Layer-specific cell differentiation in bi-layered vascular grafts under flow perfusion

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Layer-specific cell differentiation in bi-layered vascular grafts under flow perfusion

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Supplementary material for this article is available [online](#)

Abstract

Bioengineered grafts have the potential to overcome the limitations of autologous and non-resorbable synthetic vessels as vascular substitutes. However, one of the challenges in creating these living grafts is to induce and maintain multiple cell phenotypes with a biomimetic organization. Our biomimetic grafts with heterotypic design hold promises for functional neovessel regeneration by guiding the layered cellular and tissue organization into a native-like structure. In this study, a perfusable two-compartment bioreactor chamber was designed for the further maturation of these vascular grafts, with a compartmentalized exposure of the graft's luminal and outer layer to cell-specific media. We used the system for a co-culture of endothelial colony forming cells and multipotent mesenchymal stromal cells (MSCs) in the vascular grafts, produced by combining electrospinning and melt electrowriting. It was demonstrated that the targeted cell phenotypes (i.e. endothelial cells (ECs) and vascular smooth muscle cells (vSMCs), respectively) could be induced and maintained during flow perfusion. The confluent luminal layer of ECs showed flow responsiveness, as indicated by the upregulation of COX-2, KLF2, and eNOS, as well as through stress fiber remodeling and cell elongation. In the outer layer, the circumferentially oriented, multi-layered structure of MSCs could be successfully differentiated into vSM-like cells using TGF β , as indicated by the upregulation of α SMA, calponin, collagen IV, and (tropo)elastin, without affecting the endothelial monolayer. The cellular layers inhibited diffusion between the outer and the inner medium reservoirs. This implies tightly sealed cellular layers in the constructs, resulting in truly separated bioreactor compartments, ensuring the exposure of the inner endothelium and the outer smooth muscle-like layer to cell-specific media. In conclusion, using this system, we successfully induced layer-specific cell differentiation with a native-like cell organization. This co-culture system enables the creation of biomimetic neovessels, and as such can be exploited to investigate and improve bioengineered vascular grafts.

1. Introduction

Replacements of small-diameter blood vessels (<6 mm) are in clinical demand, for example in coronary artery

bypass grafting and for hemodialysis procedures [1]. Autologous conduits are the current clinical gold standard substitute because of their biocompatibility, anti-thrombogenic properties, and match of mechanical

properties with the native vessels [2]. However, autologous conduits are restricted by limited material availability, e.g. due to existing vascular disease or previous harvest. As a surrogate, synthetic vessels are frequently used in these procedures [3]. Nevertheless, synthetic vessels often occlude *in vivo* due to the development of intimal hyperplasia, i.e. progressive smooth muscle cell (SMC) ingrowth and proliferation, eventually leading to stenosis [1, 2, 4]. To overcome these issues and to address the clinical need for vascular substitutes, bioengineered grafts have been proposed as an alternative [5].

Bioengineered grafts should mimic the native vessel structure with the appropriate tissue characteristics to withstand the hemodynamic *in vivo* environment. The inherent architecture of a native vessel comprises at least a luminal layer (*tunica intima*), which is lined with an anti-thrombogenic monolayer of endothelial cells (ECs), and a secondary medial layer (*tunica media*) containing stacked SMCs with a concentric organization that is critical for vasoconstriction and vasodilatation [6]. Strategies to engineer vascular grafts have often been directed at recreating these two specific vessel layers, typically by using (bi-layered) tubular scaffolds containing a *tunica intima* with highly dispersed fibers and/or a *tunica media* with circumferentially aligned fibers [6–9]. Recently, we created biomimetic bi-layered scaffolds with a heterotypic topology by means of a unique hybrid fabrication approach that combines solution electrospinning (SES) and melt electrowriting (MEW) [10]. The heterotypic scaffold was specifically designed to direct multi-layered vSM-like cells to orient in the near-circumferential direction on the outer side, along with a confluent functional endothelium on the luminal side, both derived from human progenitor cells. However, in these statically cultured bioengineered vessels, tissue maturation and further evaluation of the endothelial functionality was hampered due to the absence of layer-specific biochemical stimuli and physiologically relevant hemodynamic loads.

The biomechanical *in vivo* forces, to which cells in bioengineered grafts are exposed, consist of blood flow, which imposes wall shear stress on the lumen, and blood pressure, which imposes circumferential wall strain across the vascular wall. It is well established that ECs are mechanosensitive, where shear stress stimulates the endothelium that acts as an anti-thrombotic barrier, to control lumen diameter, and to regulate vascular permeability [11, 12]. Similar to ECs with a mature phenotype, endothelial progenitor cells have been shown to exhibit a response to shear stress. In fact, shear stress contributes to the advancement of these cells into mature ECs [13–16]. However, *in vitro* studies investigating the shear-responsiveness of ECs (both mature and progenitor cell types) on fibrous 3D or 2D substrates are often limited to relatively short perfusion times of up to 24 h [17–23], i.e. the characteristic time period for cells to adapt to new hemodynamics (reorganization of the cytoskeleton, cellular

junctions and adaption on the gene expression level) [24, 25]. Though studies investigating EC response to shear stress of up to multiple days exist [26–31], detailed analyses at later time points of EC morphology and marker expression on the protein level, especially on fibrous substrates, are lacking. To have a better indication of graft performance with respect to ECFC functionality and retention, a prolonged exposure to flow in a physiological 3D environment is necessary.

The performance of bioengineered grafts further depends on the communication between the shear stress-stimulated ECs in the *tunica intima* and the vascular smooth muscle cells (vSMCs) in the *tunica media*. This cross-communication is necessary to regulate vasoconstriction and vasodilatation, as well as the formation and remodeling of the extracellular matrix (ECM) in response to trauma and (changing) hemodynamics, thereby maintaining a vascular homeostasis [32]. Thus, vSMCs are indispensable for the creation of functional bioengineered grafts, and should therefore be included with the required phenotype in the engineered *tunica media*. A challenge herein is to induce and maintain the respective phenotype when co-cultured with other progenitor-derived cell types within one culture system. For example, where mesenchymal stromal cells (MSCs) or vSMCs typically require TGF β to differentiate to the contractile phenotype, ECs will undergo Endothelial-to-Mesenchymal-Transition (EndoMT) when exposed to TGF β [33, 34], hampering the effective cell-specific differentiation when EC and vSMC are co-cultured on vascular grafts.

In summary, the major objective for progressing our biomimetic bi-layered graft into a neovessel is the simultaneous layer-specific maturation of the cells in a physiologically relevant environment. In this work we address this challenge by designing and validating a perfusable, two-compartment bioreactor chamber that allows for prolonged culture (i.e. more than 24 h) the bi-layered vascular grafts under luminal shear stress, with a compartmentalized exposure of the involved cell types to specific culture media. We validated the approach by co-seeding human ECFCs and MSCs in our vascular grafts and perfusing the constructs for 2 d at a physiological shear rate, with the inner and outer sides of the graft exposed to their corresponding cell-specific culture media. The established cell phenotypes of the ECs and vSMCs as well as matrix production were evaluated after perfusion.

2. Materials and methods

2.1. Bioreactor design

A two-compartment bioreactor system was designed to be able to apply physiological shear rates at the luminal side of the vascular scaffold, while also allowing the exposure of both cell layers of the

Figure 1. Bioreactor design. (A) Exploded view of the flow chamber with the most important parts indicated. (B) Photograph of the flow chamber connected to the perfusion loop (inset shows a mounted scaffold, pink arrows indicate flow direction).

bi-layered graft (i.e. the inner and the outer compartments) to cell-specific media. The platform consisted of a custom-made culture chamber in which a bi-layered tubular scaffold ($\varnothing$ 3 mm, 2 cm length) was mounted (figure 1(A)). The luminal side of the scaffold was connected to a flow loop for unidirectional flow application, whereas the outer side of the scaffold was exposed to static culture conditions. During perfusion, flow was recorded with an ultrasonic flow meter, while temperature (37°C), CO_2 level (5%), and sterility were maintained. The flow was controlled by an Ibidi pressure pump (Ibidi GmbH, Martinsried, Germany) by tuning the pump pressure (figure 1(B)). Assuming Poiseuille flow, the shear stress at the luminal wall τ_w was computed from the flow magnitude [35]:

$$\tau_w = \frac{4\mu Q}{\pi r^3}$$

with μ the dynamic viscosity, Q the applied flow rate, and r the inner radius of the scaffold. Assuming a constant medium viscosity ($\mu = 0.7 \text{ mPa s}$ at 37°C) and using a scaffold inner radius of 1.5 mm, the shear stress at the luminal wall (in Pa) scales linearly with the applied flow rate (in ml min^{-1}) via: $Q = 227\tau_w$. To apply 80 mPa shear stress, we applied a flow rate of $\sim 18 \text{ ml min}^{-1}$ (supplementary figure 1 is available online at stacks.iop.org/BF/12/015009/mmedia), which corresponds to a physiological shear rate (i.e. τ_w/μ) of $\dot{\gamma} = 114 \text{ s}^{-1}$.

2.2. Scaffold preparation

Bi-layered tubular scaffolds ($\varnothing$ 3 mm, 2 cm length) were fabricated from poly(ϵ -caprolactone) (PCL) by combining SES and MEW as previously described [10]. In short, randomly oriented fiber on the luminal side were created with SES, followed by deposition of oriented MEW fibers that served as a guidance for MSCs to adhere to and grow on. To assess their microarchitecture, scaffolds were sputter-coated with a platinum coating (4 nm, EM ACE600, Leica) and analyzed using scanning electron microscopy (SEM;

Crossbeam 340, Carl Zeiss). The resulting scaffolds were sterilized by gamma irradiation, dipped in 70% ethanol, and sutured onto male luers. After suturing, the scaffolds were once again sterilized by UV exposure (15 min on two sides). Prior to cell seeding, the scaffolds were first washed with phosphate-buffered saline (PBS) and afterwards coated in a collagen I/fibronectin solution ($30 \mu\text{g ml}^{-1}$ rat tail collagen type I, Corning 354249; $50 \mu\text{g ml}^{-1}$ fibronectin from bovine plasma, Sigma-Aldrich F1141 in PBS) for 1 h at 37°C to enhance cell attachment and retention to the scaffold. As a final step, scaffolds were incubated in complete endothelial growth medium-2 (EGM-2) containing Endothelial Basal Medium-2 + Single-Quots (Lonza), 100 U ml^{-1} penicillin and $100 \mu\text{g ml}^{-1}$ streptomycin (PenStrep; Gibco), and 10% heat-inactivated FBS (FBS-HI; Biowest S14068S1810) for 1 h at 37°C before seeding.

2.3. Cell culture and characterization

2.3.1. Endothelial colony forming cells

ECFCs were isolated and characterized as described previously [36]. In short, ECFCs were isolated from the cord blood of two different donors (the procedure was approved by the medical research ethics committee, University Medical Center Utrecht; informed consent was obtained from the mothers). The isolates are hereafter referred to as ECFC1 and ECFC2. Confirmation of their potency was performed by phenotypic characterization with flow cytometry as described and presented previously [36]. After isolation, the ECFCs were either frozen or further expanded in complete EGM-2 medium. The cells were used for scaffold seeding at passage 7 or 9 (ECFC1) and 9 or 10 (ECFC2).

2.3.2. Multipotent mesenchymal stromal cells

MSCs were isolated and characterized as described previously [36]. In short, MSCs from human bone marrow aspirates were obtained from consenting

patients ($n = 2$; the procedure was approved by the local medical research ethics committee, University Medical Center Utrecht). These isolates are referred to as MSC1 and MSC2. The multipotency of the MSCs was confirmed via differentiation towards adipogenic, osteogenic, and chondrogenic lineages, as well as via phenotypical characterization by cell surface marker expression profiles with flow cytometry as described elsewhere [36, 37]. MSCs with confirmed multipotency were further expanded in MSC expansion medium, consisting of α -Minimum Essential Medium (Gibco Paisley, 22561), supplemented with 10% FBS-HI, 100 U ml $^{-1}$ –100 μ g ml $^{-1}$ PenStrep, 0.2 mM L-ascorbic acid-2-phosphate (ASAP, Sigma), and 1 ng ml $^{-1}$ basic fibroblast growth factor (rh-FGF-2; R&D Systems). The MSCs were used for scaffold seeding at passage 4.

2.3.3. Cell seeding

Prior to seeding, the medium was removed from the scaffolds and a suspension (100 μ l) of complete EGM-2 medium containing 3.8 million ECFCs ml $^{-1}$ was carefully injected into the lumen using a syringe with a 25G blunted needle. To promote uniform cell attachment, the cell-loaded constructs were rotated by 90° every 15 min for 1 h at 37 °C. Subsequently, excess medium was removed from the lumen and a total volume of 80 μ l EGM-2 medium containing 3.8 million MSCs ml $^{-1}$ was carefully pipetted onto the outer scaffold layer. The MSCs were allowed to adhere for 1 h at 37 °C, during which the constructs were rotated by 180° after 30 min. After cell seeding, the constructs were cultured on a continuous rotating orbital shaker (50 rpm) at 37 °C and 5% CO $_2$ for 7 d in petri dishes (ϕ 60 mm) containing 10 ml complete EGM-2 medium, which was refreshed at day 3 (figure 2(A)).

2.3.4. Perfusion and vSMC differentiation

After the 7 d pre-perfusion period, the constructs were mounted into the bioreactor and connected to the flow loop to start the 2 d perfusion period. The constructs

were divided into four groups: perfusion with vSMC differentiation medium in the outer compartment (p/tgf); perfusion with complete EGM-2 medium in the outer compartment (p/egm); static culture with vSMC differentiation medium in the outer compartment (s/tgf); and static culture with complete EGM-2 medium in the outer compartment (s/egm) (figure 2(B)). For groups p/tgf and p/egm, the flow loop was filled with 13 ml of complete EGM-2 medium supplemented with 0.1 mg ml $^{-1}$ Fluorescein isothiocyanate–inulin (FITC, 3 kDa) (TdB Consultancy) in 0.1% DMSO for determining the permeability of the cultured scaffold (section 2.4.2). To increase the flow in a stepwise fashion to 80 mPa (with a shear rate of $\sim$ 100 s $^{-1}$), the pump pressure was increased from 3 to 25 mbar within a period of 12 h (with intermediate steps at 5 and 8 mbar), and maintained at 25 mbar for the following 36 h (supplementary figure 1). The static groups s/tgf and s/egm were not exposed to this flow protocol, but were only shortly flushed with complete EGM-2 medium without FITC–inulin once an hour (at 5 mbar for 60 s) to refresh the culture medium in the lumen. For groups p/tgf and s/tgf, the outside of the culture chamber was filled with 13 ml of vSMC differentiation medium (DMEM high glucose (Gibco Paisley, 31966), 10% FBS-HI, 100 U ml $^{-1}$ –100 μ g ml $^{-1}$ PenStrep, 30 μ M ASAP, and 5 ng ml $^{-1}$ TGF β 1 (PeproTech 100-21C)) for induction of vSMC differentiation. The groups p/egm and s/egm were cultured with 13 ml of complete EGM-2 medium in the outer chamber. All experiments were performed with two donor combinations ($n = 4$ per donor combination and condition).

2.4. Analyses

2.4.1. Sample processing

After the 2 d perfusion period, the constructs were collected, partitioned as illustrated in supplementary figure 2, and processed for further analysis (figure 2(C)). Sample pieces designated for immunohistochemistry and whole mount fluorescence staining were stored at 4 °C after 60 min fixation in 3.7%

formaldehyde and 3×5 min washing in PBS. Samples for total cellular RNA extraction were resuspended in 0.5 ml Trizol Reagent (Thermo Fischer Scientific) for 5 min at room temperature and subsequently stored at -80°C till further use. The constructs were processed for assessment of cell morphology and characterization of phenotype (stainings and qPCR), and to investigate tissue formation (stainings). Additionally, medium samples were collected from the flow loop during perfusion ($200\ \mu\text{l}$ at 30 min, 4, 24, and 48 h) and from the outer chamber after perfusion (at 48 h) and stored at -20°C until further analysis to determine the construct permeability.

2.4.2. Permeability

To monitor the exchange of media between the two compartments of the culture chamber, and thus the permeability of the constructs, FITC-inulin was supplemented to the flow loop prior to the perfusion period (figure 3(A)). To evaluate the influence of the cell coverage on this permeability, the results of the cell-seeded constructs were compared against unseeded scaffolds (i.e. 'empty scaffolds'). Medium ($200\ \mu\text{l}$) from the empty scaffolds was collected after 30 min, 90 min, 4 h, and 48 h of perfusion. Since FITC-inulin is a relatively small fluorescent molecule (3 kDa), of which the fluorescence scales linearly with its concentration, it can be used to quantify the

permeability of the construct to inulin). Fluorescence was detected at an emission wavelength of 535 nm in the collected medium samples after excitation at 485 nm while using a microplate reader (Synergy HTX; Biotek).

2.4.3. Gene expression

2.4.3.1. RNA isolation

Samples were mixed with 20% Chloroform (Millipore) for 3 min at room temperature and subsequently centrifuged at 12 000 G for 15 min (4°C). The colorless upper aqueous phase was mixed with the same amount of 2-propanol (Sigma) (~ 0.25 ml) and $0.5\ \mu\text{l}$ glycogen (R0551, Thermo Fisher Scientific). The mixture was then left at room temperature for 10 min and centrifuged at 12 000 G for 10 min (4°C). The resulting pellet was washed in 75% ethanol and again centrifuged at 7500 G for 5 min (4°C). After drying, the pellet was re-suspended in $10\ \mu\text{l}$ of RNase-free water and exposed to DNase treatment ($1\ \mu\text{l}$ of Turbo DNase buffer (AM2238, Ambion) and $0.5\ \mu\text{l}$ DNase ($2\ \text{U}\ \mu\text{l}^{-1}$)).

2.4.3.2. Quantitative polymerase chain reaction (QPCR)

Total RNA was reverse-transcribed into cDNA with an iScript cDNA synthesis kit (Biorad, 170-8890). The RNA input concentration was set to $5\ \text{ng}\ \mu\text{l}^{-1}$ for all samples, providing the same cDNA end concentration

for all samples used in this data set. FastStart SYBR green master (Sigma, 04673484001) was used for the qPCR reaction mix, with a total end volume of 10 μ l containing 10 ng of cDNA and 500 nM primer (forward and reversed). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as optimal housekeeping gene among a set of candidates, based on its expression stability tested with the algorithm from Normfinder. Primers were optimized for their amplification efficiency (calculated via: $E = -1 + 10^{-1/\text{slope}}$). Primer sequences and annealing temperatures can be found in supplementary table 1. Gene expression was evaluated for three main outcomes: vSMC associated markers (α smooth muscle actin (α SMA), smooth muscle protein 22 α (SM22 α), Calponin (CALP), and (tropo)elastin (ELN)), EC associated markers (CD31, vascular endothelial-cadherin (VE-cadherin), and von Willebrand factor (vWF)), and the shear responsive markers (COX-2, eNOS, and KLF2).

2.4.3.3. Relative gene expression analysis

C_t values were normalized for the housekeeping gene and relative fold gene expression was calculated according to the $2^{-\Delta\Delta C_t}$ method. To test for vSMC induction, the relative gene expression ($2^{-\Delta C_t}$) of the TGF β -stimulated samples (p/tgf and s/tgf) was normalized to their matched controls (i.e. cultures from same donor for p/egm and s/egm). To test for the influence of shear stress on the endothelium, $2^{-\Delta C_t}$ of the perfused samples was normalized to their matched controls (i.e. static samples). Finally, to confirm that the differentiation medium in the outer compartment did not influence the endothelium, $2^{-\Delta C_t}$ of the perfused, TGF β -stimulated samples (p/tgf) were normalized to the perfused, undifferentiated controls (p/egm). For statistical analysis, the $2^{-\Delta C_t}$ of the matched controls was normalized to its own group average.

2.4.4. Stainings

Whole mount staining and stained section (5 μ m paraffin sections) were analyzed to assess cell phenotypes and matrix composition.

2.4.4.1. Immunocytochemistry

Formalin-fixed whole mount samples were permeabilized in 0.5% Triton-X 100 in PBS. Non-specific binding was blocked for 30 min using 5% (w/v) bovine serum in PBS. Primary antibodies were incubated overnight at 4 $^{\circ}$ C or for 1 h at room temperature (supplementary table 2). After washing in PBS (3 $\times$ 5 min), fluorescently labeled secondary antibodies were added overnight at 4 $^{\circ}$ C or for 1 h at room temperature with possible addition of phalloidin (FITC or TRITC, supplementary table 2). In case of CD31 or calponin identification, the ABC method was used for amplification of the signal with secondary biotinylated antibodies, detected with fluorescently labeled streptavidin-conjugated tertiary antibodies.

Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI, 100 ng ml $^{-1}$) for 15 min. Whole mount constructs were kept in PBS and visualized by confocal laser scanning (SP8X Leica DMI8 with a 10 $\times$ /0.4 APO lens, 20 $\times$ /0.4 fluotar lens, or 63 $\times$ /1.40 APO CS2 lens).

2.4.4.2. Immunohistochemistry

Deparaffinized and rehydrated sections were permeabilized in 0.5% Triton-X 100 in PBS. Depending on the antibody, antigen retrieval was performed by incubation in trypsin (30 min 37 $^{\circ}$ C for laminin α 5 subunit), pepsin (12 min 37 $^{\circ}$ C for collagen III), or citrate (20 min 97 $^{\circ}$ C for collagen I). Non-specific binding was blocked for 30 min using 5% (w/v) bovine serum in PBS. Primary antibodies were incubated overnight at 4 $^{\circ}$ C or for 1 h at room temperature (supplementary table 2). After washing in PBS (3 $\times$ 5 min), fluorescently labeled secondary antibodies were added overnight at 4 $^{\circ}$ C or for 1 h at room temperature, together with DAPI (supplementary table 2). In case of staining for laminin α 5 subunit, goat anti-mouse conjugated HRP-labeled polymer was added for 1 h (Dako, Envision, K400011). Addition of DAB substrate solution resulted in a brown staining, facilitated by HRP. Nuclei were counterstained with Mayer's hematoxylin (Merck). Finally, the sections were dehydrated, mounted with DEPEX, and visualized with a bright field microscope (Leica BX51). The fluorescently labeled sections were mounted in mowiol and visualized with an inverted fluorescence microscope (Leica DMI8, 1 pixel = 0.65 μ m or 0.32 μ m with a 20 $\times$ /0.4 or 40 $\times$ /0.95 HC PL Fluotar lens, respectively).

2.4.4.3. Histochemistry

Deparaffinized sections were stained with routine hematoxylin and eosin (H&E) or with Weigert's hematoxylin (10 min). Collagenous structures in hematoxylin-stained sections were visualized with PicroSirius red stain (1 h in 0.1 g Sirius red (F3B Klinipath No.80115) in 100 ml saturated aqueous picric acid (Riedel-deHaën No.36011) and 2 min in 0.01M HCl. After dehydration, samples were mounted in DEPEX and imaged with a bright field microscope (Leica BX51). Collagenous structures were also imaged under polarized light.

2.4.5. Cellular orientation

The in-plane orientation of the cells was quantified from the F-actin images using in-house developed software as described elsewhere [38]. In short, the actin fiber angle distribution in the constructs was calculated by binning the principal direction in each pixel of the F-actin images into a histogram ($n_{\text{bin}} = 45$ between 0 rad and π rad). The fiber distributions were assessed for alignment induced by flow (ECs) and by the contact guiding cue of the MEW fibers (vSMCs). Cellular orientation of the ECs was quantified from

maximum intensity confocal projections at 6 locations per sample. For the vSMCs, three different z-stacks ($\sim 350\ \mu\text{m}$) per sample were used for the orientation analysis.

2.4.6. Statistics

Statistical analyses were performed with GraphPad Prism 6.01. Permeability measurements were tested for the influence of cell coverage on the maintenance of an inulin concentration difference between the inner and outer compartment. Gene expression outcomes were tested for the influence of vSMC phenotype induction on the SMCs, shear stress on the ECs, and vSMC differentiation medium on the endothelium. The inulin concentration differences and fold changes of each gene of interest were tested for significant differences using a student's t-test or a non-parametric Mann-Whitney U test depending on the normality of the groups (checked with the Pearson normality test). Differences between the experimental groups were considered statistically significant when $p < 0.05$.

3. Results

3.1. Cell-laden scaffolds form a leak-free construct

PCL was processed into bi-layered tubular scaffolds with an inner diameter of 3 mm and wall thickness of $\sim 250\ \mu\text{m}$ (figure 3(B), left panel). The inner layer, with a thickness of $\sim 50\ \mu\text{m}$, exhibited an isotropic microstructure with fiber diameter of $1.4 \pm 0.2\ \mu\text{m}$. The outer layer contained stacked fibers of $15.2 \pm 4.8\ \mu\text{m}$ diameter with two predominant orientations ($\pm 71.6^\circ \pm 0.6^\circ$ with respect to the longitudinal axis). Next, the capacity of the cells and matrix to seal the scaffold was assessed, which is necessary to retain the cell-specific media in the respective compartments (figure 3(A)). SEM images indicated that the cells and matrix completely covered the inner and outer layer (figure 3(B), right panel). FITC-inulin diffusion measurements revealed that the concentration of inulin in the cell-laden constructs approached a plateau between 4 and 24 h of perfusion, which significantly differed from the concentration in the outer compartment after 48 h ($p = 0.004$). In contrast, the media from the inner and outer compartments of the empty scaffolds were completely mixed after 48 h (figures 3(C), (D)). The limited diffusion between the inner and outer chamber in the cell-laden constructs validated our approach to separately providing cell-specific media to each cell type.

3.2. The endothelial layer on the luminal side is responsive to shear stress

The SES layer on the luminal side provided a substrate for the ECs to form a confluent endothelium, with CD31 expression localized at the cell adherens junctions (figure 4(A)). The cytoskeletal F-actin in the

static groups was mainly confined to the cell periphery in the absence of shear stress, but remodeled to thick dorsal stress fibers under shear stress. Along with the endothelium, a collagen IV-positive matrix was present, known to be one of the main components of the basement membrane (figure 4(A)). The ECs elongated under perfusion, as expected due to the flow responsiveness of endothelium (figure 4(A)). The endothelium integrity was retained over the complete length of the scaffold, demonstrating the endothelium's robustness to withstand the flow, and the cells appeared in a 'wave-like' organization without preferred global orientation in the direction of flow (figure 4(B)). Quantification of the F-actin distribution confirmed this (figure 4(C)). After perfusion, the monolayer still showed CD31 localization at the cell borders as well as VE-cadherin-positive adherens junctions (figure 4(D)), indicating that it preserved its selective barrier function. Localization of vinculin confirmed the linkage between the actin cytoskeleton and the ECM via focal adhesion complexes (figure 4(E), supplementary figure 3(B)).

The responsiveness of the endothelium to flow was also observed at the gene expression level. Perfused endothelium showed significant upregulation of the shear stress-associated enzymes eNOS, COX-2 and KLF2, compared to static endothelium (figure 4(F)). Furthermore, the markers CD31 and VE-cadherin, known for their role in shear responsive pathways and permeability function, were found to be upregulated after flow perfusion. Shear stress did not have a clear effect on the expression of the platelet adhesion molecule vWF on a gene expression level ($p = 0.2323$), but a conformational change of the vWF protein under shear stress was observed whereby the unfolded active form of the glycoprotein appeared in strand-like multimers (figure 4(D)), compared to a globular appearance in static control cultures (supplementary figure 3(A)). Presence of TGF β in the differentiation medium of the outer compartment did not affect the appearance and gene expression of the endothelium, in either the static or perfused condition, indicating that the sealing capacity of the construct prevented TGF β to diffuse to the endothelial compartment (supplementary figure 3(C), (D)).

3.3. MSCs differentiate to vSM-like cells

To evaluate if the addition of TGF β to the outer compartment resulted in differentiation of MSCs into vSM-like cells, the contractile vSMC markers calponin and αSMA were examined. Indeed, differentiation was confirmed by the appearance of calponin⁺/ αSMA ⁺ cells (figure 5(A) and supplementary figure 4) and by the significant upregulation of these markers at the gene expression level (figure 5(B)). No significant difference was found in the expression of the SM22 α gene ($p = 0.117$). After differentiation, the matrix surrounding the vSM-like

cells contained the required vSMC basement membrane component collagen IV (figure 5(E)), and (tropo)elastin also showed significant upregulation on the mRNA level (figure 5(B)). In addition to the expected marker expression of the cells, the cells also elongated and aligned in the circumferential orientation (figures 5(C), (D)), guided by the MEW fibers.

3.4. Co-cultured bi-layered grafts resemble the native vessel structure

Finally, the effects of the differentiation medium in the outer compartment on *de novo* tissue formation by the vSM-like cells were studied. The cells adopted an elongated morphology and organized into a multi-layered fashion around the bi-layered scaffold (figure 6(A)). Most strikingly, several matrix components in the TGFβ-stimulated constructs were more abundantly expressed compared to in the EGM-

cultured controls (figure 6(B)). Specifically, the TGFβ-stimulated constructs showed a laminin α5-positive matrix with thicker collagen bundles composed of collagen type III, but mainly collagen type I. This was the other way around in the non-differentiated control samples, where more collagen type III was present than collagen type I. The cross-sectional view shows the presence of a CD31-positive continuous monolayer together with a multi-layered αSMA-positive *tunica media* mimic, confirming that the correct phenotype, morphology, and organization of both cell types could be induced simultaneously within one co-cultured bi-layered graft (figure 6(C)). Furthermore, the low MEW fiber density resulted in large pores, allowing for close cell–cell contact of the vSM-like cells surrounded by their own ECM. The cross-section demonstrates that the SES layer separated the cell types from each other, important for prevention of intimal hyperplasia by vSMC ingrowth into the luminal side.

4. Discussion

We designed and validated a two-compartment bioreactor system for further maturation of our recently bioengineered vascular grafts, with maintenance of vSM-like cells and progenitor-derived ECs via compartmentalized exposure to their respective media. MSCs and ECFCs were selected as cell sources, with MSCs having the ability to differentiate into vSMCs and the ECFCs for their inherent vasculogenic capacity and participation in post-natal vasculogenesis. Both cell types can be isolated from autologous sources, and therefore have therapeutic potential. We demonstrated that during the period of perfusion, the cells together with their deposited matrix seal off the open, porous scaffold, resulting in the retention of cell-specific differentiation media in the lumen and outer compartment. Moreover, the vascular integrity and the endothelium's attachment to the substrate were preserved throughout the period of perfusion. Using bi-layered biomimetic scaffolds, we successfully recapitulated the architecture of native vessels, consisting of circumferentially oriented, multi-layered structures of vSM-like cells and a flow-responsive confluent luminal layer of ECs.

In our setup, the ECs were exposed to increasing levels of fluid shear stress up to 80 mPa (at a physiological shear rate of $\sim 100 \text{ s}^{-1}$). This level of shear stress is

slightly lower than that found in veins (0.1–0.6 Pa [39]), but could be increased to match arterial shear levels (1–7 Pa [39]) through the addition of medium thickening components [40], and/or application of higher flow rates if desired. However, since freshly seeded ECs initially form a weak adhesion to the graft material, they would detach from the graft when directly exposed to *in vivo* levels of high shear stress. Here, using a gradually increasing flow protocol after static pre-culturing, complete endothelial retention was achieved during an extended period of perfusion. In our pressure-driven bioreactor system, the flow protocol can be customized for other applications as well in terms of flow rate, pulsatility, pressure, and duration, and can be monitored during the experiment.

The integrity of the monolayer was also maintained, with localization of VE-cadherin in the adherens junctions and CD31 expression in the cell periphery (figure 4). Both VE-cadherin and CD31 play an important role in the vascular permeability barrier and stabilization of cell–cell junctions [41]. They also play a role in shear stress response via the formation of a multimolecular complex, together with β -catenin and VEGFR3, that is mechanoresponsive [41–43]. This mechanosensory complex is involved in a number of well-known shear stress responses by ECs (as well as their progenitor cells [16]), such as stress fiber

Figure 6. Tissue formation after 7 d of pre-culture and 48 h of perfusion. (A) Cross-sectional overview of the graft with a completely populated *tunica media* (hematoxylin and eosin (H&E) staining). (B) Cross-sections of representative samples cultured with p/egm (top rows) or p/tgf (bottom rows) indicate multiple layers of elongated vSM-like cells (H&E). The TGF β -stimulated samples show laminin α 5 expression (LAMA5) in the ECM, whereas control samples show only laminin α 5 expression in the cells. Picrosirius red staining shows collagen bundles (under polarized light), further specified with stainings for collagen type I and collagen type III (dashed lines indicate the scaffold borders, SES; solution electrospinning, MEW; melt electrowriting). (C) An α SMA/CD31-stained cross-section shows the localization and the different phenotypes of the two cell layers within one construct.

remodeling and cell alignment [17]. Consistent with reports by others [17, 44], the ECs appeared more elongated under perfusion while their F-actin remodeled from cortical stress fibers located at the cell periphery to dorsal stress fibers throughout the cytoplasm. However, we did not observe a predominant global cell alignment, which could indicate either that a certain threshold of shear stress was not reached with the applied flow protocol [45], that re-orientation was hindered by the high density of the cells [46], or that adhesion of the monolayer to the underlying basement membrane was not sufficiently strong [47].

Indeed, we used a high cell seeding density to ensure the formation of a tightly connected network of ECs. However, it has been previously shown that over-confluence can impede the alignment of cells in the monolayer in the direction of flow within the observation period of 48 h [46]. Regulation of cell morphology and F-actin organization in response to shear stress has been additionally shown to require a firm anchorage to the basement membrane via focal adhesion-associated proteins, such as vinculin [48, 49]. In our endothelium, vinculin was mainly located at the basal side, linking the F-actin stress fibers to the underlying ECM, but without obvious re-localization towards the ‘upstream’ end of the cells. The localization of this focal adhesion component determines the direction of

stress fiber formation and could therefore be related to the elongated morphology of the shear stress-stimulated cells [48]. It will be interesting to investigate the relation between vinculin, focal adhesions complexes, and morphological changes (i.e. cellular elongation and re-orientation) under the influence of shear stress using more in-depth mechanobiological analyses in the future.

Responsiveness to perfusion by the endothelium was also confirmed at the gene and protein expression levels, hinting at the biological functionality of our endothelium. The anti-thrombotic function of the endothelium is partly regulated by the glycoprotein vWF, which is the main responder to vascular injury by initiating coagulation via platelet adhesion. It has been shown that vWF is responsive to shear stress, resulting in a conformational change from the inactive globular conformation into unfolded multimeric streaks [50–52]. This activation of vWF was observed in this study, with the appearance of streaks of vWF in the perfused constructs (figure 4(D) and supplementary figure 3(A)). Moreover, the shear stress-induced genes COX-2, KLF2, and eNOS were found to be significantly upregulated under the influence of flow (figure 4(F)). These genes are inducible by shear stress [53], where especially KLF2 is found to be consistently expressed in cultures exposed to prolonged laminar flow and fails to be induced under the influence of a

disturbed flow profile [13, 54]. As such, the results indicate that the luminal layer of the bi-layered scaffold was covered by a shear responsive endothelial lining.

The vasoregulatory agents produced by the ECs under the influence of shear have to be transported to the vSMCs, residing in the *tunica media*, to be able to exert their vasoregulatory function. Not only is this communication important for the vasomotion of the vessel, it has also been previously shown that ECs promote phenotype modulation of vSMCs through signaling mechanisms [55–57]. Synthetic vSMCs can rapidly proliferate and synthesize ECM (collagen type I and fibronectin) [58], which is desired directly after seeding of cells to ensure complete colonization of the scaffold. In our study, this was mimicked by using MSCs for the initial seeding and pre-culture period. On the other hand, contractile vSMCs only produce small amounts of ECM (collagen IV, (tropo)elastin, laminin $\alpha 5$), proliferate at low rates, and can be differentiated from MSCs by the addition of biochemical factors, such as TGF β [59–62]. Specific for their function is the expression of contractile proteins α SMA, SM22 α , calponin, and SMMHC [58, 63–66]. In the present study, within a time-frame of 2 d after induction of differentiation, the MSCs already showed an upregulation of the proteins α SMA and calponin, and a significant upregulation of α SMA, calponin, and (tropo)elastin on a gene expression level compared to EGM controls. The early differentiation marker SM22 α was not upregulated. However, this could be attributed to the high SM22 α (and calponin) expression levels in the MSCs from one donor of the control group (p/egm, data not shown), probably caused by an early differentiation that is initiated through EC signaling or via confluency-induced differentiation [10].

The impact of TGF β on the differentiation of the vSMCs is further clarified by stainings of vascular-specific ECM proteins, including collagen types I, III and IV, and laminin $\alpha 5$. Our results revealed that the addition of differentiation medium resulted in elevated collagen type I secretion compared to the EGM controls, indicative of a more mature matrix [67]. Furthermore, we found that laminin $\alpha 5$, which enables cell adhesion to the surrounding basement membrane and promotes a contractile cell phenotype [68], was only located in the matrix surrounding the vSM-like cells when exposed to differentiation medium, corroborating with our result that the MSCs adopted a vSMC-like phenotype.

Together, our bioengineered graft not only holds potential to address the unmet clinical need for small-diameter blood vessels, but it could also be used as an *in vitro* model for cardiovascular research [69]. Whilst a large number of bioengineered grafts with different levels of complexity has been developed, in the approach taken here we uniquely combine mechanical and biochemical stimuli (i.e. flow-induced shear stress and TGF β) with structural components (i.e. a

co-culture of vSM-like cells and ECs in biomimetic scaffolds with a fast colonization of the *tunica media* mimic), enabling the *in vitro* study of new drugs, medical devices, and disease modeling [70]. It should be noted that the current setup has some experimental restrictions. Due to the large volume of culture medium circulating in the perfusion loop, our analysis of the endothelium did not go beyond gene expression analysis and stainings. Future assessment should include functional read-outs, for example through analysis of NO and PGI $_2$ secretion of the ECs into the culture medium, as well as anti-thrombogenic properties of perfused constructs by exposure to whole blood or plasma. In addition, evaluations could be extended with functional read-outs of vSMC contractility in response to vasoactive agents. Finally, future directions should address the gradually and uni-directionally applied flow in the bioreactor system. To precondition the ECs to adapt to and withstand *in vivo* flow conditions, shear stress can be increased and applied in a pulsatile fashion for an even longer duration. Pulsatile flow could also benefit the maturation of the vSM-like cells via the resulting circumferential strain. This, however, would first require the mechanical optimization of the scaffold material with more elastic polymers to match its compliance with natural blood vessels.

5. Conclusion and outlook

In this study, we have developed a bioreactor system to culture bi-layered vascular grafts under shear stress, with a compartmentalized exposure of the graft's luminal and outer layer to cell-specific media. We demonstrated that the bioreactor allows for simultaneous layer-specific cell differentiation in our biomimetic bi-layered vascular graft, in which the natural architecture and cell phenotypes of a native vessel, in particular the *tunica intima* and *tunica media*, were successfully recapitulated by the unique scaffold design. Our results highlight that, using this co-culture system, vascular grafts from clinically relevant progenitor cell sources can be created and investigated to further improve the next generation of bioengineered vascular grafts.

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Author contributions

Author contributions according to the CRediT Taxonomy are as follows: Conceptualization: IP, EH, AS, NK, DG; Formal Analysis: IP, EH, AS, NK, DG; Investigation: IP, EH, TJ, JB; Funding Acquisition: CB, DG; Supervision: CB, AS, NK, DG, AR, JG; Visualization: IP, EH; Writing—Original Draft Preparation: IP, EH; Writing—Review & Editing: All. All authors have approved the final article.

Declaration of interest

None.

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References

- Haruguchi H and Teraoka S 2003 Intimal hyperplasia and hemodynamic factors in arterial bypass and arteriovenous grafts: a review *J. Artif. Organs* **6** 227–35
- Salacinski H J, Goldner S, Giudiceandrea A, Hamilton G, Seifalian A M, Edwards A and Carson R J 2001 The mechanical behavior of vascular grafts: a review *J. Biomater. Appl.* **15** 241–78
- Chlupáč J, Filová E and Bačáková L 2009 Blood vessel replacement: 50 years of development and tissue engineering paradigms in vascular surgery *Physiol. Res.* **58** 119–40
- Collins M J, Li X, Lv W, Yang C, Protack C D, Muto A, Jadowiec C C, Shu C and Dardik A 2012 Therapeutic strategies to combat neointimal hyperplasia in vascular grafts *Expert Rev. Cardiovascular Ther.* **10** 635–47
- Gui L and Niklason L E 2014 Vascular tissue engineering: building perfusable vasculature for implantation *Curr. Opin. Chem. Eng.* **3** 68–74
- Hasan A, Memic A, Annabi N, Hossain M, Paul A, Dokmeci M R, Dehghani F and Khademhosseini A 2014 Electrospun scaffolds for tissue engineering of vascular grafts *Acta Biomater.* **10** 11–25
- Woods I and Flanagan T C 2014 Electrospinning of biomimetic scaffolds for tissue-engineered vascular grafts: Threading the path *Expert Rev. Cardiovascular Ther.* **12** 815–32
- Thottappillil N and Nair P D 2015 Scaffolds in vascular regeneration: current status *Vascular Health Risk Manage.* **11** 79–91
- Goins A, Webb A R and Allen J B 2019 Multi-layer approaches to scaffold-based small diameter vessel engineering: a review *Mater. Sci. Eng.* **97** 896–912
- Jungst T, Pennings I, Schmitz M, Rosenberg A, Groll J and Gawlitta D 2019 Heterotypic scaffold design orchestrates primary cell organization and phenotypes in co-cultured small diameter vascular grafts *Adv. Funct. Mater.* **29** 1905987
- Davies P F, Zilberberg J and Helmke B P 2003 Spatial microstimuli in endothelial mechanosignaling *Circ. Res.* **92** 359–70
- Lu D and Kassab G S 2011 Role of shear stress and stretch in vascular mechanobiology *J. R. Soc. Interface* **8** 1379–85
- Egorova A D, DeRuiter M C, De Boer H C, Van De Pas S, Gittenberger-De Groot A C, Van Zonneveld A J, Poelmann R E and Hierck B P 2012 Endothelial colony-forming cells show a mature transcriptional response to shear stress *Vitr. Cell Dev. Biol.—Anim.* **48** 21–9
- Obi S, Yamamoto K and Ando J 2014 Effects of shear stress on endothelial progenitor cells *J. Biomed. Nanotechnol.* **10** 2586–97
- Obi S et al 2008 Fluid shear stress induces arterial differentiation of endothelial progenitor cells *J. Appl. Physiol.* **106** 203–11
- Yamamoto K, Takahashi T, Asahara T, Ohura N, Sokabe T, Kamiya A and Ando J 2003 Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress *J. Appl. Physiol.* **95** 2081–8
- Levesque M J and Nerem R M 1985 The elongation and orientation of cultured endothelial cells in response to shear stress *J. Biomech. Eng.* **107** 341–7
- Koo M-A, Kang J, Lee M, Seo H, Kwon B-J, You K, Kim M, Kim D and Park J-C 2014 Stimulated migration and penetration of vascular endothelial cells into poly (L-lactic acid) scaffolds under flow conditions *Biomater. Res.* **18** 7
- Inoguchi H, Tanaka T, Maehara Y and Matsuda T 2007 The effect of gradually graded shear stress on the morphological integrity of a huvec-seeded compliant small-diameter vascular graft *Biomaterials* **28** 486–95
- Owatverot T B, Oswald S J, Chen Y, Wille J J and Yin F C P 2005 Effect of combined cyclic stretch and fluid shear stress on endothelial cell morphological responses *J. Biomech. Eng.* **127** 374–82
- Kwak B R, Silacci P, Stergiopulos N, Hayoz D and Meda P 2005 Shear stress and cyclic circumferential stretch, but not pressure, alter connexin43 expression in endothelial cells *Cell Commun. Adhes.* **12** 261–70
- Nackman G B, Fillinger M F, Shafritz R, Wei T, Graham A M, Freischlag J A and Kent K C 1998 Flow modulates endothelial regulation of smooth muscle cell proliferation: a new model *Surgery* **124** 353–61
- Whited B M and Rylander M N 2014 The influence of electrospun scaffold topography on endothelial cell morphology, alignment, and adhesion in response to fluid flow *Biotechnol. Bioeng.* **111** 184–95
- Zhang J and Friedman M H 2012 Adaptive response of vascular endothelial cells to an acute increase in shear stress magnitude *AJP Heart Circ. Physiol.* **302** H983–91
- Zeng Y and Tarbell J 2014 The adaptive remodeling of endothelial glycocalyx in response to fluid shear stress *PLoS One* **9** 1–15
- Kaushal S et al 2001 Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo *Nat. Med.* **7** 1035–40
- Ju Y M, Ahn H, Arenas-Herrera J, Kim C, Abolbashari M, Atala A, Yoo J J and Lee S J 2017 Electrospun vascular scaffold for cellularized small diameter blood vessels: a preclinical large animal study *Acta Biomater.* **59** 58–67
- Lee S J, Liu J, Oh S H, Soker S, Atala A and Yoo J J 2008 Development of a composite vascular scaffolding system that withstands physiological vascular conditions *Biomaterials* **29** 2891–8
- Niklason L E, Gao J, Abbott W M, Hirschi K K, Houser S, Marini R and Langer R 1999 Functional arteries grown in vitro *Science* **284** 489–93
- Ott M J and Ballermann B J 1995 Shear stress-conditioned, endothelial cell-seeded vascular grafts: Improved cell adherence in response to in vitro shear stress *Surgery* **117** 334–9

- [31] Dekker R J R, van Soest S, Fontijn R R D, Salamanca S, de Groot P, VanBavel E, Pannekoek H and Horrevoets A J G A 2002 Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Krüppel-like factor (KLF2) *Blood* **100** 1689–98
- [32] Elliott M B and Gerecht S 2016 Three-dimensional culture of small-diameter vascular grafts *J. Mater. Chem. B* **4** 3443–53
- [33] de Jonge N, Muylaert D E P, Fioretta E S, Baaijens F P T, Fledderus J O, Verhaar M C and Bouten C V C 2013 Matrix production and organization by endothelial colony forming cells in mechanically strained engineered tissue constructs *PLoS One* **8** e73161
- [34] Fioretta E S, Fledderus J O, Burakowska-Meise E A, Baaijens F P T, Verhaar M C and Bouten C V C 2012 Polymer-based Scaffold designs for *in situ* vascular tissue engineering: controlling recruitment and differentiation behavior of endothelial colony forming cells *Macromol. Biosci.* **12** 577–90
- [35] White F M 2010 *Fluid Mechanics* (New York: McGraw-Hill) pp 272–3 (https://academia.edu/32134233/Fluid_Mechanics_seventh_edition_by_Frank_M._White.pdf)
- [36] Pennings I et al 2019 Effect of donor variation on osteogenesis and vasculogenesis in hydrogel cocultures *J. Tissue Eng. Regen. Med.* **1**–13
- [37] Klotz B J, Lim K S, Chang Y X, Soliman B G, Pennings I, Melchels F P W, Woodfield T B F, Rosenberg A J W P, Malda J and Gawlitta D 2018 Engineering of a complex bone tissue model with endothelialised channels and capillary-like networks *Eur. Cells Mater.* **35** 335–49
- [38] van Haaften E E, Wissing T B, Rutten M C M, Bulsink J A, Gashi K, van Kelle M A J, Smits A I P M, Bouten C V C and Kurniawan N A 2018 Decoupling the effect of shear stress and stretch on tissue growth & remodeling in a vascular graft *Tissue Eng. C* **24** 418–29
- [39] Malek A M, Alper S L and Izumo S 1999 Hemodynamic shear stress and its role in atherosclerosis *JAMA* **282** 2035–42
- [40] van den Broek C, Pullens R, Frøbert O, Rutten M, den Hartog W and van de Vosse F 2008 Medium with blood-analog mechanical properties for cardiovascular tissue culturing *Biorheology* **45** 651–61
- [41] Privratsky J R and Newman P J 2014 PECAM-1: regulator of endothelial junctional integrity *Cell Tissue Res.* **355** 607–19
- [42] Tzima E, Irani-Tehrani M, Kiosses W B, Engelhardt B, Cao G, DeLisser H and A S M 2005 A mechanosensory complex that mediates the endothelial cell response to fluid shear stress *Nature* **437** 426–31
- [43] Baeyens N and Schwartz M A 2016 Biomechanics of vascular mechanosensation and remodeling *Mol. Biol. Cell* **27** 7–11
- [44] Barry A, Wang N and Leckband D 2015 Local VE-cadherin mechanotransduction triggers long-ranged remodeling of endothelial monolayers *J. Cell Sci.* **128** 1341–51
- [45] Baeyens N et al 2015 Vascular remodeling is governed by a VEGFR3-dependent fluid shear stress set point *Elife* **4** 1–16
- [46] Ohta S, Inasawa S and Yamaguchi Y 2015 Alignment of vascular endothelial cells as a collective response to shear flow *J. Phys. D: Appl. Phys.* **48** 245401
- [47] Di Russo J et al 2017 Endothelial basement membrane laminin 511 is essential for shear stress response *EMBO J.* **36** 1464
- [48] Girard P R and Nerem R M 1995 Shear stress modulates endothelial cell morphology and f-actin organization through the regulation of focal adhesion-associated proteins *J. Cell. Physiol.* **163** 179–93
- [49] Verma D, Meng F, Sachs F and Hua S Z 2015 Flow-induced focal adhesion remodeling mediated by local cytoskeletal stresses and reorganization *Cell Adhes. Migr.* **9** 432–40
- [50] Siedlecki C A, Lestini B J, Kottke-Marchant K K, Eppell S J, Wilson D L and Marchant R E 1996 Shear-dependent changes in the three-dimensional structure of human von Willebrand factor *Blood* **88** 2939–50
- [51] Tsai H 2003 Shear stress and von willebrand factor in health and disease *Semin. Thromb. Hemost.* **29** 479–88
- [52] Auton M, Zhu C and Cruz M A 2010 The mechanism of VWF-mediated platelet GPIIb/IIIa binding *Biophys. J.* **99** 1192–201
- [53] Senbanerjee S et al 2004 KLF2 is a novel transcriptional regulator of endothelial proinflammatory Activation *J. Exp. Med.* **199** 1305–15
- [54] Dekker R J, van Soest S, Fontijn R D, Salamanca S, de Groot P, VanBavel E, Pannekoek H and Horrevoets A J G 2002 Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Krüppel-like factor (KLF2) *Blood* **100** 1689–98
- [55] Brown D J, Rzuclido E M, Merenick B L, Wagner R J, Martin K A and Powell R J 2005 Endothelial cell activation of the smooth muscle cell phosphoinositide 3-kinase/Akt pathway promotes differentiation *J. Vasc. Surg.* **41** 509–16
- [56] Hirschi K and D'Amore P A 1996 Pericytes in the microvasculature *Cardiovascular Res.* **32** 687–98
- [57] Lilly B 2014 We have contact: endothelial cell-smooth muscle cell interactions *Physiology* **29** 234–41
- [58] Wang G, Jacquet L, Karamariti E and Xu Q 2015 Origin and differentiation of vascular smooth muscle cells *J. Physiol.* **593** 3013–30
- [59] Gu W, Hong X, Le Bras A, Nowak W N, Bhaloo S I, Deng J, Xie Y, Hu Y, Ruan X Z and Xu Q 2018 Smooth muscle cells differentiated from mesenchymal stem cells are regulated by microRNAs and suitable for vascular tissue grafts *J. Biol. Chem.* **293** 8089–102
- [60] Gong Z, Calkins G, Cheng E, Krause D and Niklason L E 2009 Influence of culture medium on smooth muscle cell *Tissue Eng. A* **15** 319–30
- [61] Beamish J A, He P, Kottke-Marchant K and Marchant R E 2010 Molecular regulation of contractile smooth muscle cell phenotype: implications for vascular tissue engineering *Tissue Eng. B* **16** 467–91
- [62] Seeger T, Hart M, Patarroyo M, Rolauffs B, Aicher W K and Klein G 2015 Mesenchymal stromal cells for sphincter regeneration: role of laminin isoforms upon myogenic differentiation *PLoS One* **10** e0137419
- [63] Owens G K 1995 Regulation of differentiation of vascular smooth muscle cells *Physiol. Rev.* **75** 487–517
- [64] Owens G K, Kumar M S and Wamhoff B R 2004 Molecular regulation of vascular smooth muscle cell differentiation in development and disease *Physiol. Rev.* **84** 767–801
- [65] Liu Y, Deng B, Zhao Y, Xie S and Nie R 2013 Differentiated markers in undifferentiated cells: Expression of smooth muscle contractile proteins in multipotent bone marrow mesenchymal stem cells *Dev. Growth Differ.* **55** 591–605
- [66] Bacakova L, Travnickova M, Filova E, Metecka R, Stepanovska J, Musilkova J, Zarubova J and Molitor M 2018 The role of vascular smooth muscle cells in the physiology and pathophysiology of blood vessels *Muscle Cell and Tissue—Current Status of Research Field* ed Kunihiro Sakuma (Rijeka: IntechOpen) pp 229–57
- [67] Hanson A N and Bentley J P 1983 Quantitation of type I to type III collagen ratios in small samples of human tendon, blood vessels, and atherosclerotic plaque *Anal. Biochem.* **130** 32–40
- [68] Yousif L F, Di Russo J and Sorokin L 2013 Laminin isoforms in endothelial and perivascular basement membranes *Cell Adhes. Migr.* **7** 101–10
- [69] van Haaften E E, Bouten C V C and Kurniawan N A 2017 Vascular mechanobiology: towards control of *in situ* regeneration *Cells* **6** 1–24
- [70] Wolf F, Vogt F, Schmitz-Rode T, Jockenhoevel S and Mela P 2016 Bioengineered vascular constructs as living models for *in vitro* cardiovascular research *Drug Discov. Today* **21** 1446–55