescence images and relative abundance of CoIV in the ECM

The immunofluorescence images of CoIV on ECM/HAP and ECM/TiOH was displayed in Fig. S2(A), and the ECs on TiOH/HAP (labeled as ECs/HAP), ECs on TiOH (labeled as ECs/TiOH), TiOH/HAP samples and TiOH samples were stained as references. The green fibers-like and/or web-like on the images represented the CoIV which is the specific component of the vascular endothelial cells ECM. It could be seen from the images both the ECM/HAP and ECM/TiOH surfaces possessed a lot of CoIV, indicating the survived vascular endothelial ECM on the two surfaces. Nevertheless, the CoIV on the ECM/HAP surface did not show a patterned distribution, suggesting the ECMs cover on the entire surface, but not alternate with HAP as a patterned surface. The relative abundance of CoIV (Fig. S2-B) showed that there were more CoIV on the ECM/HAP surface compared with that on the TiOH surface, indicating more ECM secreted by ECs on the TiOH/HAP samples.

3.2. FGN attachment and conformational change of FGN on ECM/HAP

The attached FGN on ECM/HAP were tested to evaluate its blood compatibility preliminarily, and the results were presented in Fig. 4A. The amount of the attached FGN on the ECM/HAP and TiOH surfaces were significantly less than that on the Ti, ECM/TiOH and TIOH/HAP. The amount of attached FGN on different samples decreased in the order TiOH/HAP > ECM/TiOH and Ti > TiOH and ECM/HAP. The conformational change of FGN plays a critical role in platelet activation and aggregation, and the mechanism has been elaborated by Li et al. [11]. The conformational changes of FGN on ECM/HAP were performed to test the thrombosis tendency, and the

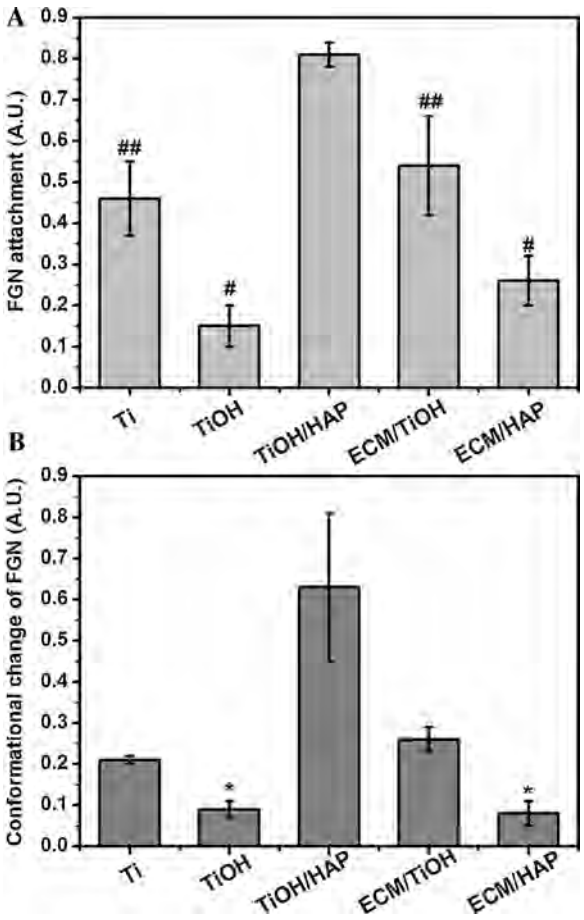

Fig. 4. (A) FGN attachment on Ti, TiOH, TiOH/HAP, ECM/TiOH and ECM/HAP samples ($^{\#}p < 0.05$ compared with Ti, TiOH/HAP and ECM/TiOH, mean ± SD, $N = 6$); (B) Conformational change of FGN on Ti, TiOH, TiOH/HAP, ECM/TiOH and ECM/HAP samples ($^{*}p < 0.05$ compared with Ti, TiOH/HAP and ECM/TiOH, mean ± SD, $N = 6$).

result was displayed in Fig. 4B. It could be seen that the absorption of the conformational changes of FGN on the ECM/HAP and TiOH surfaces were significantly less than that on the Ti, ECM/TiOH and TIOH/HAP. The conformational changes of FGN on different samples decreased in the order TiOH/HAP > ECM/TiOH > Ti > TiOH and ECM/HAP. The result indicated that the ECM/HAP on TiOH surface possess a better hemocompatibility, and the patterned HA sample has been improved on hemocompatibility by preparing ECM of EC on the surface.

3.3. Rapid endothelialization

EPCs are circulating bone marrow derived cells able to home to sites of vascular damage retaining the ability to differentiate into a vascular endothelial cell like phenotype, which contribute to the revascularization [34]. Thus, EPCs adhesion and proliferation for 1 day on the ECM/HAP surface was investigated to evaluate the ability on endothelialization of the sample. The morphology and skeleton arrangement of EPCs on ECM/HAP surfaces were detected by the immunofluorescence staining for actin expression (Fig. 5A). The results showed that EPCs seeded on the TiOH and ECM/TiOH samples exhibited polygonal or elongated morphology, while EPCs cultured on the TiOH/HAP and ECM/HAP exhibited mainly elongated morphology, which indicated the elongated effect of the HA micro-pattern on the cells. Additionally, ECM/HAP showed a significantly larger coverage of EPCs compared with ECM/TiOH, TiOH/HAP, TiOH and Ti. The amount of attached EPCs on different samples decreased in the order ECM/HAP > TiOH > TiOH/HAP and Ti > ECM/TiOH as shown in Fig. 5B. These results indicated that the attachment and proliferation of EPCs on the sample of ECM/HAP was enhanced, EPCs was elongated and regulated along the micro-pattern, the ECM micro-pattern immobilized surface performed better endothelialization than Ti, TiOH, TiOH/HAP and even the ECM covered surface.

Anti-coagulation factors release of the attached cells is very important for the cardiovascular implanted biomaterials. Thus, we detected the nitric oxide (NO) and prostacyclin (PGI_2) release of the EPCs on each samples. The NO and PGI_2 release results (Fig. S3) showed that the EPCs on the ECM/HAP samples released more NO and PGI_2 compared with other samples (NO amount: ECM/HAP > ECM/TiOH and TiOH/HAP > TiOH > Ti (Fig. S3A); PGI_2 amount: ECM/HAP > ECM/TiOH and TiOH/HAP > TiOH and Ti (Fig. S3B)), suggesting better anti-coagulation function.

3.4. SMCs culture

To evaluate the effect of inhibiting the proliferation of SMCs on the ECM/HAP surface, 3rd passage of SMCs were seeded on the ECM/HAP, ECM/TiOH, TiOH/HAP, TiOH and Ti samples surface, respectively, and cultured for 1 day. The immunofluorescence staining images and CCK-8 detection results are shown in Fig. 6, respectively. It could be seen from Fig. 6A that the SMCs on the patterned samples surface exhibited a contractile phenotype and grew along the micro-strips in orientation while the cells on the flat surfaces exhibited more spreading morphology and distributed in random. Fig. 6B shows the cell counting result of SMCs by a CCK-8 assay, the HA micro-pattern samples represented significantly smaller absorbance values than the flat samples, and the absorbance value was positive correlated to the cell numbers. Fig. 7 displayed the quantitative characterization of α-SMA and SM-myosin expressions which are considered as the specific proteins in the contractile SMCs, and the results showed the tendency: for α-SMA expression, ECM/HAP and TiOH/HAP > ECM/TiOH, TiOH and Ti (Fig. 7A); for SM-myosin expression: ECM/HAP > TiOH/HAP > ECM/TiOH, TiOH and Ti (Fig. 7B). All these results suggested that the ECM/HAP surface not only possess good ability of inhibiting the adhesion and proliferation of the SMCs, but also could regulate the SMCs morphology to the contractile phenotype approaching physiological.

3.5. Macrophage culture

The macrophage is one of cells which arrived at the tissue-implant interface at first [35]. The immunofluorescence photographs of macrophages on ECM/HAP, ECM/TiOH, TiOH/HAP, TiOH and Ti surfaces *in vitro* after incubation of 3 h, 6 h and 12 h, respectively, are shown in Fig. 8A. The actin fibers were stained green using SABC-FITC kit. The morphology of macrophages was similar to that reported by Li et al. [14]. The macrophages showed spreading and irregular shape or extended pseudopods on Ti, TiOH, TiOH/HAP and ECM/TiOH surfaces, and round or dilapidated shape on ECM/HAP surface. The cell counting results (Fig. 8B) showed that ECM/HAP sample exhibited significantly smaller amount of attached macrophages than the other samples ($p^{***} < 0.001$), and the result was consistent with the fluorescence graphic. This result suggests better anti-inflammatory properties of ECM micro-patterned TiOH than Ti, TiOH, TiOH/HAP and flat ECM monolayer.

4. Discussion

A functional stent used in vascular implantation has been anticipated to inhibit thrombus formation while accelerate endothelial regeneration. Up to now, many methods have been applied to modify the biomaterials surface for improving the hemocompatibility

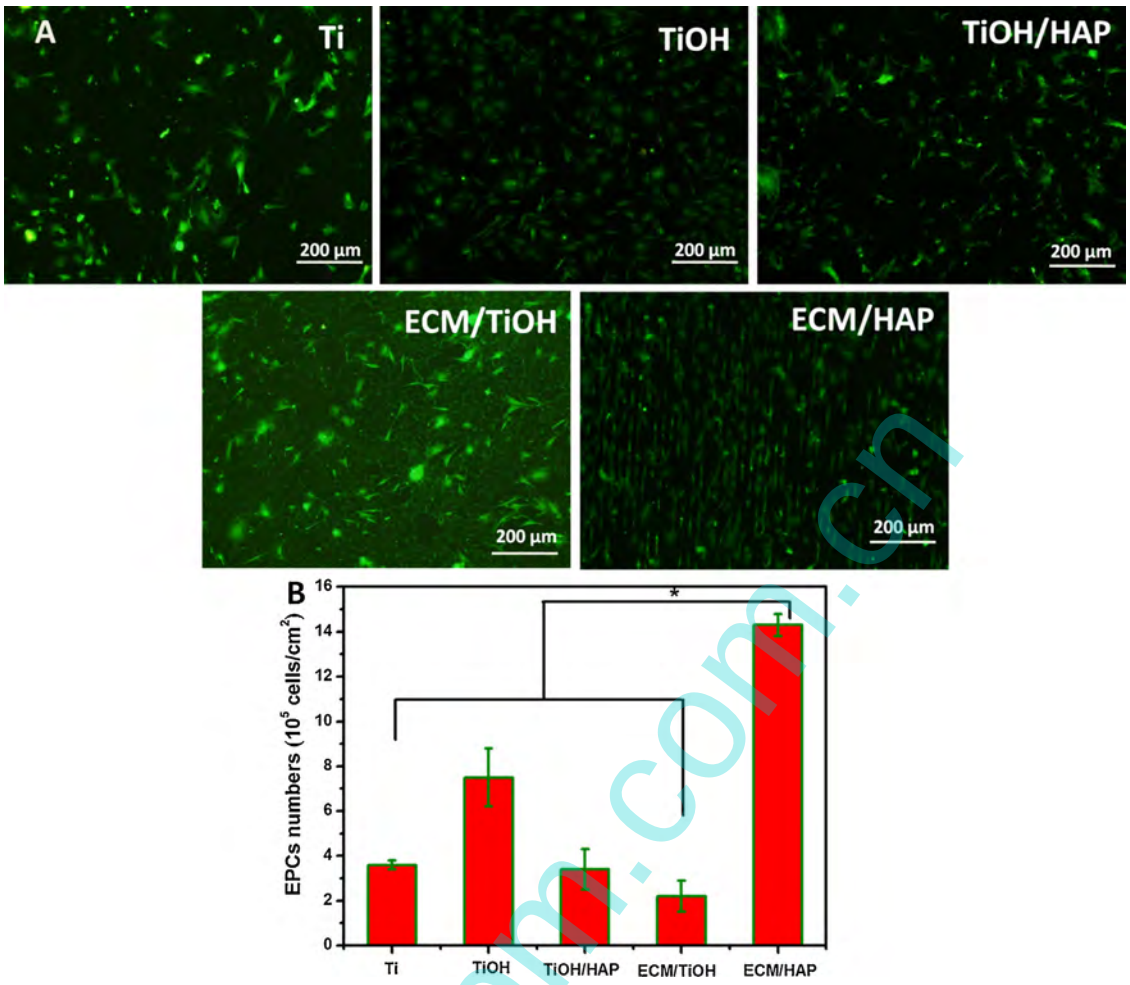

Fig. 5. (A) Immunofluorescence images of EPCs on Ti, TiOH, TiOH/HAP, ECM/TiOH and ECM/HAP surfaces; (B) Amount of attached EPCs on TiOH, TiOH/HAP, ECM/TiOH and ECM/HAP surfaces by CCK-8 assay ($^*p < 0.05$, mean ± SD, $N = 3$).

as well as endothelialization [11,36,37]. In these methods, direct biomolecules immobilization aiming at different targets on biomaterials surface has attracted a lot of researches [11,36]. Components or products of ECM, including proteins, glycosaminoglycans, glycoproteins or intact matrix have been used as the immobilized biomolecules. Such an abundant material in nature, the simplest way of collagen production is extracting from animal tissues [38]. Importantly, collagen chemistry is species-specific, so that human and animal collagens are biologically different [25,38,39]. HA has been considered to have the protection effect on ECM from immune rejection by maintaining an open, hydrated and stable extracellular space and the significant advantage of constructural conservation regardless of the source, showing non-allergenic and no immune rejection, although it just forms a smaller part of ECM [25,38]. Thus, the introducing of HA on the biomaterials surface is reasonable and necessary.

In our study, it has been anticipated to obtain a functional surface would inhibit thrombus formation while accelerate endothelial regeneration. HA micro-pattern on Ti surface can effectively elongate cell morphology, induce more anticoagulant factors secreted, and limit the SMCs in contractile phenotype. Therefore, good hemocompatibility of HA micro-pattern was anticipated to confirm to provide a new approach of bio-modification for vascular implanted devices. However, the introduction of HA induced the poor hemocompatibility of Ti in our preliminary work (Fig. S1). The reason may be the link between HA and FGN [40]. Then, the problem how to enhance the hemocompatibility of the HA micro-pattern on Ti surface became the title in the subsequent work. The research reported by Tu [41] offered an inspiration and improved strategies for our work. It showed that canine aortas patches could be derived by decellularization and the ECM integrity were still maintained, and their further work demonstrated excellent hemocompatibility of the human umbilical vein endothelial cell ECM on Ti surface *in vivo* test [29,30]. However, the anti-inflammatory evaluation of this ECM cultured *in vitro* has not been performed. Thus, we cultured ECs on HA micropattern and prepared a bionic surface coated ECM of the elongated ECs by decellularization to promote the hemocompatibility and anti-inflammatory ability of the biomaterials surface.

ECM integrity is an important aspect for the function expression and maintain of the bionic surface. Until now, the perfect ECM integrity has only been found on the natural ECM which was obtained from the animal or human tissues. Although many researches declared their materials coated one or more components of the ECM own good biocompatibility *in vitro* trials [3,11,36], the natural cells and ECM were still considered as the best for their effects of integrity. HA has been considered to have the effect of integrity for enhancing ECM remodeling and make collagen deposition more ordered [38]. A lot of studies have declared that cell adhesion and migration within the ECM also appear closely related to HA [38,42]. The high molecular weight values and strong intermolecular interactions of HA determine that it is difficult to degrade under the neutral condition [25]. *In vivo*, the hyaluronidase is responsible for the decomposition of HA to ensure a physiological

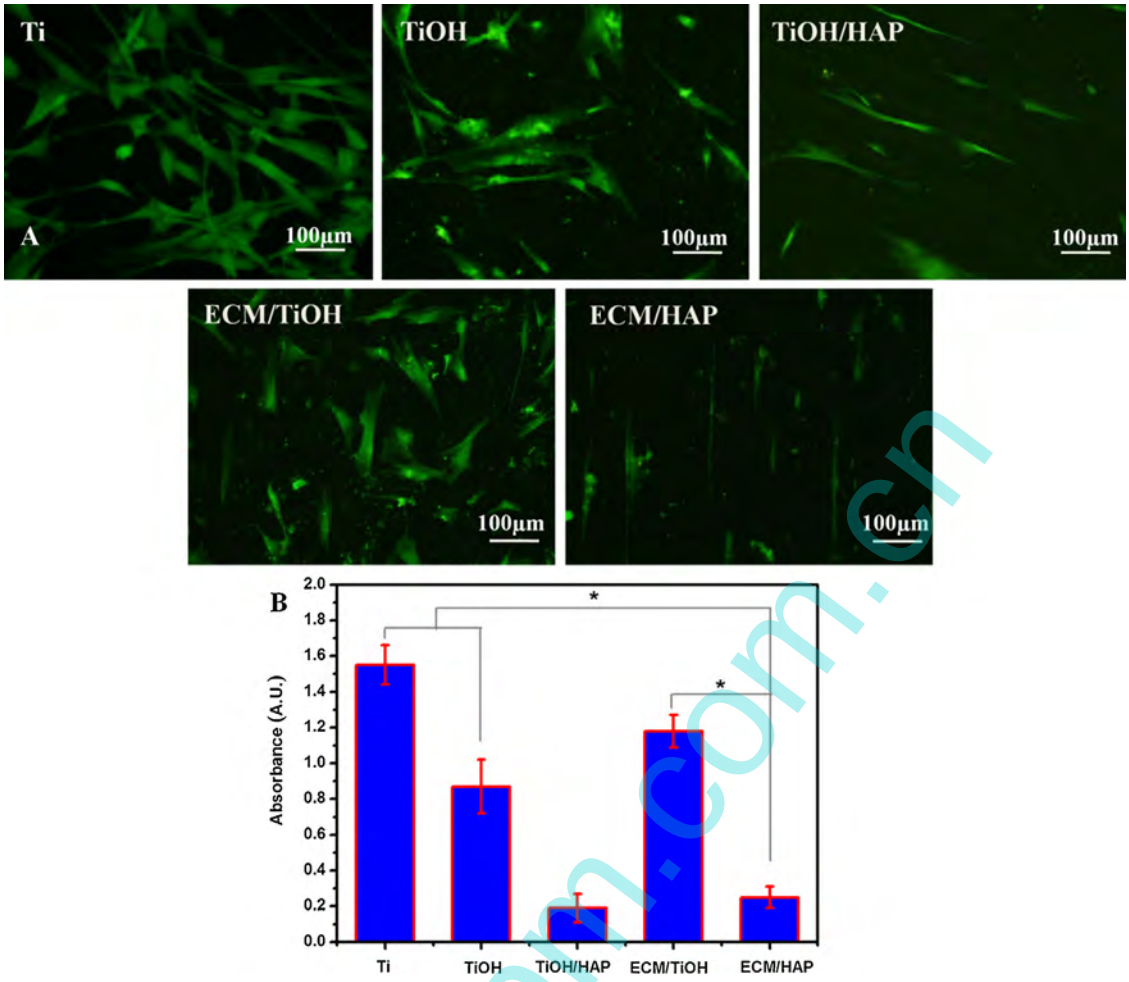

Fig. 6. (A) Immunofluorescence images of SMCs on Ti, TiOH, TiOH/HAP, ECM/TiOH and ECM/HAP surfaces; (B) Amount of attached SMCs on TiOH, TiOH/HAP, ECM/TiOH and ECM/HAP surfaces by CCK-8 assay (*$p < 0.05$, mean ± SD, $N = 3$).

ECM maintaining [25,38]. In summary, the outstanding regulation acting of the HA on the overall ECM make it indispensable.

In this improved work, HA micro-strips was first prepared onto Ti surface, because HA is known to have excellent cell and ECM regulation properties, while Ti is widely used as biomaterial substrates because of its good biocompatibility. Human umbilical vein endothelial cells were elongated by the HA micro-strips and left their ECM covered the entire surface after decellularization (Fig. 3). This ECM/HAP surface displayed anticoagulation (Fig. 4) and endothelialization (Fig. 5) properties as demonstrated by the evaluation experiments of hemocompatibility and EPCs culture. Moreover, ECM/HAP coating was proven to inhibit the excessive proliferation of SMCs (Figs. 6 and 7) and the adhesion of the macrophages (Fig. 8) by culturing these two cells, respectively. The successful preparation of ECM/HAP on Ti surfaces was monitored by AFM and CoIV immunofluorescence staining (Fig. S2).

Conformational change of FGN on ECM/HAP, ECM/TiOH, TiOH/HAP, Ti and TiOH were tested to evaluate the anticoagulant

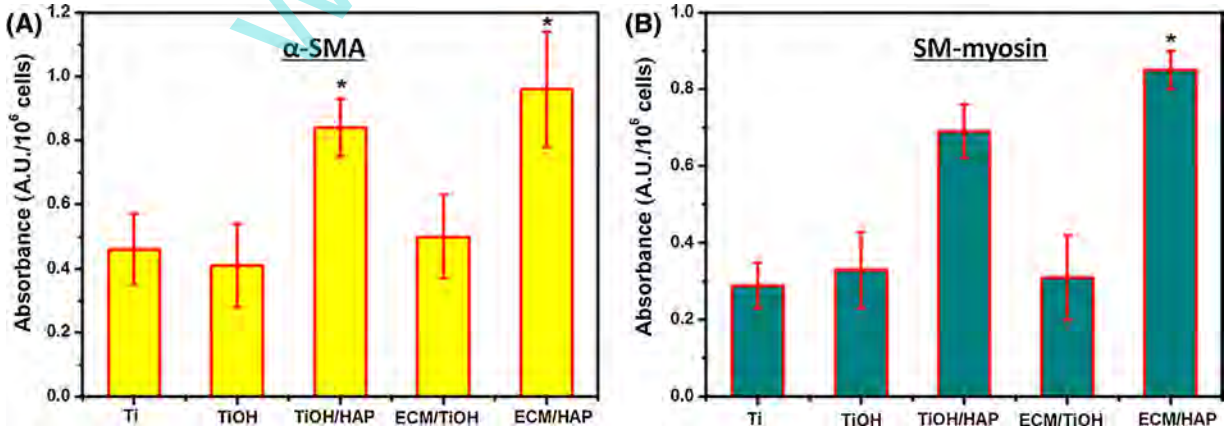

Fig. 7. Quantitative characterization of (A) α-SMA and (B) SM-myosin using an ELISA assay (*$p < 0.05$ compared with other samples, mean ± SD, $N = 3$).

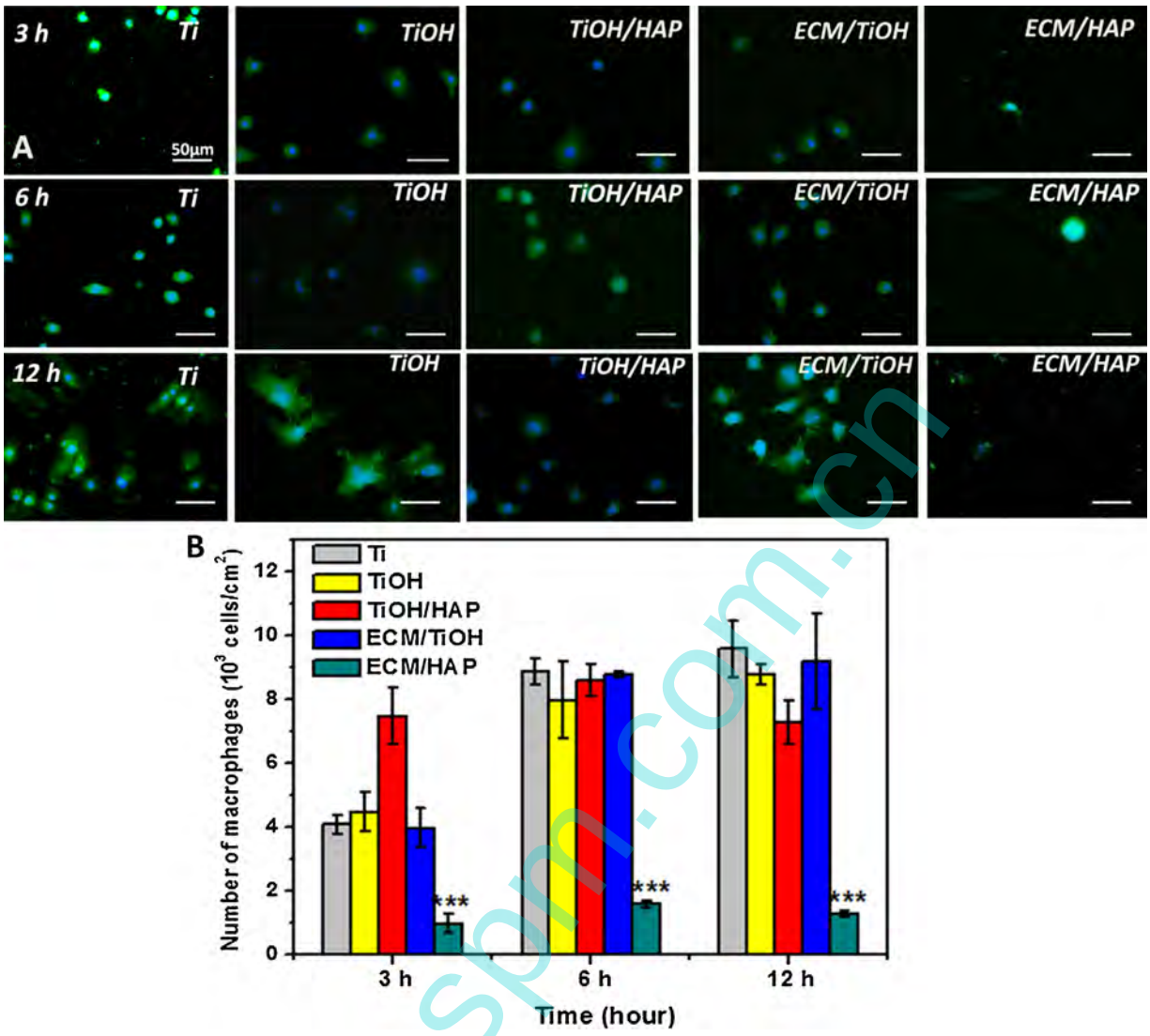

Fig. 8. (A) Fluorescence images of macrophages on Ti, TiOH, TiOH/HAP, ECM/TiOH and ECM/HAP surfaces (actin: green; nucleus: DAPI); (B) Amount of attached macrophages on TiOH, TiOH/HAP, ECM/TiOH and ECM/HAP surfaces (***$p < 0.001$ compared with other samples, mean ± SD, $N = 3$).

property. It has been reported that FGN binds to HA by linking to C3 binding sites of FGN to HA [41,43]. Then, the denatured FGN promote the platelet adhesion and aggregation to the TiOH/HAP surface, several red blood cells are also involved in this progress and finally the thrombosis formats. The preparation of ECM/HAP can introduce more anticoagulant factors, such as NO and PGI_2 which has been demonstrated in our previous study [33]. These factors could reduce the conformational change of FGN (Fig. 4), and further inhibit the platelet and red blood cells. Thus, it could be concluded that the ECM/HAP surface got excellent hemocompatibility.

Rapid endothelialization is one of the most important aspects for blood contacting vascular devices. This process could be realized through ECs migration from the surrounding vessel wall [44] and/or induction of EPCs homing from the blood stream [45]. Nevertheless, Vartanian et al. have demonstrated that EPCs are an advantageous cell source for endothelializing vascular constructs because EPCs deposited and remodeled the ECM to a greater extent compared with ECs [1]. In this work, we choose EPCs derived from bone marrow mesenchymal of SD rats as the cell source to evaluate the endothelializing function of the ECM/HAP surface. The EPCs staining and counting results proved the good endothelializing function of the ECM/HAP surface compared with ECM/TiOH, TiOH, Ti and TiOH/HAP (Fig. 5). The morphology of EPCs was elongated and the EPCs coverage was also considerable. The reason might be greater producing of the proteins which promote EPCs adhesion and proliferation such as fibronectin, laminin, collagen IV and VEGF. The anti-coagulation factors, NO and PGI_2 released from the EPCs were also detected (Fig. S3). The EPCs on the ECM/HAP surface significantly released more NO and PGI_2 compared with ECM/TiOH, TiOH, Ti and TiOH/HAP.

SMCs and macrophages represent major cellular components in vascular disorders such as atherosclerosis, in-stent restenosis, and vein graft disease [46]. Thus, enhancing the function of inhibiting the excessive proliferation of SMCs and the attachment of macrophages is very important for the blood implanted stent. The evaluation of SMCs proliferation (Figs. 6 and 7) and macrophage adhesion (Fig. 8) were also performed in this work. It seemed that the micro-pattern surfaces could effectively regulate the SMCs proliferation to a lower value while only ECM/HAP surfaces showed an effective function of inhibiting macrophage adhesion. The SMCs regulation function of HA micro-pattern may be attributed to the inhibiting effect of HMW-HA on SMCs, and the macrophage adhesion result indicated the non-immunogenic property and structural protection of the HMW-HA. The great amount

of adherent macrophages on TiOH/HAP may be due to the exposed TiOH which can cause immune rejection.

Incontrovertibly, continuous anticoagulant, anti-inflammatory, inhibiting hyperplasia as well as rapid endothelialization of the implants surface will be the most ideal situation for their long-term implantation. Further investigation will focus on the bio-evaluation of the ECM/HAP coating under flow conditions *in vitro* and *in vivo* experiments. Therefore, the combining of HA micro-pattern and endothelial ECM provides us a potential and promising method to develop the cardiovascular implants with a bionic properties.

5. Conclusion

To enhance the biocompatibility of the vascular implanted biomaterials, a bionic coating composed of endothelial ECM and the HA micro-pattern are prepared on the Ti surface. The bio-evaluation results consistently demonstrate that the HA-patterned endothelial ECM simultaneously improves the hemocompatibility and endothelialization of biomaterials, and inhibits excessive proliferation of SMCs and attachment of the macrophages, which may show great significance for the implantation of biomedical devices. In our opinion, this bionic surface modification technology may offer a potential application of implanted biomaterial devices that are directly in contact with blood and tissue.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2015.01.010.

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