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# Human-derived extracellular matrix from Wharton's jelly: An untapped substrate to build up a standardized and homogeneous coating for vascular engineering

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## abstract

One of the outstanding goals in tissue engineering is to develop a natural coating surface which is easy to manipulate, effective for cell adhesion and fully biocompatible. The ideal surface would be derived from human tissue, perfectly controllable, and pathogen-free, thereby satisfying all of the standards of the health authorities. This paper reports an innovative approach to coating surfaces using a natural extracellular matrix (ECM) extracted from the Wharton's jelly (WJ) of the umbilical cord (referred to as WJ-ECM). We have shown by atomic force microscopy (AFM), that the deposition of WJ-ECM on surfaces is homogenous with a controllable thickness, and that this easily-prepared coating is appropriate for both the adhesion and proliferation of human mesenchymal stem cells and mature endothelial cells. Furthermore, under physiological shear stress conditions, a larger number of cells remained adhered to WJ-ECM than to a conventional coating such as collagen – a result supported by the higher expression of both integrins  $\alpha 2$  and  $\beta 1$  in cells cultured on WJ-ECM. Our data clearly show that Wharton's jelly is a highly promising coating for the design of human biocompatible surfaces in tissue engineering as well as in regenerative medicine.

## Statement of Significance

Discovery and design of biomaterial surface are a hot spot in the tissue engineering field. Natural matrix is preferred to mimic native cell microenvironment but its use is limited due to poor resource availability. Moreover, current studies often use single or several components of natural polymers, which is not the case in human body. This paper reports a natural extracellular matrix with full components derived from healthy human tissue: Wharton's jelly of umbilical cord. Reconstituting this matrix as a culture surface, our easily-prepared coating provides superior biocompatibility for stem and mature cells. Furthermore, we observed improved cell performance on this coating under both static and dynamic condition. This novel human derived ECM would be a promising choice for regenerative medicine.

## 1. Introduction

The main goal of the tissue engineering triad is to create combinations of cells, scaffolds and environment (i.e. soluble mediators and/or mechanical stresses). For vascular tissue engineering, finding appropriate cells and scaffolds remain the two major challenges. Stem cells, and in particular, mesenchymal stem cells (MSCs), have emerged as the most promising source of suitable cells as a result of their good availability and ability to differentiate into vascular cells, as well as their immunosuppressive properties which are particularly interesting for allogeneic applications [1,2]. As to the scaffold, synthetic materials are most commonly used for engineered vascular grafts, but they are poor substrates for cells. Therefore, substantial effort has been made to develop new coatings, ranging from synthetic polymers to isolated natural extracellular matrix (ECM) to: (i) provide a solid base for cell anchorage to allow resistance to blood flow; (ii) favor endothelial differentiation of stem cells; and (iii) facilitate cell expansion to mimic the endothelial layer [3,4].

Synthetic polymers have the advantage of being precisely tailored, and they can be combined with other biological interfaces, allowing for the construction of desired tissues [5]. However, those which have been studied to date have often exhibited poor biocompatibility and/or immunogenicity, limiting their clinical use. In the case of ECM, decades of extensive research on its structure and biological influence on cell behavior and fate have shown that its physical properties (elasticity, stiffness, resilience to the cellular environment, etc.), specific chemical signals exhibited by its peptide epitopes, and the nanoscale topography of micro-environmental adhesive sites, are all relevant to the design of biomimetic surfaces [6]. Nonetheless, current studies usually exploit one or several purified ECM components to construct surfaces [7], a practice which does not faithfully replicate the native ECM entire environment [8].

Complete ECM derived from human tissue could be an ideal source for natural matrices. In this context, tissues such as placenta and umbilical cord which are obtained from non-pathological medical procedures, could provide a good supply. For example, placenta has recently been treated using a chemical procedure (urea solubilization) to furnish a complex of ECM for tissue engineering [9]. Wharton's jelly of human umbilical cord contains various types of collagen and glycosaminoglycan, as well as growth factors and/or cytokines synthesized by mesenchymal stem cells, and thus may be viewed as a natural source for ECM-based coating [10]. Indeed, Hao et al. previously showed that mechanically-obtained ECM from Wharton's jelly can support MSC culture [11].

Here we describe a novel enzymatic method to isolate complete ECM from Wharton's jelly (this material will be referred to hereafter as WJ-ECM), which boasts many benefits relative to existing procedures. The most important advantages of our coating are its human tissue origins and a method of preparation that does not involve chemicals which are toxic to cells. To test our material, we prepared, characterized, and determined the in vitro response to WJ-ECM of MSCs and endothelial cells, which are currently the two most-used cell sources in vascular tissue engineering, under both static and dynamic conditions. Our data show that WJ-ECM-

derived coating enhanced proliferation of both human MSCs (hMSCs) and human umbilical vein endothelial cells (HUVECs). This new surface also exhibits improved cell resistance to flow under shear stress, a phenomenon which is of particular interest in vascular tissue engineering. The role of integrins in the possible mechanism underlying cell retention under shear stress was also investigated.

## 2. Methods

### 2.1. Preparation of WJ-ECM coating

Fresh human umbilical cords were obtained after full-term births with informed consent using the guidelines approved by the Cell Therapy Unit of the University Hospital Center (CHRU) of Nancy (Authorization number: TCG/11/R/011). Briefly, whole umbilical cord was washed 3 times with Hank's balanced salt solution (HBSS) and cut into 5-cm explants, and Wharton's jelly was carefully isolated without taking umbilical cord membrane or vessels. The WJ of umbilical cord from 3 separate donors was then pooled prior to sample homogenization. The isolated WJ was further cut into 0.5-cm pieces using sterile surgical scissors. The WJ was then digested in trypsin (0.025%, Bioblock, 1158-0626, France) at 37 °C for 24 h with magnetic stirring (ratio of wet WJ/trypsin was 1 g: 5 ml), the stirred suspensions were centrifuged at 16,000g for 10 min, and the collected supernatants were filtered (100 µm filter). Fetal bovine serum (FBS) (Dutscher, S1900) was added into the solution (final concentration of 10%) to inhibit the trypsin reaction. The concentration of WJ (mg/ml) was calculated using lyophilized WJ tissues dissolved in final phosphate buffered saline (PBS). The obtained WJ-ECM solution was conserved at 4 °C for further use. To coat culture surfaces, the freshly-obtained or conserved WJ-ECM was deposited onto glass coverslips which had been carefully pre-treated with SDS-HCl [12]. The glass coverslips coated with WJ-ECM were incubated for 12 h at 37 °C, then washed twice with culture medium.

### 2.2. Characterization of the surface coating by AFM

Before each measurement, fresh samples were extensively rinsed with milli-Q water and then slowly and completely dried with nitrogen. AFM experiments were carried out using a MFP3D-BIO instrument (Asylum Research Technology, Atomic Force F&E GmbH, Mannheim, Germany). Silicon nitride cantilevers of conical shape were purchased from Olympus (OTR-400 PSA, Bruker-nano AXS, Palaiseau, France). The spring constants of the cantilevers measured using the thermal noise method were found to be 0.020–0.024 nN/nm. Experiments were performed both in air and in PBS buffer at room temperature. AFM images were recorded at room temperature (20 °C) in contact mode. The applied force between the tip and the surface was carefully monitored and minimized at approximately 0.2nN for experiments performed in aqueous medium. All images were collected with a resolution of 512 × 512 pixels at a scan rate of 1 Hz. The nanoindentation method was used to quantify the mechanical properties. Young's modulus of the thin films was calculated from force vs. indentation curves. Mechanical properties were obtained by recording a grid map of 32-by-32 force curves at different locations on the thin deposits over the 10 µm × 10 µm surface area. The maximal loading force was 0.5 nN, the piezodriven was fixed to 1 µm and the approach rate was 1000 nm s<sup>-1</sup>. The Young's modulus was estimated from the analysis of the approach curves according to the Sneddon model [13].

$$F = \frac{2E \cdot \tan(\alpha)}{\pi(1 - \nu^2)} R^{1/2} d^2 \cdot f_{BECC}$$

where  $d$  is the indentation depth,  $\nu$  the Poisson coefficient,  $R$  the curvature radius of the AFM-tip apex and  $f_{BECC}$  is the Bottom Effect Cone Correction function that takes into account the presence of substrate stiffness [14]. All the force curves were analyzed by means of an automatic Matlab algorithm described elsewhere [15].

### 2.3. Biomolecular composition analysis

WJ-ECM solution (10mg/ml) was analyzed using a human angiogenesis array kit (R&D systems, ARY007, France) according to the manufacturer's protocol, and the pixel density of detected spots was measured with Image J software.

### 2.4. Culture of hMSC and HUVEC cells

To isolate hMSCs from umbilical cord (UC), Wharton's jelly was sliced into 5-mm explants after removing the umbilical vessels and the umbilical outer layer membrane, and the slices were subsequently attached and cultured in minimum essential medium Eagle-alpha modification (α-MEM) (Lonza, BE12-169F) supplemented with 10% FBS, Fungizone<sup>1</sup> (100mg/ml, Fisher, 11520496), penicillin (100 IU/ml, Sigma, P0781) and L-glutamine (200 mM, Sigma, G7513) on culture plates. The medium was changed every 3 days, and hMSC populations appeared as outgrowths from the UC fragments at day 6. After 15 days, the UC fragments were discarded, and the cells were passaged with trypsin and expanded until they reached sub-confluence. To isolate HUVECs, full length UC was taken, the umbilical vein was washed carefully with HBSS to remove residual blood, an appropriate volume of trypsin was injected into the vein, the trypsin was harvested after 20 min of incubation at 37 °C, the suspension was centrifuged at 300g for 5 min, and finally, the pellet was suspended in endothelial basic medium (EBM<sup>TM</sup>-2, Lonza) supplemented with 10% FBS, Fungizone<sup>1</sup> (100 mg/ml, Fisher, 11520496), penicillin (100 IU/ml, Sigma, P0781) and L-glutamine (200 mM, Sigma, G7513). Cells were cultivated on culture plates for 10 days until 90% confluence was reached.

### 2.5. Bioactivity of soluble factors in WJ-ECM

hMSCs at passage 4 were seeded onto 12-well plates at 3000 cells/cm<sup>2</sup>. One milliliter of culture medium  $\alpha$ -MEM supplemented with different percentages of WJ-ECM solution (2.5%, 10% and 40%, respectively) was added to different wells to observe its influence on cell proliferation. In order to ensure that the influence on cell proliferation was not due to FBS in the WJ-ECM solution but to active soluble factors in the solution, cells were seeded into different culture mediums with or without FBS. The various culture conditions were: hMSCs cultured  $\pm$ 10% FBS (the usual concentration in cell culture); WJ-ECM  $\pm$  FBS, where (1) 40% of WJ-ECM solution (containing 10% of FBS) corresponds to 10% FBS in the cell culture medium and (2) 10% of WJ-ECM solution (containing 2.5% of FBS) which corresponds to 2.5% FBS in the cell culture medium. (Note, as the 10% WJ-ECM contains 2.5% FBS, we also evaluated a 2.5% FBS control). After 5 days of cell culture, cells were stained with Live/Dead assay (Sigma-Aldrich, 04511, France) and observed using a fluorescence microscope.

## 2.6. Cell viability and proliferation

The viability of both hMSCs and HUVECs on different surfaces was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The MTT assays were carried out as per the manufacturer's protocol (Sigma-Aldrich, M5655). Briefly, cells were seeded onto a 24-well plate containing cover slides coated with WJ-ECM at different viscosities; collagen (0.1 mg/ml) was used as a positive control, and cover slips alone as a negative control. Cells were seeded at a density of 5.10<sup>3</sup> cells/cm<sup>2</sup>. After 1, 3 and 5 days of culture, medium was removed, 10% MTT in fresh medium was added, and the cells were incubated for 4 h. The MTT solution was then carefully removed after incubation, and 200  $\mu$ l of DMSO per well were added to dissolve the produced formazan, followed by a further incubation for 5 min. 100  $\mu$ l of solution from each well were transferred to a new 96-well plate, and the absorbance at 570 nm read using an automated plate reader (Varioskan Flash, Thermo scientific, France).

## 2.7. Cell proliferation by DNA quantification

In parallel, at each time point, total DNA of each sample was collected and quantified using a Hoechst 33258 fluorescent dye assay (Invitrogen, H3569, France). Briefly, cells cultivated on different surfaces were washed twice with PBS and the cell pellet was harvested, after which the total DNA content was extracted with two freeze (liquid nitrogen)-thaw (37C) cycles. Fluorescence was measured with a microplate fluorescence reader (Varioskan Flash, Thermo scientific, France) using equal volumes of cell lysates and Hoechst 33258 dye solution (100  $\mu$ g ml<sup>-1</sup>). The number of viable cells per scaffold was calculated by extrapolation from a calibration curve that was generated as described in Rago et al. [16].

## 2.8. Endothelial differentiation of hMSCs

On passage 4, hMSCs were seeded at a density of 3000 cells/cm<sup>2</sup> onto glass coverslips coated with WJ-ECM or collagen or without coating. When cell confluence reached 40%, a commercial medium (EGM<sup>TM</sup>-2) which is used for EC differentiation was added into the culture plates. hMSCs were cultivated in EGM<sup>TM</sup>-2 for 14 days, with the medium being changed every 3 days. Protein and RNA were harvested for assessment of differentiation markers by Western-blot and quantitative real-time PCR, respectively.

## 2.9. Laminar shear stress assessment

To assess the cell adherence of both hMSCs and HUVECs under dynamic conditions, the cell nuclei were stained with Hoechst 33258 (Invitrogen, H3569) and the membrane with DiI (Invitrogen, D282), before being seeded onto coated glass coverslips. When the cell confluence reached 70%, the glass coverslips were assembled into a parallel-chamber flow system (Masterflex, USA). The shear stress that cells experienced was gradually increased to 20 dyn/cm<sup>2</sup>, and applied for 12 h. After application of shear stress, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, France), photos were taken by fluorescence microscopy (Leica DMI 3000B). 10 randomly chosen areas were investigated before and after shear stress, and the resulting images analyzed using the software Image J to count cell numbers [17]. Cells were then stained using crystal violet (Sigma-Aldrich, France) for large-scale images. For gene expression assessment, RNA was harvested directly after shear stress.

## 2.10. Quantitative real-time PCR

Total RNA from cell samples was isolated using the RNeasy Mini Kit (Qiagen). cDNA was synthesized from 100 ng total RNA using iScript Reverse Transcription Supermix (Bio-Rad). Quantitative Real-Time PCR (RT-PCR) was carried out using iQ<sup>TM</sup> SYBR<sup>1</sup> Green Supermix (Bio-Rad) and primers designed by us for human cluster of differentiation (CD)31, vascular endothelial (VE)-cadherin, vascular endothelial growth factor receptor 2 (VEGFR2), integrin  $\alpha$ 2,  $\alpha$ 3, and  $\beta$ 1 and the ribosomal protein S29 (RP 29) with the use of the StepOnePlus<sup>TM</sup> device (ThermoFisher, France). Cycling parameters were 3 min at 95 C; 40 cycles of 30 s denaturation at 95 C and 1 min of elongation at the annealing temperature (60 C for CD31, VE-Cadherin, VEGFR2 and RP29, 54C for integrin  $\alpha$ 2, 56 C for integrin  $\alpha$ 3 and 58 C for integrin  $\beta$ 1). Data were normalized using RP29 as a housekeeping gene. Analyses and fold differences were determined using the comparative CT method. Fold change was calculated from the  $\Delta\Delta$ CT values with the formula  $2^{-\Delta\Delta CT}$  and data are reported relative to control values.

## 2.11. Statistical analysis

Results are presented as mean  $\pm$  standard deviation. Statistical significance was determined by ANOVA followed by a t-test between each group; p-values less than 0.05 were considered as statistically significant.

### 3. Results

#### 3.1. Extraction of WJ-ECM from Wharton's jelly

As an initial step, we compared chemical, mechanical and enzymatic procedures to extract the ECM from the WJ of the umbilical cord. These procedures included acetic acid, type II collagenase, PBS (mechanical) and trypsin-mediated methods. Acetic acid was chosen as it was previously used to isolate and purify collagen I [18], one of the WJ-ECM components. When applied to the WJ, one week was required to achieve complete digestion of the ECM, and the procedure failed to produce enough ECM to coat glass substrates. Type II collagenase is commonly used for isolation of MSCs from the connective tissue of umbilical cord [19], but atomic force microscopy (AFM) analysis showed that this procedure was too aggressive for ECM extraction, as no collagen fibers could be detected after digestion. Similarly, analysis by AFM showed that Wharton's jelly stirred in PBS did not generate enough products to coat glass substrates. Taken together, these data demonstrated that acetic acid, type II collagenase, and PBS were unsuitable for obtaining an appropriate coating surface (Fig. S1).

We next investigated whether trypsin is able to digest WJ-ECM, as it is a common enzyme used in routine cell culture. Dissociated WJ tissue was digested in 0.025% trypsin at 37 °C for 24 h under magnetic stirring, the supernatant was collected after centrifugation, and FBS was added to stop the trypsin reaction. This method allowed us to obtain a viscous solution, with the viscosity between different batches remaining stable (Fig. 1a). Twenty-four hour digestion and further centrifugation removed cells or other debris in the WJ-ECM (Fig. 1b, c). The complete WJ-ECM solution was lyophilized to obtain a dry powder, and the precise concentration of WJ-ECM in the solution was calculated according to this dry powder and original solution volume. The influence of FBS, trypsin and PBS in the mixed solution was also taken into account during the calculations.

Previous studies have demonstrated that WJ contains abundant growth factors, a beneficial feature as this would likely stimulate angiogenesis [20]. Therefore, following the enzymatic extraction procedure, the biomolecular composition of the WJ-ECM was determined using a human angiogenesis array (Fig. S2). Out of the 55 proteins that were screened for, 16 were detected in WJ-ECM (Figs. 1d, S2). These included several essential growth factors for vascular cell differentiation such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). According to the results, there was little variability between healthy donors. When WJ-ECM solution was used as a cell culture supplement, increased hMSCs proliferation was observed (Fig. S3). This result indicated that the soluble factors in WJ-ECM remained active.

#### 3.2. Fabrication of WJ-ECM derived coating

We tested the coating capacity of WJ-ECM on positively and negatively charged glass coverslips. Our obtained AFM images showed that WJ-ECM extract could not coat an electrically-neutral glass coverslip, but could produce a non-continuous thin layer on a positively-charged surface. On the other hand, WJ-ECM reliably formed a continuous coating on a negatively-charged surface (Figs. S4S6).

To visualize the morphological features of the WJ-ECM coated surface, and in particular the thickness of the WJ-ECM-based coating, different concentrations of WJ-ECM solution were pipetted onto negatively-charged glass coverslips and incubated for 12 h at 37 °C before imaging by AFM. Depending on the concentration, WJ-ECM formed a homogeneous coating from  $13 \pm 5$  nm to  $74 \pm 16$  nm thick (Table 1 and Figs. S7S12). At 5 mg/ml, a thickness of  $45 \pm 12$  nm was obtained (Fig. 2a, b), similar to that produced by collagen (0.1mg/ml) which was used as positive control (Fig. 2c, d). As the stiffness of the coated material has a significant effect on cell adhesion, proliferation and/or differentiation [21], the Young's modulus of the WJ-ECM was determined by the nanoindentation method using AFM. Mechanical measurements performed with AFM revealed an inverse correlation between the coating's Young modulus and its thickness. At the lowest concentration (1 mg/ml), the stiffness of the coating reached a maximal value of  $1.34 \pm 0.19$  MPa ( $n = 1024$ ). At 5 mg/ml, the stiffness was  $1.18 \pm 0.15$  MPa ( $n = 1024$ ), a value below that obtained for a collagen coating used as positive control ( $2.04 \pm 0.17$  MPa ( $n = 1024$ )) (Fig. 2e). Such an increase in stiffness is likely due to a decrease in the water content of the layer, as well as greater crystalline packing of WJ-ECM components during the adsorption process.

#### 3.3. Static cell adhesion and proliferation

To examine the behavior of the cells seeded on this surface, the viability of the hMSCs and HUVECs was examined. Human MSCs showed a significantly higher mitochondrial activity when cultured on 5–8mg/ml WJ-ECM than on glass coverslips alone (Fig. 3a). In the case of the HUVECs, no difference between WJ-ECM concentrations was observed. However, cellular mitochondrial activity was also significantly enhanced when compared to the activity of cells cultured on glass coverslips (Fig. 3b).

We next evaluated at different time points the adhesion and proliferation of HUVECs and hMSCs on WJ-ECM coating, when applied at a concentration of 5 mg/ml. Commercially available collagen I at 0.1 mg/ml and glass coverslips were used as positive and negative controls, respectively. Cells cultured on a collagen-coated surface showed a slightly higher viability than those cultured on coverslips at all 3 time points (day 1, day 2 and day 3 for hMSCs, day 1, day 3 and day 7 for HUVECs, taking into account their different proliferation rates), but this difference was not statistically significant. Interestingly, however, the WJ-ECM coated surface afforded a significantly higher mitochondrial activity, when measured at the third time point (Fig. 3c, d). Total DNA quantification was also performed to evaluate cell proliferation on different surfaces (glass, collagen and ECM coating). In agreement with the viability observation, the WJ-ECM-coated surface promoted cell proliferation both for hMSCs and HUVECs (Fig. 3e, f). The difference in proliferation rates became significant between the glass and ECM coatings at days 3 and 7 for hMSCs and HUVECs, respectively.

#### 3.4. Differentiation of hMSCs to endothelial-like cells

Published studies, as well as our previous data, suggest that cell culture surface and medium can contribute to variations in the differentiation capacity of hMSCs [1,22]. As VEGF has been detected in the WJ-ECM, we thought to further investigate the endothelial differentiation potential of hMSCs. For this, we treated hMSCs expanded on WJ-ECM with Endothelial Growth Medium-2 (EGM™-2). Fourteen days after the induction of differentiation, Western-blot and RT-PCR revealed the expression of endothelial cell specific markers such as CD31, VEGFR2 and VE-Cadherin (Fig. 4a, b). These results confirmed that the functionalization of the cell culture surface is mandatory to induce differentiation of hMSCs, and show that the WJ-ECM coating is suitable for both the expansion and differentiation of hMSCs.

### 3.5. Cell retention under dynamic stimulation

Biomaterials in vascular tissue engineering must provide appropriate cell anchorage to resist physiological shear stress. To evaluate cell retention on WJ-ECM, we expanded MSCs and HUVECs onto 3 different surfaces (WJ-ECM coating, collagen and glass coverslips), then subjected them to 12 h of laminar shear stress at  $20 \text{ dyn/cm}^2$ . Crystal violet staining showed that more cells remained on WJ-ECM coating than the other two surfaces after shear stress (Fig. 5a). Cells from both sheared and static samples were also stained for membranes and nuclei (Fig. 5b), images of 10 randomly chosen fields on these coatings were taken using a fluorescence microscope, and the cells were then counted using Image J Software [17]. Notably, the WJ-ECM-coated surface maintained  $92 \pm 2\%$  of MSCs after shear stress, whereas the collagen-coated surface and control glass coverslips retained  $78 \pm 3\%$  and  $74 \pm 5\%$  of MSCs, respectively (Fig. 5c). The retention of HUVECs on these different surfaces after 12 h of shear stress showed a similar pattern: the WJ-ECM coated surface retained  $92 \pm 3\%$  of the HUVECs whereas  $75 \pm 11\%$  and  $74 \pm 13\%$  were retained on collagen and on glass coverslips, respectively (Fig. 5c). These results clearly suggest that WJ-ECM coating allows a better cell anchorage than commercially-available collagen I coating or glass, with the consequence that WJ-ECM may be a suitable coating to build up a continuous layer to mimic the endothelium during vascular tissue engineering.

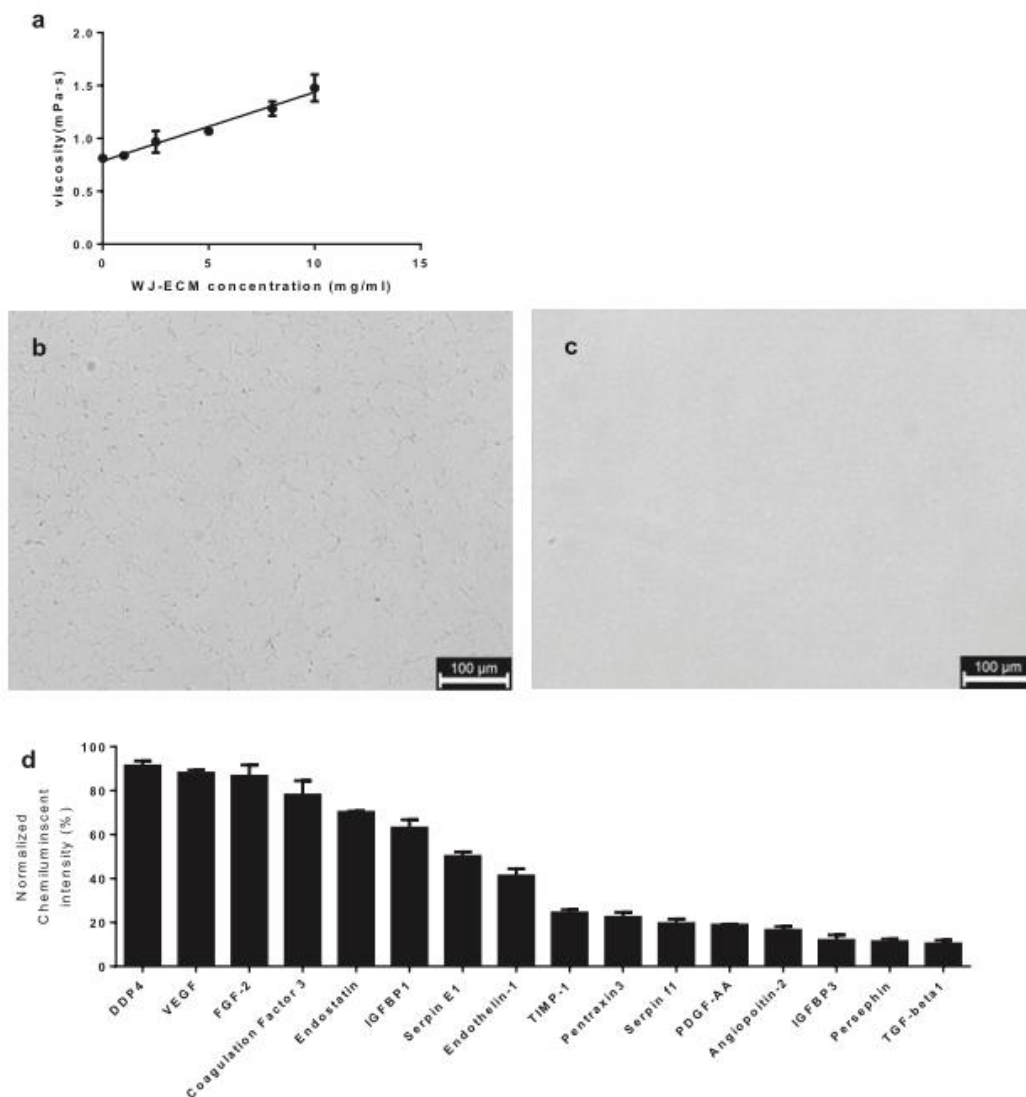


Fig. 1. Characterization of WJ-ECM solution. (a) The viscosity of WJ-ECM depended on its concentration. (b-c) Optical microscopic images showing the appearance of commercial collagen (0.1 mg/ml) (b) and WJ-ECM (5 mg/ml) (c) deposited on glass coverslips. (d) Cytokine analysis of WJ-ECM solution (10 mg/ml) was performed using a human angiogenesis array, and data were normalized on a scale ranging from negative control values (0%) to positive control values (100%). Data are representative of 3 independent batches, where each batch contained WJ tissue collected from 3 different healthy donors.



**Table 1**  
Concentration-dependent thickness and Young's modulus of WJ-ECM.

Surface coating solution (mg/ml)	Thickness of surface (nm)	Young's modulus (MPa)
Collagen	0.1 37 ± 15	2.04 ± 0.17
WJ-ECM solution	1 13 ± 5	1.34 ± 0.19
	2.5 34 ± 9	1.27 ± 0.12
	5 45 ± 12	1.18 ± 0.15
	7.5 61 ± 17	0.82 ± 0.10
	10 74 ± 16	0.61 ± 0.07

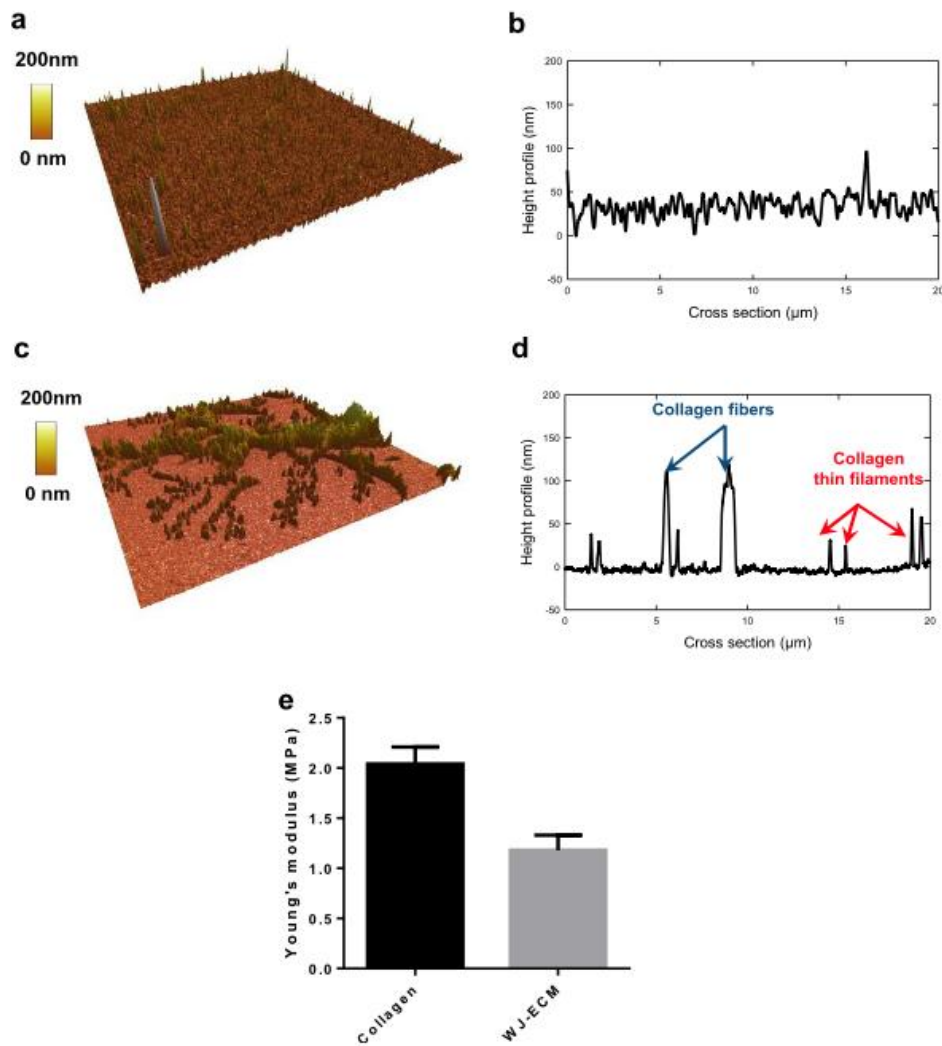


Fig. 2. Characterization of WJ-ECM derived coating. (a) Atomic force microscopy image of WJ-ECM at 5 mg/ml coated onto negatively-charged coverslips showing formation of a homogenous surface. (b) Cross-section of WJ-ECM coating, revealing a measured thickness of  $45 \pm 12$  nm and an even distribution. (c) Commercial collagen (0.1 mg/ml) coating which served as a positive control. Collagen fibers are observed and they showed a random distribution. (d) The average thickness of the collagen coating was  $37 \pm 15$  nm. (e) Young's modulus of collagen coating at 0.1 mg/ml and WJ-ECM coating at 5 mg/ml.

### 3.6. Gene expression responding to shear stress

Human MSCs and HUVECs are known to express a large range of integrin subunits [17,23,24]. Here we selected 3 representative integrins (integrins  $\alpha 2$ ,  $\alpha 3$  and  $\beta 1$ ) [25,26], to evaluate their expression in cells cultivated on different surfaces in the presence or absence of shear stress. Under static conditions, hMSCs expressed significantly higher levels of integrin  $\alpha 2$  on the WJ-ECM surface than on glass coverslips or collagen (Fig. 6a). Furthermore, following 6 h of physiological shear stress, a significantly increased expression of integrins  $\alpha 2$  and  $\beta 1$  on WJ-ECM was observed compared to the static condition on WJ-ECM (Fig. 6b).

Under the same dynamic conditions, a slight decrease of  $\alpha 2$  and  $\beta 1$  expression was observed in hMSCs cultured on collagen, and a significant increase of  $\alpha 2$  expression was observed in hMSCs on glass. As for the HUVECs, higher  $\beta 1$  expression was observed on WJ-ECM relative to the other two surfaces under static condition (Fig. 6c). Under shear stress, a significant increase in expression of integrins  $\alpha 2$  and  $\beta 1$  was observed in HUVECs cultured on WJ-ECM. On collagen, this increase was only significant for  $\beta 1$  (Fig. 6d). Thus overall, both hMSCs and HUVECs exhibited a significant increase in expression of integrins  $\alpha 2$  and  $\beta 1$  due to shear stress on WJ-ECM relative to collagen. In the case of integrin  $\alpha 3$  expression, no difference was detected under static culture conditions irrespective of the surface employed.

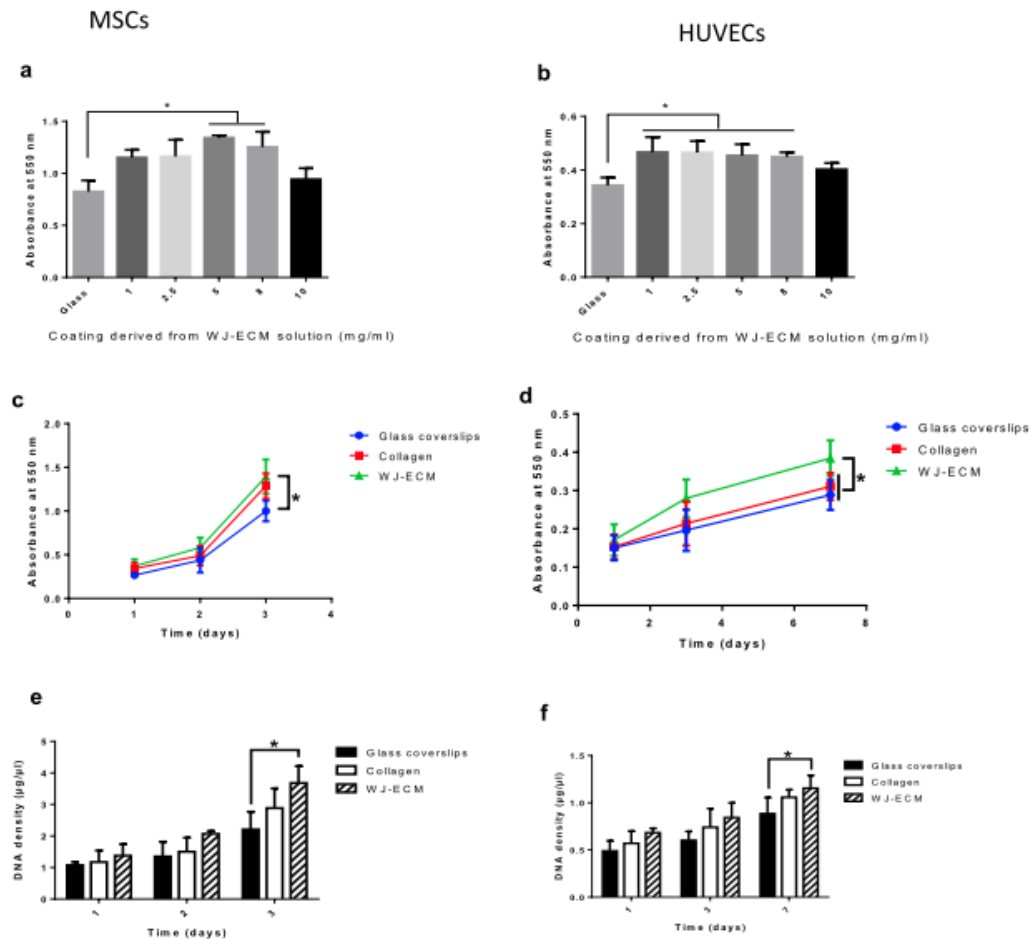


Fig. 3. Cell behavior on different surfaces. Cells were expanded on different coating surfaces formed from various concentrations of WJ-ECM. Cell adhesion was evaluated by MTT assay for mesenchymal stem cells (MSCs) after 72 h (a) and for endothelial cells (ECs) after 9 days (b). A single factor ANOVA test allowed rejection of the null hypothesis for all assays and a Tukey test between all groups was performed (SD,  $n = 4$ ,  $^* p < 0.05$ ). Cell proliferation of MSCs (c) and ECs (d) on glass coverslips, collagen (0.1 mg/ml) and WJ-ECM-coated slips (5 mg/ml) was measured at three time points by MTT assay. The DNA content of each sample was also measured at those time points for MSCs (e) and ECs (f) (SD,  $n = 4$ ,  $^* p < 0.05$  compared to glass coverslip surfaces). In all of the assays, the WJ-ECM coating at 5 mg/ml produced the best results in terms of both cell adhesion and proliferation.

Furthermore, a significant decrease in integrin  $\alpha 3$  was observed after subjecting HUVECs cultured on all three surfaces to shear stress. Taken together, these results suggest that integrins  $\alpha 2$  and  $\beta 1$  might be involved in helping cells cultured on WJ-ECM to resist shear stress.

In order to investigate the interaction between cells and WJ-ECM, we assessed the expression levels of mechanosensory complex of endothelial cells: CD31, VE-cadherin and VEGFR2 in HUVECs on this surface, and compared them with those obtained on collagen and glass coverslips under both static and dynamic conditions. Gratifyingly, higher levels of CD31, VE-cadherin and VEGFR2 were detected in HUVECs cultured on WJ-ECM than from cells cultured on collagen or glass coverslips (Fig. 6e).

Even more interestingly, dynamic conditions (20  $\text{dyn}/\text{cm}^2$  laminar shearing for 6 h), increased expression of these genes in cells cultured on collagen, but significantly decreased expression of these genes in cells culture on glass (Fig. 6f). Therefore, dynamic conditions enhance expression of the mechanosensory complex in cells grown on both collagen and WJ-ECM.



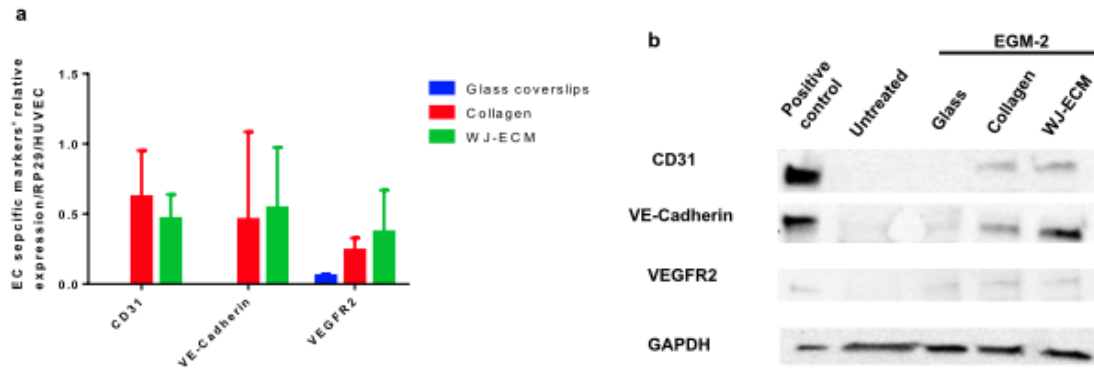


Fig. 4. Mesenchymal stem cells differentiate into an endothelial-like cell lineage. MSCs cultured on 3 different surfaces were treated with EGM<sup>TM</sup>-2 for 14 days. (a) Endothelial specific gene expression was normalized to the house keeping gene RP29 and then normalized to positive HUVECs. (b) Expression of endothelial specific markers by Western-blot after EGM<sup>TM</sup>-2 treatment. HUVECs were taken as a positive control, while untreated MSCs cultured on glass served as a negative control.

#### 4. Discussion

Cardiovascular diseases, particularly atherosclerotic coronary artery diseases are still the leading cause of mortality the world over [27]. Tissue engineered vascular grafts which incorporate cells into a biodegradable scaffold, have emerged as a promising alternative for those patients who lack appropriate autologous graft candidates [28]. The main hurdle in small-diameter (<6 mm) vascular tissue engineering is the thrombogenicity of newly designed grafts. Studies aimed at resolving this challenge have to date focused on investigating cell sources and discovering new scaffold/coating that promote full cellularization. MSCs are now considered as the most promising source for vascular tissue engineering due to their excellent availability, ability to differentiate into smooth muscle cells and endothelial cells under appropriate stimulation, and their immunosuppressive properties [29,30]. On the other hand, mature endothelial cells are still the first choice for the purpose of mimicking the native environment [9]. Only grafts that are well cellularized or which can recruit circulating cells, can avoid long-term graft failure [31,32]. To this end, new synthetic and natural isolated scaffolds/coatings have been extensively studied. Unlike traditional approaches which isolate one or few components of tissue extracellular matrix, the full complement of ECM components is preferred in order to mimic the native cell environment. However, non-pathological human source is often limited. We describe in detail here a novel full ECM derived from healthy human Wharton's jelly (WJ-ECM).

The isolation of ECM from human tissue can be challenging in terms of restoring all of the functional components. Mechanical, chemical and enzymatic methods are most typically employed in ECM isolation. For example, ECM has been extracted from human placenta by chemical procedures and shown to contain various functional cytokines [9]. In this study, WJ tissue simply stirred in PBS failed to generate enough product to form a coating, while ECM derived from tissue dissolved in acetic acid was also unsatisfactory. Furthermore, this chemical treatment is a time-consuming procedure, and is typically used for collagen I isolation but not for obtaining full ECM. Here we choose to isolate complete ECM using enzymatic methods to simplify the isolation procedure, and more importantly, to maintain the ECM-containing tissue in a relatively physiological condition. We next compared collagenase II with trypsin, as both are routinely used in cell culture. Collagenase II generates a viscous solution, but which was unable to form a coating for *in vitro* cell culture, whereas trypsin readily yielded a viscous solution from human Wharton's jelly which was easily coated onto surfaces. Furthermore, abundant biological molecules such as VEGF, FGF-2, TGF- $\beta$ 1, endothelin-1, etc., which are considered important in vascular function, were detected in the solution [33]. As we used a semi-quantitative method to measure the growth factors and cytokines in the current study which allowed us to detect a large panel of factors, further quantitative assays and characterization of WJ-ECM will be required to fully characterize its composition in solution. These active growth factors and cytokines may allow WJ-ECM solution to be utilized in other applications, such as cell culture supplement and the formation of a gel for cell assays.

For studies *in vitro*, biomaterials are typically coated onto glass coverslips in order to obtain a surface with appropriate thickness. ECM proteins such as collagen and fibronectin can form a coating directly on glass coverslips and polystyrene-based culture surfaces [34], and even polyelectrolyte films exhibiting a certain charge can coat surfaces [12]. Inspired by this idea, we tested the influence of different surface charge on the coating efficiency. AFM results revealed that negatively-charged glass coverslips can facilitate WJ-ECM coating. Furthermore, compared to commercial collagen coating, WJ-ECM yielded a homogenous surface without collagen fibers. The thickness and stiffness of WJ-ECM coating depended on ECM concentration.

It has been previously shown that modified ECM coating can enhance cell adhesion and proliferation [35], probably due to several features such as its thickness and stiffness, and the incorporation of biological molecules [6]. In this study, the collagen-modified surface demonstrated higher cell adhesion and proliferation than the glass surface, but the WJ-ECM coating exhibited the highest cell attachment and proliferation with both hMSCs and HUVECs. Because the WJ-ECM is derived from human tissue, and the isolation procedure is completely physiological, WJ-ECM did not show any toxicity to cells. These features should make WJ-ECM a versatile tool in various type of tissue engineering to support cell cultures. Furthermore, WJ-ECM was capable of facilitating the differentiation of hMSCs into endothelial-like cells by expressing endothelial cell specific markers. This approach could allow the construction of a tissue engineered vessel using a single cell source [31].

In native blood vessels, the endothelium is exposed to stable shear stress that is generated by the flow of blood. Therefore, tissue engineered vascular grafts must support cell attachment under physiological shear stress. Surface components, and in particular, topography and stiffness, can influence cell attachment under dynamic condition [24]. A major limitation of current vascular tissue

engineering is the low number of endothelial cells (ECs) or EC-replacing cells remaining after exposure to fluid shear stress representative of blood flow. Nonetheless, studies with modified substrates such as collagen coating have shown an amelioration in cell retention under shear stress conditions [36,37]. However, our results have shown that a collagen coating failed to improve cell resistance to laminar shear stress (although this behavior might be attributed to the use of a relatively lower concentration of collagen (0.1 mg/ml) relative to previous studies), whereas WJ-ECM significantly retained cells exposed to 6 h of shear stress. The

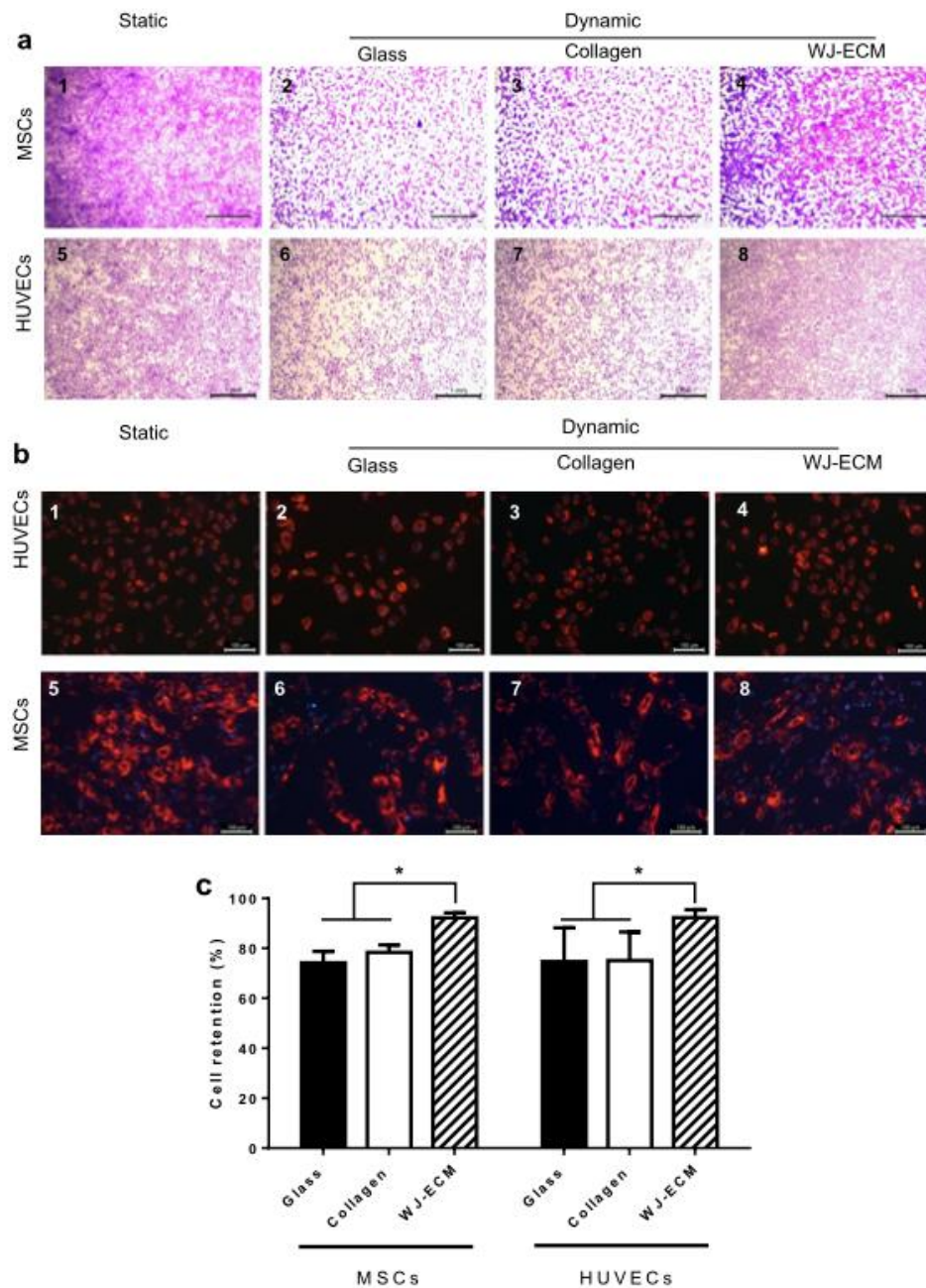


Fig. 5. Cell behavior under dynamic conditions. (a) and (b), Confluent cells cultured on 3 different surfaces (Static) were subjected to shear stress at  $20 \text{ dyn/cm}^2$  for 12 h (Dynamic). (a) Cells were stained with crystal violet. Scale bar, 1 mm. (b) Fluorescence microscopy images in which the cell membrane was stained with DiI (red) and the nucleus was stained with Hoechst 33258 (blue). Scale bar, 100  $\mu\text{m}$ . (c) Cell retention after 12 h of shear stress ( $n = 4$ ). Statistical significance, indicated by (\*), was determined with a one-way ANOVA followed by Tukey pairwise comparisons between all groups,  $\alpha = 0.05$ . Cell retention was calculated by counting cell numbers before and after shear stress. Cells were counted by Hoechst staining on 10 random areas (0.721  $\times$  0.54 mm<sup>2</sup>/area) on each culture surface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

underlying mechanism of the increased cell retention might be related to the stiffness of WJ-ECM surface, adhesive elements incorporated in the coating, and an interaction between cells, culture surface and shear stress [38]. Among the molecules involved in cell retention, integrins are a large family of transmembrane, heterodimeric cell-surface molecules, believed to be the principle adhesion factors for cells to attach to their surfaces. They establish cytoskeletal dynamics [23]. Integrins can thus be considered as cell bonds with a range of ligands existing on ECM, and regulate important indicators of the interaction between cells, ECM and dynamic forces. It has been reported that integrins of ECs were responsible for cell retention under shear stress [25], as well as improved retention of MSCs with concomitant upregulation of integrins [39]. Our results show that both hMSCs and HUVECs exhibit

a significant increase in expression of integrins  $\alpha 2$  and  $\beta 1$  due to shear stress. On the other hand, once shear stress is applied to cells seeded on a defined support, the cells transmit a signal from their apical surface through the cytoskeleton to points of attachment, in order to foster cell-matrix adhesions [40]. It has been proposed that CD31, VE-cadherin and VEGFR2 serve as a mechanosensory complex for the attachment of ECs in response to shear stress [41]. Indeed, these three junctional adhesion molecules play crucial roles in cell-cell and cell-matrix attachment. In addition, cell culture on different surfaces has been reported to modify the expression level of EC functional markers [42]. Our results have revealed that the expression of the mechanosensory complex by ECs cultured on WJ-ECM coating was relatively high and was maintained with or without shear stress, which may explain the retention of the cells on the coating. On the other hand, HUVECs cultured on collagen under shear stress increased expression of mechanosensory complex, whereas expression of mechanosensory complex by cells grown on glass coverslips decreased significantly. Taken together, these results indicate that the WJ-ECM coating may find application in cell culture under both static and dynamic conditions.

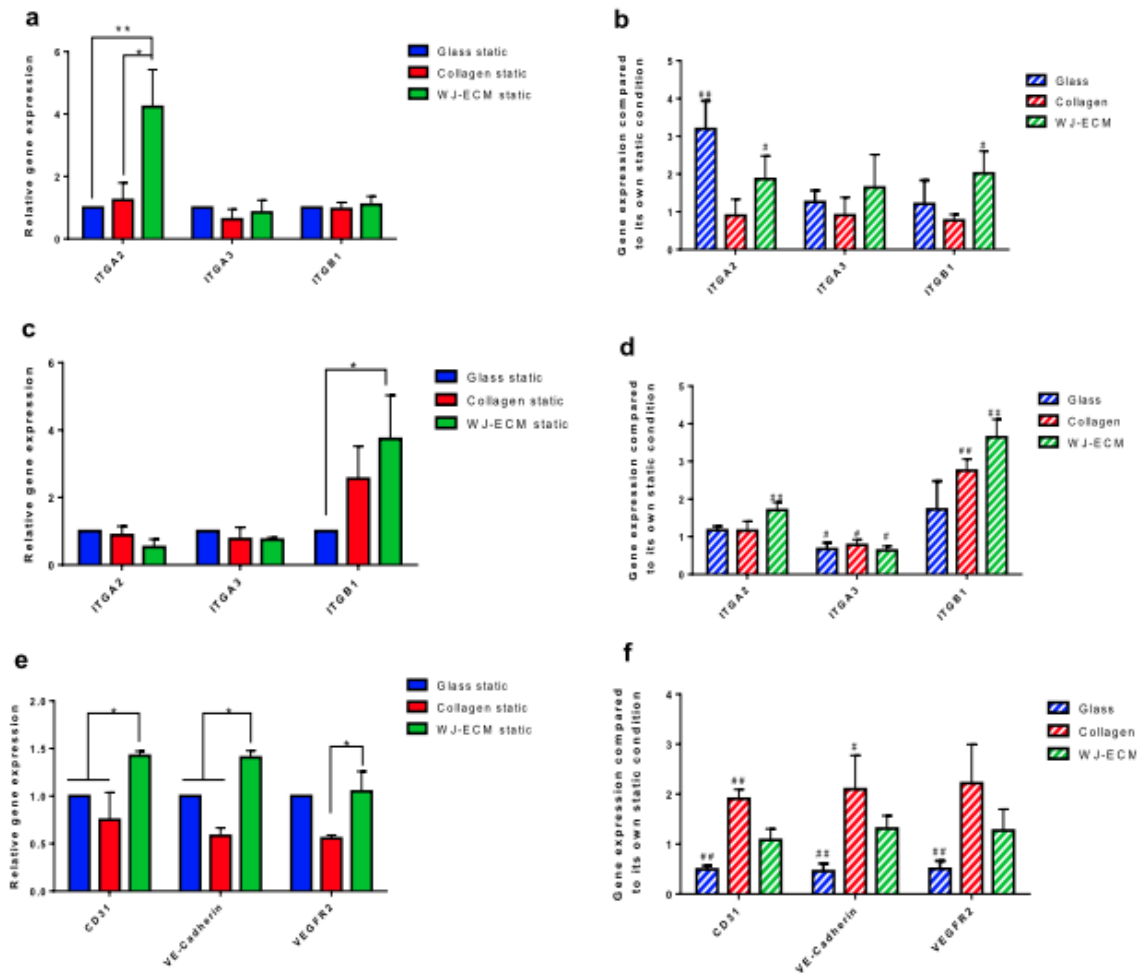


Fig. 6. Gene expression of MSCs and HUVECs cultured on different surfaces under static and dynamic conditions. Expression of integrins in hMSCs (a) and HUVECs (c) under static culture on glass coverslips, collagen and WJ-ECM. Expression levels are normalized to cells on glass coverslips (blue bars, \*  $p < 0.05$ , \*\*  $p < 0.01$ ). Changes in integrin expression levels after 6 h of shear stress at  $20 \text{ dyn/cm}^2$  for hMSCs (b) and HUVECs (d); expression levels were normalized to static conditions on glass coverslips, collagen and WJ-ECM, respectively. # indicates significant changes ( $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$ ) compared to static expression levels. (e) Expression of mechanosensory complex proteins (CD31, VE-cadherin and VEGFR2) in HUVECs under static culture; mRNA levels are normalized to those in cells grown on glass coverslips (blue bars, \*  $p < 0.05$ ). (f) Changes in the mechanosensory complex after 6 h exposure to shear stress at  $20 \text{ dyn/cm}^2$ ; statistical analysis was performed as for integrins, where # indicates significant changes ( $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$ ) compared to static expression levels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 5. Conclusion

In summary, we have demonstrated that our new, easily-prepared WJ-ECM coating is appropriate for both the adhesion and proliferation of the two cell types most commonly employed in vascular tissue engineering, human mesenchymal stem cells and mature endothelial cells. Furthermore, under conditions of physiological shear stress, a larger number of cells remained adherent to WJ-ECM than onto a conventional coating such as collagen—a result supported by a higher expression of both integrins  $\alpha 2$  and  $\beta 1$  in hMSCs and HUVECs cultured on WJ-ECM. Thus, overall, our data clearly show that Wharton's jelly derived-ECM is a highly promising coating for cell culture surfaces in tissue engineering and regenerative medicine.

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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.actbio.2016.10.018>.

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