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Original Research

Shear stress and circumferential stretch by pulsatile flow direct vascular endothelial lineage commitment of mesenchymal stem cells in engineered blood vessels

Dong Hwa Kim[1,2] · Su-Jin Heo[2] · Yun Gyeong Kang[1] · Ji Won Shin[1] · So Hee Park[1] · Jung-Woog Shin[1,3]

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Abstract Understanding the response of mesenchymal stem cells (MSCs) in the dynamic biomechanical vascular environment is important for vascular regeneration. Native vessel biomechanical stimulation in vitro is thought to be the most important contributor to successful endothelial differentiation of MSCs. However, the appropriate biomechanical stimulation conditions for differentiating MSCs into ECs have not been fully investigated. To accomplish an in vivo-*like* loading environment, a loading system was designed to apply flow induced stress and induce hMSC differentiation in vascular cells. Culturing MSCs on tubular scaffolds under flow-induced shear stress (2.5 dyne/cm^2) for 4 days results in increased mRNA levels of EC markers (vWF, CD31, VE-cadherin and E-selectin) after one day. Furthermore, we investigated the effects of 2.5 dyne/cm^2 shear stress followed by 3 % circumferential stretch for 3 days, and an additional 5 % circumferential stretch for 4 days on hMSC differentiation into ECs. EC marker protein levels showed a significant increase after applying 5 % stretch, while SMC markers were not present at levels sufficient for detection. Our results demonstrate that the expression of several hMSC EC markers cultured on double-layered tubular scaffolds were upregulated at the mRNA and protein levels with the application of fluid shear stress and cyclic circumferential stretch.

✉ Jung-Woog Shin
sjw@bme.inje.ac.kr; biomechshin@gmail.com

[1] Department of Biomedical Engineering, Inje University, Rm #309, BLDG-A, 607 Obang-Dong, Gimhae, Gyeongnam 621-749, Republic of Korea

[2] Department of Orthopaedic Surgery, McKay Orthopaedic Research Laboratory, University of Pennsylvania, 36th Street and Hamilton Walk, 424 Stemmler Hall, Philadelphia, PA 19104-6081, USA

[3] Department of Health Science and Technology, Institute of Aged Life Redesign, Cardiovascular and Metabolic Disease Center, UHRC, Inje University, Gimhae, Gyeongnam 621-749, Republic of Korea

1 Introduction

Large-diameter engineered polymer vessels have been successfully used as replacement blood vessels. However, the use of engineered small diameter blood vessels less than 5 mm usually causes several problems including aneurysm, calcification, thrombosis and vessel occlusion [1]. Additionally, vascular autografts commonly used in the clinical replacement of smaller vessels are in limited supply [2]. To address such problems, several tissue-engineering approaches to fabricate functional, small-diameter blood vessels have been introduced [3–6]. Achieving successful clinical results in vascular tissue engineering requires cells to be collected, bioactive scaffolds to be fabricated, and vascular micro-biophysical environments to be simulated in vitro [7, 8]. Therefore, an understanding of the in vivo environment, enabling accurate in vitro replication, is key to successful tissue engineering and clinical translation [9, 10].

Anatomically, a blood vessel consists of three layers, with different cell types residing in each layer [11]. Endothelial cells (ECs) are the main cellular component of the intimal surface and play an important role in vessel function, including the maintenance of tissue hemostasis, and preventing coagulation in small-diameter vessels [12]. The growth of engineered vessels requires ECs to be isolated from tissues, and then cultured in vitro for an extended period. Currently, obtaining ECs in sufficient quantity, and maintaining their phenotype is challenging [13]. To overcome these problems, in this study we utilized

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bone-marrow derived human mesenchymal stem cells (hMSCs) that can differentiate into a vascular EC lineage [14–18].

Along with chemical induction, accurate simulation of biomechanical stimulation of native vessels in vitro is thought to be the most important contributor to successful endothelial differentiation of MSCs. However, the biomechanical stimulation conditions appropriate for differentiating MSCs into ECs have not been fully investigated. For example, O'Cearbhaill et al. [18] reported that when MSCs seeded on tubular silicone substrates were exposed to both pulsatile flow and stretching for 24 h, SMC-associated markers such as α-smooth muscle actin (α-SMA) and calponin were upregulated, while EC-associated markers such as vWF showed no significant change. Kobayashi et al. [16] applied shear and compressive stress to rat stromal cells. Changes in only SMC differentiation markers were reported; MSC differentiation into ECs was not confirmed. Dong et al. [19] reported success in differentiating MSCs seeded on tubular decellularized substrates into ECs by monitoring changes in the von Willebrand factor, an endothelial cell marker, under pulsatile flow for 4 days. However, to confirm MSC differentiation into the EC lineage, further analysis of differentiation markers should be carried out.

Multiple studies have shown that appropriate biomechanical stimuli enhance the differentiation of MSCs into ECs [16, 18, 20]. Specifically, ECs, a major component of the inner vessel layer, are exposed to pulsatile blood flow-induced shear stress and circumferential stretching [21]. Here, a custom bioreactor system is designed to apply fluid shear stress and cyclic stretch. A new biomechanical stimulation protocol is also proposed for the successful differentiation of MSCs in ECs. First, a low, steady shear stress is applied to MSCs to determine the effect on their differentiation into ECs. Various fluid-induced cyclic circumferential stretching conditions were then applied, followed by 1 day of low, steady shear stress to induce MSC differentiation into ECs.

Simulating the hierarchical structure and physical properties of native blood vessels is essential when fabricating synthetic vessels [22]. Poly(L-lactide-co-ε-caprolactone) (PLCL) double-layered tubular-type scaffolds for culturing MSCs were developed using an electrospinning technique. PLCL is widely used in the study of blood vessel regeneration [23–25], owing to its mechanical compliance and burst strength, properties important for engineering of small-diameter vessels. To mimic the anatomical structure of blood vessels, we fabricated tubular PLCL scaffolds, 5 mm in diameter, by electrospinning the polymer onto high-speed rotating rods. The inner tubule region was composed of non-aligned fibers, while the fibers of the outer region were well aligned.

In this study, we fabricated double-layered tubular type scaffolds using an electrospinning technique, determined the effect on MSC differentiation into ECs of a shear stress of 2.5 dyne/cm^2 in the presence of chemical factors, and investigated the effects of 2.5 dyne/cm^2 shear stress on MSC differentiation by applying various cyclic circumferential stretch conditions.

2 Materials and methods

2.1 Fabrication of tubular type scaffolds

Tubular poly(L-lactide-co-ε-caprolactone) (PLCL, 50:50, MW: 125 kDa, Lakeshore Biomaterials Inc., Birmingham, AL, USA) nanofiber scaffolds (5-mm diameter, 50-mm length, 300-μm wall thickness) were fabricated by an electrospinning technique utilizing five stainless steel mandrels (5-mm diameter, Fig. 1a–b). Briefly, the copolymer (PLCL) was dissolved in chloroform (Junsei Chemical Co., Ltd., Tokyo, Japan) by stirring at room temperature for 8 h, resulting in a 13 % (w/v) solution. Water-soluble 30 wt% polyethylene oxide (MW: 100,000; Sigma, St. Louis, MO, USA) was first pre-spun onto the mandrel for 10 min to aid the detachment of completed PLCL scaffolds. The non-aligned inner scaffold layer was fabricated by ejecting PLCL at 1.7 ml/h for 2 h, while the mandrel was rotated with a surface velocity of 0.3 m/s. The circumferentially aligned outer scaffold layer was formed by increasing the surface velocity of the mandrel to 3.1 m/s, while the feeding rate was maintained for 1 h. The scaffolds were detached from the mandrel by immersion in distilled water for 1 h. A set of five rotating mandrels, and a moving 18-G syringe enabled five scaffolds to be fabricated simultaneously (Fig. 1).

2.2 Scanning electron microscope (SEM) for scaffold characterization

Electrospun PLCL tubular scaffolds were measured using a scanning electron microscope (SEM, JSM-6700; JEOL, Japan), at an accelerating voltage of 10 kV. Before scanning, samples were sequentially dehydrated in 50, 60, 70, 80, 90 and 100 % ethanol solutions for 3 min per dehydration step, dried in a clean hood, and then sputter-coated with gold.

2.3 Compliance test

The relationship between the internal pressure and the circumferential compliance of the tubular scaffolds was evaluated using a custom device. Pressure was applied using a 50-ml syringe filled with phosphate-buffered saline (PBS, Sigma), while the pressure was monitored with 1-mmHg precision using a digital gauge (ZSE50F, SMC Korea Co., Ltd. Seoul). Scaffolds were preconditioned by injecting PBS three times, at a pressure of 120 mmHg. PBS

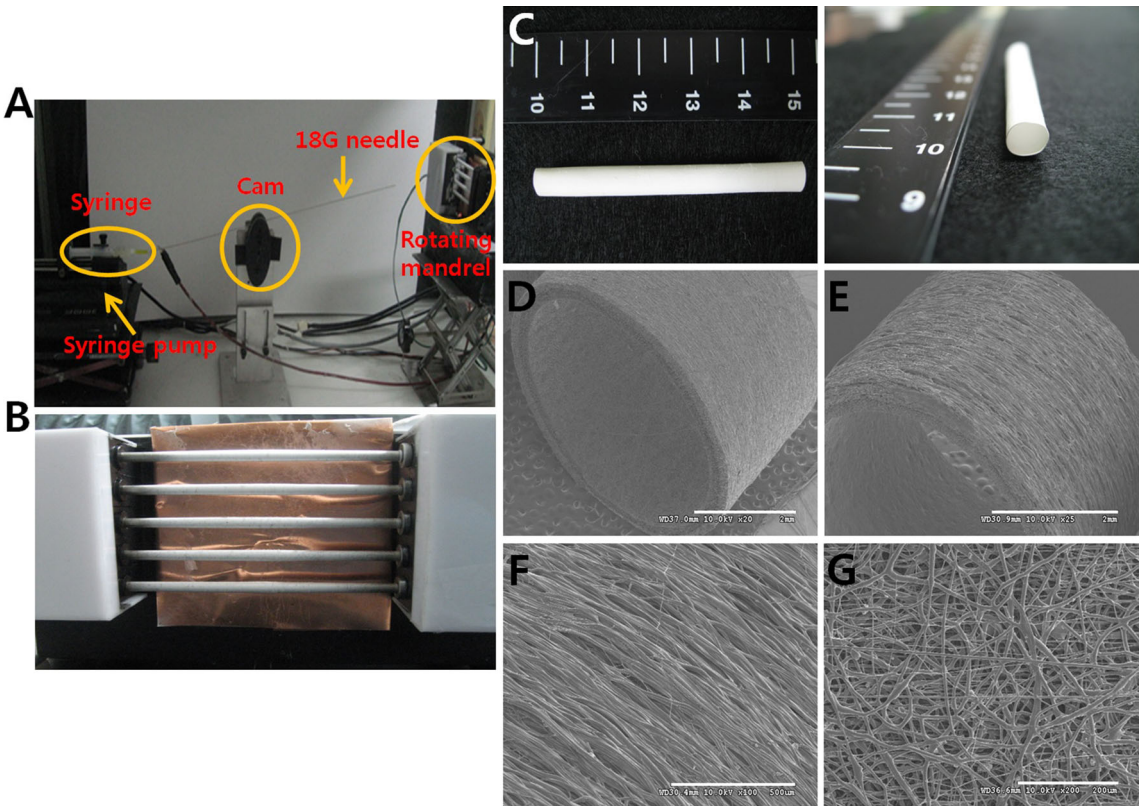


Fig. 1 Fabrication of electrospun tubular-type scaffolds for vessel regeneration. **a** Overview of the electrospinning apparatus (syringe pump, syringe, cam, 18-G needle, and rotating mandrel), **b** five rotating mandrels for forming the scaffolds, **c** digital photographs of the PLCL tubular type construct, **d** scanning electron microscope (SEM) images of the PLCL scaffolds used as a substrate for cyclic circumferential stretch; the entire circumferential section (bar = 2 mm), **e**, **f** the outer surface (**e** scale = 2 mm, **f** scale = 500 μm) and **g** the luminal surface (scale = 200 μm)

was then continuously injected at pressures from 0 to 120 mmHg, in 20-mmHg increments. Throughout this process, digital images were obtained, and the external diameter measured using Image J (National center for biotechnology information, rsb.info.nih.gov/ij/docs/index.html). The compliance (C) = $(\Delta D/D_0)/\Delta P$ (where D_0 is the initial diameter, ΔD is the change in diameter, ΔP is the change in pressure), a mechanical property of the vascular scaffold, was determined at the average native intraluminal pressure, 103.7 mmHg (n = 3) [26].

2.4 Cell seeding and culture in tubular scaffolds

In preparation for cell culture, the electrospun scaffolds were hydrated and sterilized by decreasing ethanol concentrations (100, 70, 50, 30 %; for 30 min per step), and pre-warmed with culture medium for 4 h. Scaffolds were then coated with 8 μg/ml of fibronectin (Sigma, St. Louis, MO, USA) to promote cell attachment on the surface for 24 h, after which they were rinsed in HBSS.

Human MSCs (hMSCs, PT-2501; Lonza Walkersville, MD, USA) were cultured in an MSC growth medium (BulletKit PT-3001, Lonza) at 37 °C in a humidified 5 % CO_2 incubator. MSCs (passage 2) were then suspended in endothelial cell growth medium-2 (EGM-2, Lonza, Walkersville, ML) and seeded onto the inner and outer surfaces of the scaffold at a concentration of 5×10^5 cells/cm^2, utilizing a horizontal rotating system at 1 rpm for 24 h to promote homogeneous cell adhesion, as described in our previous report [27]. MSCs (passage 2) from three donors were combined and seeded onto scaffolds.

2.5 Application of fluid shear stress and circumferential stretching to MSCs

Flow-induced shear stress, and circumferential stretching was applied to MSCs seeded on tubular scaffolds using a custom bioreactor system, the operation of which was described previously [27]. In brief, the bioreactor consisted of three major components: a flow chamber, a gear pump (Ismatec, Switzerland), and a medium reservoir. In addition, a bubble trap was used to prevent the transport of gas bubbles into the medium tube. EGM-2 was used in the scaffolds. Mean wall shear stress (τ) was calculated by the

Hagen-Poiseuille equation [28]: $\tau = 4\mu Q/\pi R^3$, where μ is the dynamic viscosity of the medium (1.8×10^{-3} Pa s), Q is the flow rate in volume, and R is the inner radius of the tubular construct (2.5 mm).

MSCs were seeded on the inner scaffold layer for 1 day to allow cell attachment, and then pre-cultured for 2 days before stress application. In step 1 (Fig. 2c), a steady shear stress of 2.5 dyne/cm^2 [27] was applied for up to 4 days to determine the mechanical stimulation period suitable for MSC differentiation.

A two-step experiment was carried out to evaluate strategies for MSC differentiation into ECs. First, MSCs were seeded on the inner scaffold layer for 1 day to allow cell attachment, and then pre-cultured for 2 days before stress application. For step 1 (Fig. 2c), a steady 2.5 dyne/cm^2 shear stress [27] was applied for up to 4 days to determine the mechanical stimulation period suitable for MSC differentiation. To investigate the synergistic effects of shear stress and cyclic circumferential stretching on differentiation, a 3 % cyclic circumferential stretch (2 Hz, 250 ml/min) was applied for 3 days followed by a 5 % circumferential stretch (2 Hz, 400 ml/min) for 4 days. The pressure was 50.01 mmHg or 83.34 mmHg when we applied for the 3 or 5 % cyclic stretch, respectively. Note that each pressure value was calculated from the compliance test. The scaffold strain was regulated by changing the flow rate of the gear pump, and was measured using Image J.

2.6 Immunofluorescence staining

Scaffolds were fixed with 4 % paraformaldehyde, and permeabilized in 0.2 % triton X-100 for 10 min. After twice being washed with PBS, the scaffolds were incubated with 1 % BSA in PBS. Cells were labeled with VE-cadherin (1:200, Santa Cruz Biotechnology Inc.), von Willebrand Factor (vWF, 1:100, Santa Cruz Biotechnology Inc.), α-smooth muscle actin (α–SMA, 1:100, Sigma-Aldrich), and calponin (1:200, Sigma-Aldrich), followed by TRITC (1:200), or FITC (1:50) conjugated secondary antibodies. The cells were observed using a Carl Zeiss microscope (LSM 510 META, Zeiss, Oberkochen, Germany).

2.7 Real-time PCR analysis

RNA was extracted from frozen samples using a Qiagen mini kit (Chatsworth, CA, USA), and cDNA cloning was performed with a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). The presence of EC or SMC markers was investigated by real-time PCR, performed on a StepOne system (Applied Biosystems, Foster City, CA, USA). The amount of target cDNA was determined using the comparative threshold (CT) method. Levels of RNA expression were determined according to the $2^{-\Delta\Delta CT}$ method. Data are presented after normalization to β-actin. Primers are described in Table 1.

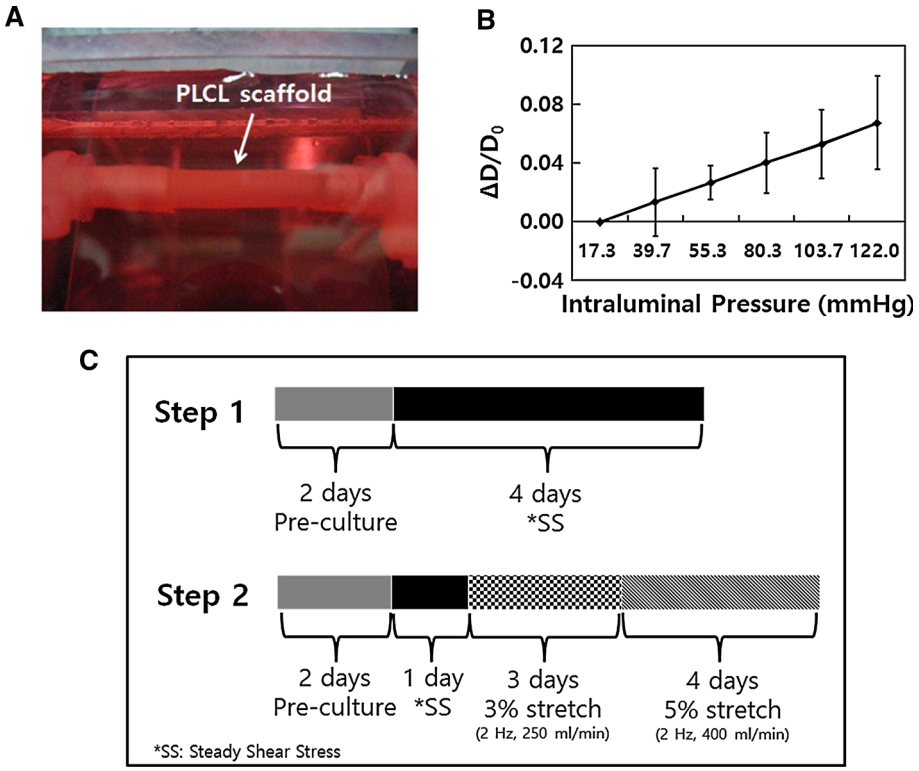


Fig. 2 Application of fluid shear stress and cyclic circumferential stretch to MSCs. **a** Digital photograph of the pulsatile loading system chamber equipped with a PLCL electrospun scaffold. **b** Compliance test: the diameters of the PLCL scaffold were measured at six loading pressures. The vessel diameter in the deformed state (D) was normalized to the initial diameter (D_0) (n = 3). **c** Timetable of the experimental procedure

Table 1 PCR primer sequences

Primer	Forward (F) and reverse (R) primer (5′ → 3′)	Accession No.	Product size (bp)
vWF	F ccctgtggactttgggaact	NM_000552	120
	R tccaccatcgtctgcttcat		
CD31 (PECAM-1)	F gtcccctaagaattgctgcc	NM_000442.4	115
	R taaaaacagctgtgctgggg		
VE-cadherin	F gacctcatcagccttgggat	NM_001795	141
	R tgggtgacagggttatgtgg		
E-selectin	F aagaggttccttcctgccaa	NM_000450.2	103
	R ttgcacacagtgccaaacac		
α-SMA	F acaatgtcctatcaggggc	X13839	95
	R gatcttcatggtgctgggtg		
Calponin	F accctccgctctgtagtgct	NM_001299	91
	R agctggaatagcgttgctca		
Caldesmon	F tggtgtcaaatcgacccatc	NM_033139.3	148
	R gcgtacaccttcagcaggaa		
β-actin	F cccaaagttcacaatgtggc	NM001101.3	103
	R gatggcaagggacttcctgt		

2.8 Flow cytometry

Using flow cytometry, the surface markers of differentiated MSCs were evaluated based on the expression of Flk-1/KDR, and vWF. Cells were stained with primary antibodies, mouse anti-Flk-1/KDR, and vWF. The cells were subsequently reacted with a goat anti-mouse secondary antibody. The stained cells were fixed with 1 % paraformaldehyde, and analyzed immediately on a FACS Canto flow cytometer equipped with FACSDiva version 6.1.3 (BD Biosciences, Franklin Lakes, NJ, USA).

2.9 Statistical analysis

One-way ANOVA was performed using SPSS version 10.0 K (SPSS Inc., Chicago, IL, USA). Where ANOVA indicated a significant difference among groups, the difference was evaluated using the least-significant difference (LSD) method. All data are presented as means ± standard deviation (SD) at a significance level of $P = 0.05$.

3 Results

3.1 Fabrication and characterization of tubular PLCL scaffolds

Tubular PLCL scaffolds (Fig. 1c) that were 50 mm in length and 5 mm in diameter were fabricated by electro spinning technique. The morphology of the aligned and random PLCL scaffold is given in Fig. 1d–g, which shows the three-dimensional structure (d), a top-side view of the outer surface (e, f) and an internal view (g) of a scaffold luminal surface, at low (d, e) and high (f, g) magnification. Scaffolds were composed of two layers, an inner layer with randomly aligned fibers, and an outer layer of circumferentially aligned fibers, formed by varying the collection mandrel rotational speed. A total wall thickness of ∼ 300 μm was determined from SEM images.

3.2 Biomechanical effects on MSC differentiation into endothelial cells

hMSC-seeded tubular constructs were connected to the pulsatile loading system as shown in Fig. 2a. Mechanical compliance tests of the fabricated tubular scaffolds showed that the scaffold diameter increased linearly with applied pressure. The fabricated scaffolds had a compliance of 5.14 % ± 2.33/100 mmHg (n = 3), comparable to that of native blood vessels (Fig. 2b), validating their utility in vascular graft applications (native arterial compliance is 0.059 ± 0.005 %/mmHg as in [38]).

3.2.1 Step 1: Real-time PCR analysis of hMSCs

The ability of hMSCs to differentiate into ECs on the inner layer of the PLCL scaffolds was analyzed by the application of a 2.5 dyne/cm^2 flow-induced shear stress for 4 days. Shear stress markedly increased the mRNA levels of the EC markers vWF, CD31, VE-cadherin, and E-selectin at day 1 ($P < 0.05$, Fig. 3a). All EC marker levels were highest 1 day after the application of shear stress, and decreased significantly thereafter. α-SMA SMC marker levels were not affected by the stress applied, while the

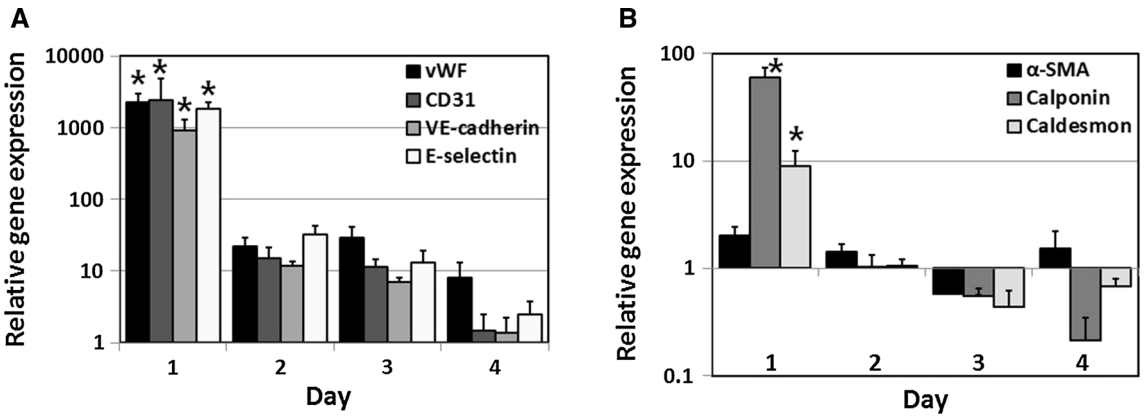


Fig. 3 Gene expression levels of hMSCs on the inner surface of tubular electrospun scaffolds under steady 2.5 dyne/cm^2 shear stress. Fluid shear stress upregulates **a** EC markers (vWF, CD31, VE-cadherin, E-selectin) and (n = 3), **b** SMC markers (α-SMA, Calponin, Caldesmon) at day 1, *: $P < 0.05$ versus (days 2, 3, and 4). All results were normalized to β-actin (n = 3)

calponin and caldesmon levels increased during day 1, and returned to baseline by day 2 (Fig. 3b). mRNA SMC marker levels were considerably lower than the mRNA levels of EC differentiation markers.

3.3 Immunostaining for hMSC-associated proteins

Figure 4 shows immunofluorescence staining of hMSCs cultured on the scaffold inner surface with 4 days of steady flow-induced shear stress at 2.5 dyne/cm^2. All cells on the tubule inner surface (where fibers were randomly oriented) were aligned parallel to the flow direction [27]. hMSCs expressed VE-cadherin through cell membrane transiently with mechanical conditioning for 2 days, but the magnitude decreased after the second day. vWF, a late marker of EC differentiation, was highly expressed at day 2, and remained so at day 4. Immunofluorescence staining for the SMC markers α-SMA and calponin was performed to observe changes in hMSCs as a function of stress. α-SMA expression gradually increased throughout the 4-day loading period, and Calponin expression was increased at 2 days after the loading.

The results showed that EC markers were upregulated by the steady shear stress within 2 days, and then decreased with culture duration. However, SMC markers gradually increased with time in shear stress. Therefore, 1-day shear stress was selected for optimal hMSC differentiation into ECs based on EC marker gene expression results.

3.3.1 Step 2: Gene expression of hMSCs in response to cyclic circumferential stretch

Figure 5 shows the mRNA levels of hMSCs cultured on the inner scaffold layer after a loading regimen which included 1 day of steady shear stress, followed by 3 % cyclic circumferential stretch for 3 days, and 5 % cyclic circumferential stretch for 4 days. EC markers (vWF, E-selectin, and VE-cadherin) showed a significant increase after the application of a 5 % stretch, while SMC markers (α-SMA, caldesmon, calponin) were not present at levels sufficient for detection.

All hMSC EC markers were significantly upregulated by 5 % circumferential stretching for 4 days, when compared to a static group (without loading). One EC-specific marker, E–selectin, was significantly upregulated after a 3 % circumferential stretch for 1 day, and continued to be upregulated after 3 days. SMC markers, α-SMA was minimally changed in response to cyclic circumferential stretch. Caldesmon and calponin expression was slightly increased with a 5 % circumferential stretch, but not significantly different from the static group.

3.4 Flow cytometry analysis of hMSCs

hMSC cell surface markers cultured on the scaffold inner layer were analyzed by flow cytometry (Fig. 6). Flow cytometry was used to determine the suitability of KDR/Flk-1 and vWF to analyze the differentiation of hMSCs into ECs. hMSC, KDR/Flk-1 and vWF expression levels were determined, and compared to ECs as a positive control. Before applying mechanical stimulation, KDR/Flk-1 and vWF were detected in 1 and 1.4 % of the hMSC population, respectively (Fig. 6a), and in 32.2 and 34.4 % of the EC-positive control population (Fig. 6b). In the 0 % stretch group at day 1, 3.2 % (KDR/Flk-1) and 5 % (vWF) of cells presented EC surface markers (Fig. 6c). One day after applying 5 % stretch, the EC markers were expressed in 13.9 % (KDR/Flk-1) and 25.6 % (vWF) of the hMSC population (Fig. 6d). Four days after applying the 5 % stretch, the number of hMSCs expressing KDR/Flk-1 (12.8 %), and vWF (27.2 %) remained at levels similar to those on day 1 (Fig. 6d). In the 0 % stretch group, the percentage of hMSCs expressing KDR/Flk-1 (8.9 %) and vWF (6.4 %) had significantly increased by day 4.

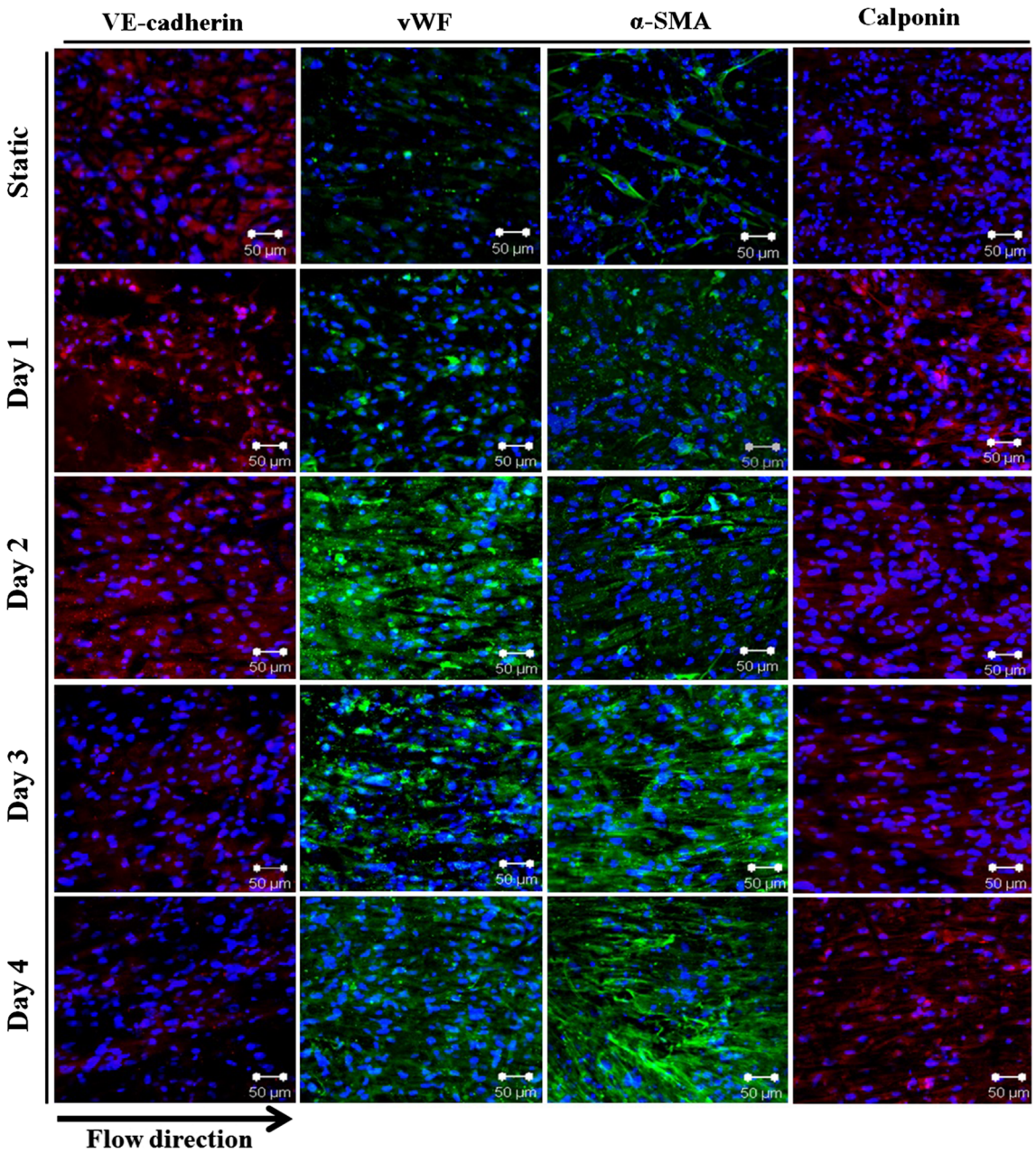


Fig. 4 Images of immunofluorescence staining of hMSCs on the luminal surface of tubular electrospun scaffolds. High VE-cadherin and vWF expression levels under shear stress were observed at day 2. Expression of α-SMA and calponin was induced by day 3 after the application of mechanical stimulus (control: without stimulation) (n = 3)

4 Discussion

The aim of this study was to investigate the effect of cyclic circumferential stretch on the differentiation into ECs of MSCs seeded on biometric engineered blood vessels. This was achieved through the fabrication of double-layered tubular scaffolds with an inner region composed of non-aligned fibers, and an outer region composed of aligned fibers, mirroring the structural microenvironment of native blood vessels. A bioreactor system was designed to apply fluid shear stress and cyclic circumferential stretch to simulate the in vivo biophysical environment of blood vessels [27]. In this study, we found that the expression of several EC markers was upregulated at the mRNA and protein levels, indicating MSCs cultured on the inner layer had developed an EC-like phenotype with cyclic circumferential stretch.

Bone-marrow-derived MSCs have the potential to differentiate along several mesenchymal lineages, including the adipogenic, osteogenic, and chondrogenic lines [29, 30].

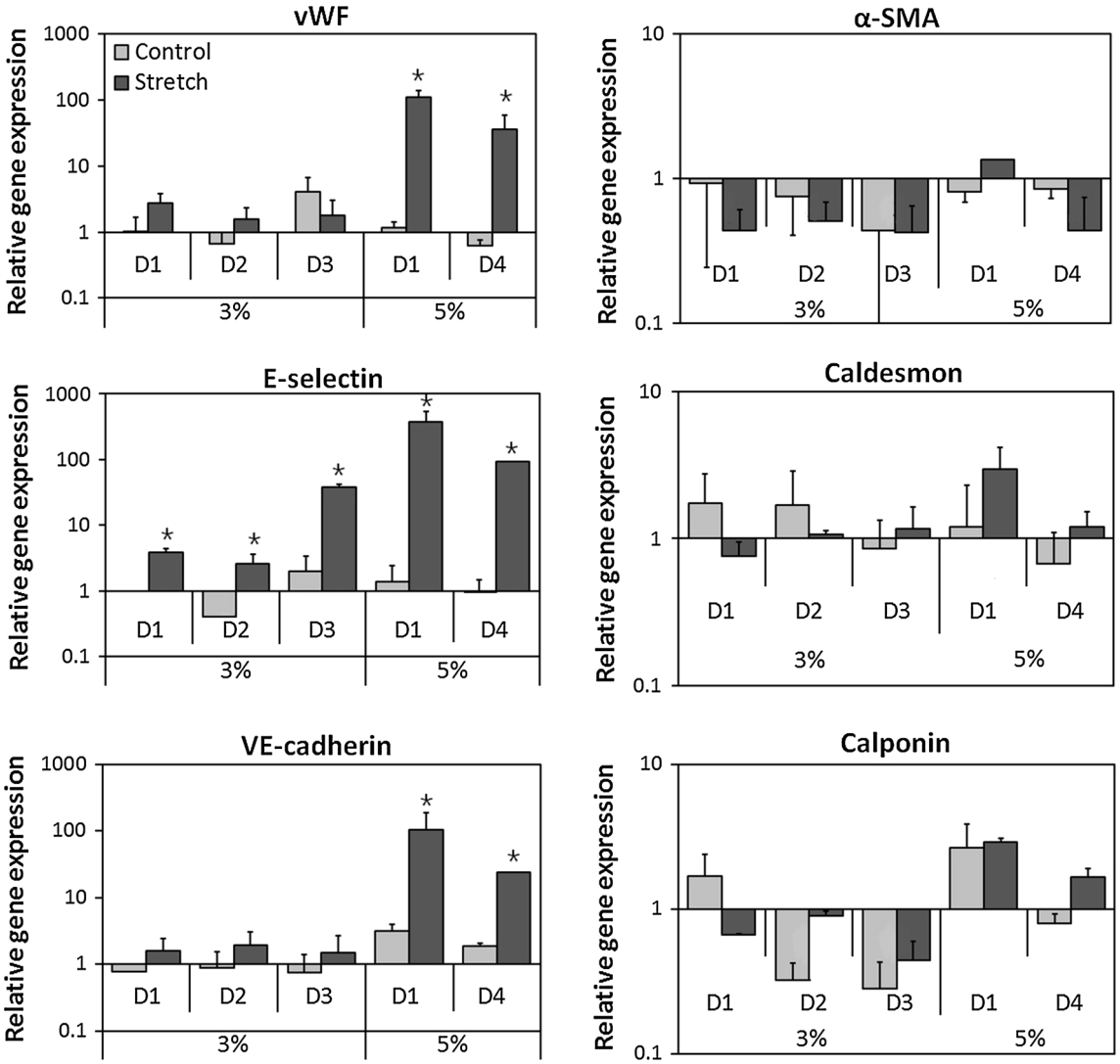


Fig. 5 Changes in gene expression in response to cyclic circumferential stretch conditions. Cyclic circumferential stretch increased the expression levels of EC markers (vWF, E-selectin and VE-cadherin, *: $P < 0.05$ vs. control, control: without stimulation, 3 % stretch followed by 5 % stretch for 3 days, D: day) in hMSCs. However, there were minimal changes in SMC makers following cyclic circumferential stretch (n = 3)

Studies have also reported that MSCs differentiate into ECs in vivo or in vitro following chemical stimulation [31, 32]. To use MSCs as a cell source for engineering vascular grafts, it is necessary to investigate the role of mechanical stimulation in MSC differentiation into the vascular cell lineage. In addition to chemical induction, mechanical stimulation plays an important role in determining the MSC phenotype. For example, especially short-term shear stress can induce the differentiation of stem cells (including embryonic stem cells and MSCs) into ECs [31], and MSCs can differentiate into an SMC lineage with cyclic mechanical stretch [33, 34]. Bai et al. [31] applied various shear stress magnitudes (10, 15, 20 and 25 dyne/cm^2) for 12, 24 and 48 h to MSCs, while supplementing growth media with various biochemical reagents. Consistent with our finding, they reported that gene expression, in relation to EC markers, was significantly increased within 24 h, but drastically reduced after 48 h; however, the expression of SMC markers was not evaluated. hMSCs expressed VE-cadherin through cell membrane transiently with mechanical conditioning for 2 days. Dong et al. [35] continuously increased shear stress from 1.0 to 15.0 dyne/cm^2 for 2 days, and held the shear stress at 15 dyne/cm^2 for the next 2 days to investigate the differentiation of MSCs into ECs. They found increased expression of EC–related markers and decreased expression of SMC-related markers in mechanically loaded MSCs compared to unloaded controls.

In the current study, two-step experiments were performed as follows: in step 1 a low and steady shear stress was applied to the MSCs to induce MSC differentiation

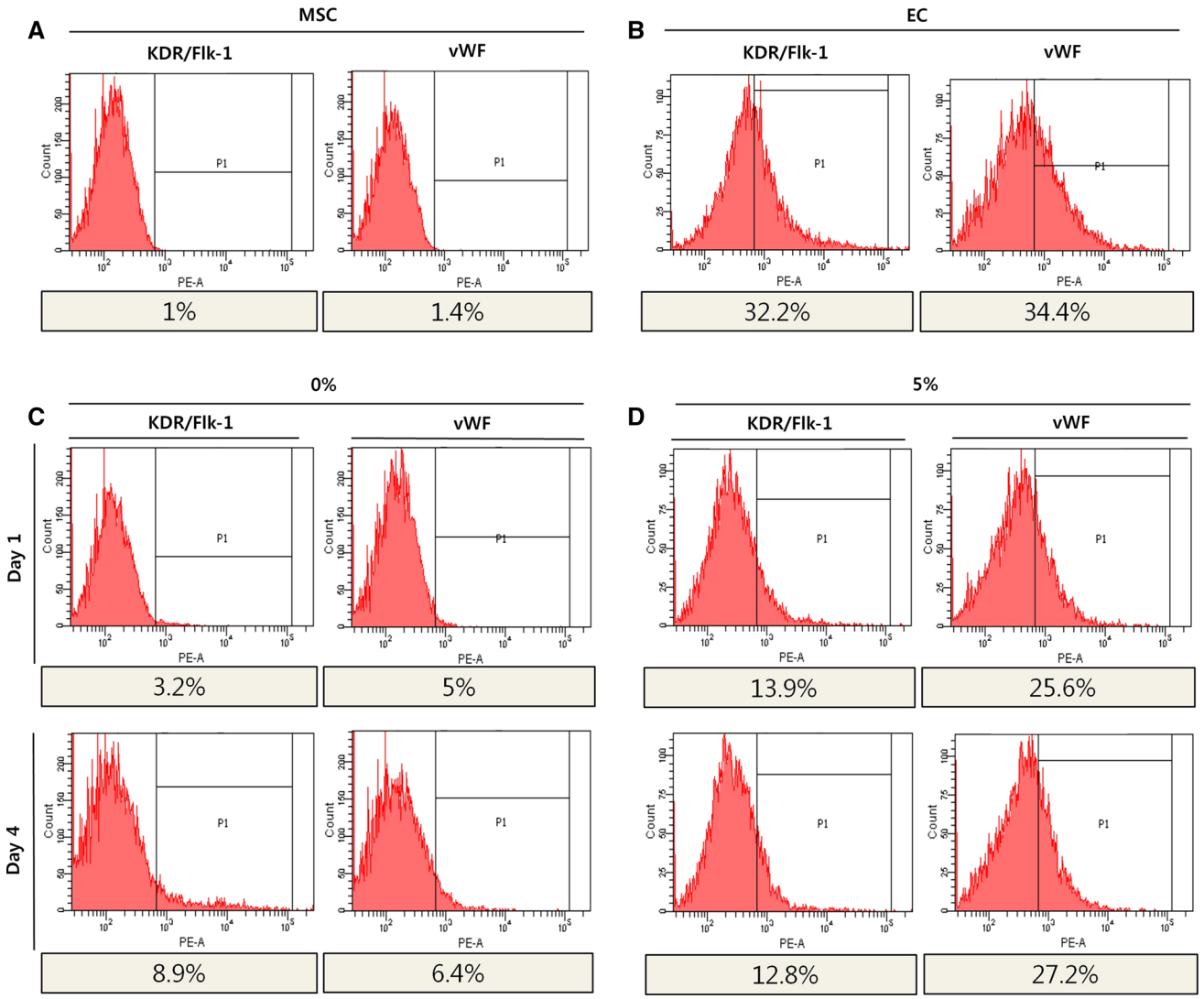


Fig. 6 Quantitative flow cytometry analysis of MSC differentiation. KDR/Flk-1 and vWF expression by MSCs was increased by a 5 % stretch. KDR/Flk-1 and vWF expression levels in **a** MSCs before stimulation, in **b** ECs as positive controls, in **c** MSCs without stimulation, and in **d** MSCs with stimulation (n = 3)

into ECs. In step 2, fluid-induced cyclic circumferential stretching, followed by 1 day of low, steady shear stress was applied, resulting in successful MSC differentiation into ECs. When a constant steady shear stress of 2.5 dyne/cm^2 was applied, we found that mRNA levels of the EC markers vWF, CD31, VE-cadherin and E-selectin markedly increased after 1 day. When steady shear stress was applied, all EC markers peaked after 1 day, and decreased significantly thereafter. The mRNA levels of SMC markers, however, were low compared to those of EC markers, although the that of the SMC marker α-SMA was increased 3 days after the stress loading. On the other hand, protein expressions related to EC makers were expressed after 2 days. It is probably because the upregulated mRNA expressions led to protein expressions after 1 day. Compared to gene expression levels of EC markers, the levels of SMC markers were very low under the steady shear stress of 2.5 dyne/cm^2 for 4 days. However, MSCs expressed SMC related proteins from day 3 or 4. It seems that the shear stress may gradually increase the expression levels and short-term shear stress (within 1 day) is sensitive for MSC differentiation into EC-like cells. As vascular cells are subject to both shear and circumferential strains in vivo [36], biomechanical conditions likely produce beneficial effects for vascular regeneration in vitro. For example, O'Cearbhaill et al. [18] demonstrated that MSCs have a greater capacity to differentiate towards an SMC phenotype, rather than an EC phenotype, after 24 h under both shear stress and cyclic circumferential stretch without biochemical stimulation. Toda et al. [37] analyzed ECs subjected to both shear stress and cyclic circumferential stretch for 24 h. The expression of ET-1 and eNOS, EC-

specific markers, was evaluated, and no significant change in ET-1 mRNA levels was observed, while those of eNOS increased. These results indicate that the expression of endothelial genes, in the context of shear stress or cyclic circumferential stretch, depends on whether the two forces are applied separately or simultaneously. Hamilton et al. [33] also demonstrated that bone-marrow-derived MSC, stimulated with 10 % strain at 1 Hz for 7 days, developed an SMC-like phenotype. Therefore, we suggest that while biomechanical factors are effective in promoting EC differentiation, vascular cell differentiation may require longer loading durations. In this study, MSCs seeded onto PLCL scaffolds were subjected to pulsatile flow, causing increased expression of EC markers on the mRNA and protein levels. EC-associated gene expression was upregulated, while vWF, a glycoprotein specific to ECs, was expressed only transiently at the protein level. In addition, no upregulation of the SMC-associated α-SMA, caldesmon, and calponin genes was detected. These factors could be the result of the combination of mechanical and biochemical stimulation, in which circumferential stretch played a synergistic and dominant role.

We have investigated the effects of combined mechanical and chemical stimuli on the differentiation of MSCs seeded on the inner scaffold layer into EC-like cells. In future, the evaluation of mechanical effects on MSCs seeded on the outer layer could further the characterization of engineered blood vessels. In addition, various mechanical stimulation conditions should be tested to optimize the differentiation of MSCs into a vascular cell lineage.

5 Conclusions

We found that the expression of several MSC EC markers in cells cultured on double-layered tubular scaffolds were upregulated at the mRNA and protein levels following the application of fluid shear stress, and cyclic circumferential stretch. This approach will facilitate the development of regeneration of small-diameter artificial blood vessels, and the results of this study will provide fundamental information to further clinical applications.

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