Chapter 14

Vascular Smooth Muscle Cells (VSMCs) in Blood Vessel Tissue Engineering: The Use of Differentiated Cells or Stem Cells as VSMC Precursors

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Abstract

Vascular smooth muscle cells (VSMCs) play important roles in the physiology and pathophysiology of the blood vessels. In a healthy adult organism, VSMCs are quiescent, but after a blood vessel injury, they undergo phenotypic modulation from the contractile phenotype to the synthetic phenotype, characterized by high activity in migration, proliferation and proteosynthesis. This behavior of VSMCs can lead to stenosis or obliteration of the vascular lumen. For this reason, VSMCs have tended to be avoided in the construction of blood vessel replacements. However, VSMCs are a physiological and the most numerous component of blood vessels, so their presence in novel advanced vascular replacements is indispensable. Either differentiated VSMCs or stem cells as precursors of VSMCs can be used in the reconstruction of the *tunica media* in these replacements. VSMCs can be obtained from blood vessels (usually from subcutaneous veins) taken surgically from the patients and can be expanded in vitro. During in vitro cultivation, VSMCs lose their differentiation markers, at least partly. These cells should therefore be re-differentiated by seeding them on appropriate scaffolds by composing cell culture media and by mechanical stimulation in dynamic bioreactors. Similar approaches can also be applied for differentiating stem cells, particularly adipose tissue-derived stem cells, toward VSMCs for the purposes of vascular tissue engineering.

Keywords: vascular replacements, adipose tissue-derived stem cells, transforming growth factor-beta, bone morphogenetic protein-4, mechanical loading, dynamic bioreactors, smooth muscle cell differentiation, tissue engineering, regenerative medicine

1. Introduction

VSMCs are the most numerous cell types in blood vessels, where they are located in the medial layer of the vascular wall, that is, in the *tunica media*. These cells are necessary for the physiological functioning of blood vessels, particularly for vasoconstriction, for vasodilatation and for synthesis of vascular extracellular matrix. These cells are also implicated in pathological changes in blood vessels during atherosclerosis, hypertension, diabetic angiopathy and other vascular disorders. After biochemical or mechanical damage to blood vessels, VSMCs undergo phenotypic modulation, that is, they make the transition from their original quiescent contractile phenotype to a synthetic phenotype, characterized by increased proteosynthesis and by activation of the migration and growth of VSMCs [1–4]. These changes often lead to irreversible damage to blood vessels, including stenosis and occlusion. Ischemia of the tissues supplied by the damaged vessels is then manifested by serious disorders, for example, heart failure, brain stroke or necrosis of leg tissues, which can result in amputation of the leg.

Low patency of arteries can be treated by balloon angioplasty or by endarterectomy. However, in cases of severe blood vessel damage, vascular replacements need to be implanted, usually in the form of bypasses spanning the damaged region of the original vessel.

Vascular bypass grafts can be obtained from four sources: autologous, allogenous, xenogenous or artificial. Autologous grafts, that is, grafts derived from the patient, have the drawbacks of limited availability, donor site morbidity, burden to the patient due to additional surgery and, in the case of implantation of a vein into an arterial position, also mechanical mismatch. Allogeneous transplants, that is, transplants derived from the same species, or xenogenous transplants, that is, transplants derived from a different species, are associated with a risk of immune rejection, disease transmission and, when they are fixed in glutaraldehyde, also potential release of cytotoxic molecules [5, 6]). In view of these problems, artificially constructed vascular grafts have been considered as very promising for future applications.

Artificial grafts currently used in clinical practice are made of synthetic polymers, namely polyethylene terephthalate (PET), expanded polytetrafluoroethylene (ePTFE) and, in some cases, also polyurethane [5, 7, 8]. The first generation of these prostheses was constructed as cell-free, that is, without the reconstruction of any layer of the natural blood vessel. However, the inner surface of the prosthesis attracted cell types participating in thrombus formation, immune reaction and prosthesis restenosis, that is, thrombocytes, inflammatory cells (leucocytes, lymphocytes, monocytes, macrophages), and also VSMCs. VSMCs migrated on the prosthesis mainly from the sites of the anastomosis of the graft with the original vessel and were prone to excessive proliferation. In addition, precursors of VSMCs, originating from the bone marrow and circulating in the blood, can adhere to the inner surface of the prosthesis and can proliferate [9]. All these events can lead to considerable stenosis, obliteration and failure of vascular prostheses, especially medium-diameter vascular grafts (up to 8 mm in diameter) and small-diameter vascular grafts (up to 4 mm in diameter). Attempts have therefore been made to cover the luminal surface of the prosthesis with a confluent, phenotypically mature and semi-permeable endothelial cell layer, which is considered optimal for preventing thrombosis, inflammatory cell adhesion and VSMC hyperplasia [5, 7, 8].

However, in advanced vascular replacements, it is necessary to reconstruct not only the endothelial cell layer, that is, the main component of the *tunica intima*, but also the other layers of the vascular wall, particularly the *tunica media* with VSMCs as the physiological component of natural blood vessels. It is necessary only to control the proliferation activity of these cells precisely and to direct them toward a differentiated quiescent contractile phenotype. In modern tissue engineering, it is also desirable to differentiate stem cells toward VSMCs, particularly stem cells derived from adipose tissue, which is relatively easily accessible and is available in sufficient quantities [10, 11].

This chapter summarizes our own experience and the experience of other authors in re-differentiating VSMCs on vascular constructs via appropriate cultivation substrates, the composition of cell culture media, cell–cell interaction and mechanical stimulation in dynamic bioreactors. Similar approaches have also been applied for differentiating stem cells, particularly adipose tissue-derived stem cells, toward VSMCs for the purposes of vascular tissue engineering.

2. Use of differentiated VSMCs in blood vessel tissue engineering

As mentioned earlier, attempts have been made to reconstruct the *tunica intima* on artificial vascular replacements, and these replacements have been used sporadically in clinical practice [7, 8]. At the same time, the *tunica media* has been reconstructed only rarely in vascular replacements, due to the tendency of VSMCs to proliferate excessively, and these attempts still remain at the experimental level. However, as was mentioned earlier, the presence of the *tunica media* enhances the functionality of artificially constructed blood vessels, if the VSMCs gain their quiescent contractile phenotype [12]. This phenotype is usually lost during the expansion of VSMCs after they have been harvested from blood vessels obtained surgically from patients. The contractile phenotype can be restored by an appropriate structure and composition of the scaffolds, by appropriate composition of cell culture media, by appropriate cell–cell interactions and by appropriate mechanical stimulation of VSMCs in dynamic cell culture systems, especially if the factors mentioned here are applied in combination.

2.1. Structure and composition of the scaffolds

As concerns the structure of the scaffolds, three-dimensional (3D) porous scaffolds are more physiological than two-dimensional (2D) scaffolds, because 3D scaffolds better mimic the architecture of the native *tunica media* and enable a multilayered arrangement of VSMCs [13, 14]. The differentiation response of VSMCs to the uniaxial stress generated by a dynamic cell culture system was more pronounced in 3D scaffolds than on 2D scaffolds [15].

As concerns the chemical composition of the scaffolds, attempts are being made to fabricate these scaffolds from degradable materials, such as synthetic polymers (e.g., polylactides, polyglycolides, polycaprolactone and their copolymers), natural polymers (collagen, elastin, fibronectin, laminin, fibrin) and combinations of these materials [14–20]. Degradable scaffolds are used for vascular tissue engineering, because the scaffolds will gradually be removed and replaced by a newly regenerated vascular tissue. In addition, some natural polymers maintain the VSMCs in a differentiated contractile phenotype, for example, elastin and proteins of the

cell basement membrane, namely type IV collagen and laminin, while other natural polymers, such as fibronectin and vitronectin, stimulate the phenotypic modulation of VSMCs toward the synthetic phenotype and VSMC migration and proliferation [18, 21, 22]. The role of type I collagen is ambiguous. Polymeric fibrillary type I collagen in a healthy blood vessel keeps the VSMCs in their quiescent state, but degraded or denatured type I collagen supports VSMC proliferation [21, 23]. Scaffolds obtained by decellularization of various tissues, including blood vessels, recently emerged as very promising structures for cardiovascular tissue engineering. After decellularization, the tissues lose most of their immunogenicity and could even be used for xenogeneic transplantation. At the same time, these scaffolds retain their original biochemical composition and mechanical properties [11, 12, 19, 20, 24].

2.2. Composition of the cell culture medium

Another important issue in the reconstruction of the *tunica media* is the composition of the cell culture medium. In the initial phase of colonization of the scaffolds with VSMCs, the migration and proliferation of these cells and their synthesis of ECM molecules is desirable, and therefore a standard serum-supplemented medium can be used. At the same time, the scaffolds should be seeded with a high number of VSMCs in order to shorten their migratory and proliferative phase as much as possible. It is known that the confluence of VSMCs and the development of cell–cell contact support the re-differentiation of VSMCs toward the contractile phenotype [25]. When the scaffolds are well populated with VSMCs, it is necessary to achieve the quiescent differentiated contractile phenotype of VSMCs. For this purpose, chemically defined serum-free or serum-low media are used, for example, media supplemented with transforming growth factor-β (TGF-β) [26–28] or with heparin [29]. At the same time, heparin supports endothelialization of the prosthesis [30], which also contributes to the development of the contractile phenotype in VSMCs, for example, by producing sulfated heparin-like glycosaminoglycans [2, 18, 31], nitric oxide [32, 33] and by developing contacts between VSMC and endothelial cells, that is, myoendothelial gap junctions [27].

2.3. Interactions of VSMCs with endothelial cells and with other VSMCs

VSMCs co-cultured in direct contact with endothelial cells showed more pronounced differentiation toward the contractile phenotype (manifested by increased expression of contractile proteins, that is, SM1 and SM2 isoforms of smooth muscle myosin heavy chain, calponin 1 and smooth muscle α-actin) than VSMCs co-cultured with endothelial cells without direct contact with these cells. This effect was mediated by connexin 43 (Cx43), an important component of myoendothelial gap junctions. Inhibition of gap junctional communication pharmacologically or by knock down of Cx43 in endothelial cells blocked TGF-β signaling and VSMC differentiation [27]. However, the gap junctions between VSMCs are a more controversial issue. On the one hand, an increased number of these junctions and upregulation of Cx43 have been shown to be associated with undesirable VSMC proliferation and vascular diseases. On the other hand, when increased expression of Cx43 in VSMCs was induced by TGF-β1, these cells enhanced the expression of smooth muscle α-actin (SM α-actin), calponin and SM1 myosin heavy chain, that is, markers of VSMC differentiation toward the contractile phenotype [34].

In comparison with other connexins, for example, Cx37, Cx43 is highly mechanosensitive. The exposure of human coronary artery smooth muscle cell to shear stress of 5 dyn/cm^2, but not

to physiological shear stress of 12 dyn/cm^2, caused the dysfunction of Cx40/Cx43 heterotypic myoendothelial gap junctions, which may be replaced by homotypic Cx43/Cx43 channels and induced the transition of VSMCs to the synthetic phenotype, which was manifested by decreased expression of smooth muscle myosin heavy chain (SM-MHC) and calponin and by increased release of platelet-derived growth factor-BB (PDGF-BB). At the same time, the VSMCs under shear stress of 5 dyn/cm^2 were randomly oriented, while under shear stress of 12 dyn/cm^2, these cells were aligned in the flow direction [35].

In our experiments, we investigated the effects of periodical uniaxial stretching of VSMCs on the Cx43 expression. Mechanical stimulation of VSMCs was performed using STREX equipment (B Bridge International, Ltd). VSMCs were seeded in flexible silicone chambers coated with type I collagen and fibronectin. After a 2-day static culture, the VSMCs were subjected to stretch at a frequency of 0.5 Hz and an amplitude of 5%. After a further period of 48 h, the frequency was changed to 1 Hz. The changes in Cx43 expression were tested by qRT-PCR. At near-physiological conditions (frequency of 1 Hz and amplitude of 5%), the expression immediately rose almost 5 fold, with the maximum in the first 30 min. At a lower degree of stimulation (at a frequency of 0.5 Hz and an amplitude of 5%), the maximal expression was delayed to about 60 min, and it was considerably lower. For longer time periods, the expression of Cx43 decreased again (**Figure 1**). VSMCs were also stained by immunofluorescence to show the changes in the arrangement and the distribution of the contractile protein SM α-actin. After stretching, the SM α-actin was more intensely stained than in the control static culture and was organized into filaments, especially in cells after 24 h of stretching (**Figure 2**).

2.4. Mechanical loading of VSMCs in a dynamic culture system

In general, dynamic cultivation of VSMCs is an important tool for restoring the contractile differentiated phenotype of these cells [16, 19]. It has been shown repeatedly that differentiation of VSMCs requires pulsatile stress and cyclic strain, that is, components of the hemodynamic stress to which blood vessels are exposed in vivo [36].

As concerns **pulsatile stress**, rabbit aortic VSMCs were seeded onto rubber-like elastic, three-dimensional poly(lactide-*co*-caprolactone) scaffolds and were exposed to a pulsatile flow of

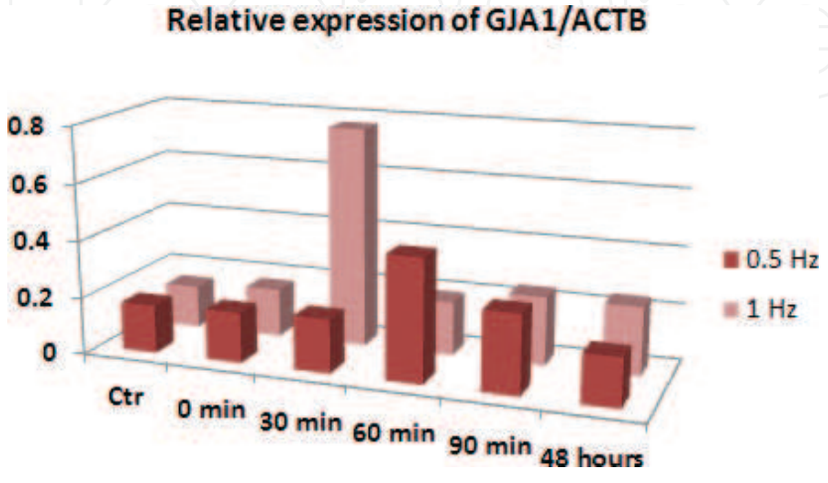

Figure 1. Relative mRNA expression of connexin 43 (GJA1) in rat aortic smooth muscle cells after uniaxial stretching in the STREX dynamic cell culture system (B bridge international, ltd.) for 0–48 h at a frequency of 0.5 Hz (dark) or 1 Hz (light).

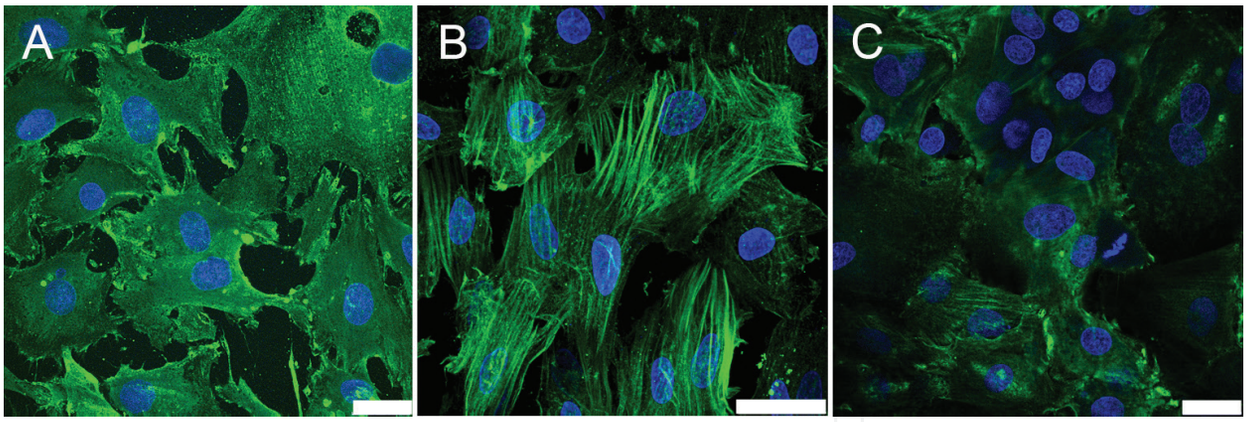

Figure 2. Immunofluorescence of SM α-actin in rat aortic smooth muscle cells in 2-day-old cultures after exposure to uniaxial stretching at a frequency of 1 Hz for 4 h (A), 24 h (B) and in control cells without stretching (C). Leica SPE confocal microscope (DM 2500 CSQ V-VIS), obj. 63×. Scale bar = 25 μm.

the culture medium (flow rate 130 ml/min, pressure 25 mmHg with a pulse of 1 Hz, amplitude of radial distention 5%, exposure 8 weeks). The pulsatile strain and the shear stress enhanced the VSMC proliferation and collagen production. However, at the same time, the expression of SM α-actin, an early marker of VSMC differentiation, was upregulated 2.5-fold in comparison with the value in VSMCs under static conditions, and the VSMCs were aligned in a direction radial to the distending direction, that is, similarly as in native blood vessels in vivo, whereas the VSMCs were randomly oriented under static conditions [37].

The behavior of VSMCs in a pulsatile bioreactor can be further modulated by the presence or absence of endothelial cells. Endothelial cells were seeded on the opposite side of a porous polycarbonate membrane and were placed in contact with a collagen gel containing VSMCs. The presence of the endothelial cells increased the VSMC size and the expression of the contractile proteins, namely SM α-actin and SM-MHC. Absence of endothelial cells decreased the expression of SM α-actin and SM-MHC without affecting the size of the VSMCs. The proliferation of VSMCs was not affected by the presence or absence of endothelial cells [38].

As concerns **cyclic strain**, collagen-based gels laden with primary human umbilical artery VSMCs were exposed to a 10% cyclic strain at 0.5 Hz for 5 days. Cyclic stimulation promoted cell-driven collagen matrix bi-axial compaction, enhancing the mechanical strength of the strained samples with respect to the static controls. Moreover, cyclic strain had a positive effect on VSMC behavior: the cells maintained their contractile phenotype and spread uniformly throughout the thickness of the walls of collagen-based tubular structures [39].

The effect of cyclic strain can be further modulated by the presence of various growth factors. For example, VSMCs in a 3D collagen type 1 matrix were exposed to a 10% circumferential strain at a frequency of 1 Hz. These conditions increased the gel compaction and the VSMC proliferation, which was further enhanced by adding PDGF into the cell culture medium. Conversely, the addition of TGF-β strongly inhibited cell proliferation and increased the expression of SM α-actin [40]. In a study by Yao et al. [41], rat aortic VSMCs in 70% confluence and after starving in a Dulbecco's Modified Eagle Medium (DMEM) without serum for 24 h were subjected to cyclic strain of 10% elongation at 1.25 Hz for 24 h in the Flexercell Tension

Plus system. The strain stimulated the secretion of TGF-β1 by VSMCs and upregulated the expression of contractile phenotype markers in these cells, namely smooth muscle protein 22-α (SM22-α), SM α-actin and calponin.

The parameters of the cyclic strain also strongly modulate the VSMC response. For example, rat aortic VSMCs were exposed to cyclic strains in vitro with defined parameters, that is, 5% strain, considered as physiological, and 15% strain, considered as pathological. Both types of strain had a frequency of 1.25 Hz and were applied for 24 h. The results showed that 15% strain significantly increased VSMC migration and proliferation in comparison with 5% strain [42].

3. Use of stem cells as a source of VSMCs for blood vessel tissue engineering

Stem cells have emerged as a promising resource for advanced tissue engineering, including vascular tissue engineering. Differentiated VSMCs are often obtained from aged and polymorbid patients. These cells show lower proliferation potential than is desirable, as the harvested cells need to be expanded in cell culture conditions. In addition, the VSMCs also show a higher tendency toward senescence. Another consideration is that these VSMCs are mostly of venous origin because it is easier and less invasive to isolate subcutaneous veins than arteries. However, venous VSMCs have different properties from those of arterial VSMCs, for example, they are adapted for lower pressure and slower blood circulation in the vein system.

Stem cells are a component of the blood vessels themselves, where they are distributed throughout the entire vascular wall, that is, in the subendothelial space of the *tunica intima*, in the *tunica media* and also in the *tunica adventitia*. Their primary function is postnatal vasculogenesis and regeneration of the vascular wall after injury, but they can also be a cell source for vascular tissue engineering [43–45]. However, harvesting stem cells and isolating differentiated VSMCs are associated with similar problems [46].

Other sources of stem cells with the potential to be differentiated into VSMCs are human pluripotent stem cells, obtained from embryonic tissues [47, 48] and induced pluripotent stem cells (iPSCs) [49–51]. However, the use of these cells, although promising, is associated with ethical and legal issues in human embryonic stem cells and with a risk of potential tumorigenicity of iPSCs. These complications can be overcome by the use of stem cells isolated from extrafetal tissues, for example, placenta [52] and umbilical cord [53] or by the use of stem cells from adult tissues, such as bone marrow [36, 54, 55], epidermis, namely hair follicles [56] or skeletal muscle [57]. In addition, adult stem cells can be applied in autologous form. However, harvesting the adult tissues mentioned here is often invasive and painful, and the tissues are obtained in relatively small quantities. Consequently, adipose tissue-derived stem cells (ASCs) seem to be the most promising source because the adipose tissue, located subcutaneously, can be obtained by a less invasive method, that is, liposuction, and in relatively large quantities.

ASCs have been used relatively widely for experimental vascular tissue engineering. The main tools for differentiating ASCs toward VSMCs include composing cell culture

media and exerting mechanical stress in dynamic cell culture systems, similarly as for the re-differentiation of VSMCs. Examples of results obtained by various authors [10, 11, 58–67] are summarized in **Table 1**.

Author	Scaffolds	Medium supplement	Cultivation system	Obtained VSMC markers
Rodríguez et al. [10]	Uncoated tissue culture polystyrene dishes or dishes coated with laminin or collagen	Medium MCDB 131 with 1% FBS plus 100 units/ml of heparin	Static	SM α-actin, calponin, caldesmon, SM22-α, SM-MHC, smoothelin
Kim et al. [58]	Tissue culture polystyrene	Angiotensin II	Static	SM α-actin, calponin, h-caldesmon, SM-MHC
Kim et al. [59]	Tissue culture polystyrene	Bradykinin	Static	SM α-actin
Kim et al. [60]	Tissue culture polystyrene	Thromboxane A_2 mimetic U46619	Static	SM α-actin, calponin, SM-MHC, smoothelin
Nincheri et al. [61]	Tissue culture polystyrene, microscope slides coated with gelatine	Sphingosine 1-phosphate	Static	SM α-actin, transgelin, cytoskeletal F-actin assembly, Ca^{2+} currents
Wang et al.. [62]	Tissue culture polystyrene dishes	TGF-β1, BMP-4	Static	SM α-actin, SM22-α, calponin, SM-MHC
Aji et al. [63]	Tissue culture polystyrene	TGF-β1, BMP-4	Static	SM α-actin, SM22α, calponin, SM-MHC
Elçin et al. [64]	8-chamber slides (Labtek)	TGF-β1, BMP-4, angiotensin II	Static	SM α-actin, calponin, h-caldesmon SM-MHC
Lachaud et al. [65]	Tissue culture polystyrene dishes	EGF	Static	SM α-actin, calponin, caldesmon, SM22α, desmin, SM-MHC, smoothelin-B
Wang et al. [66]	Polyglycolic acid mesh	TGF-β1, BMP-4	Pulsatile stress	SM α-actin, calponin, SM-MHC
Harris et al. [11]	Decellularized saphenous vein	angiotensin II, SPC, TGF-β1	Bioreactor generating: Tension Compression Pressure Perfusion	calponin, caldesmon, SM-MHC
Rashidi et al. [67]	Plasma-treated silicon membranes with collagen I	TGF-β1	Cyclic strain	SM α-actin, SM22-α, h-caldesmon, calponin3

TGF-β1: transforming growth factor-β1; BMP-4: bone morphogenetic protein-4 (a polypeptide belonging to the TGF-β superfamily); SM α-actin: α-isoform of smooth muscle actin; SM22-α: smooth muscle protein 22-α; SM-MHC: smooth muscle myosin heavy chain; Transgelin: actin cross-linking/gelling protein in fibroblasts and smooth muscle cells; SPC: sphingosylphosphorylcholine; EGF: epidermal growth factor.

Table 1. Culture conditions for differentiation of ASCs into VSMCs and the obtained markers of differentiation.

For our experiments, the ASCs were isolated from lipoaspirates obtained from patients by liposuction under their informed consent and ethical approval. Lipoaspirates of subcutaneous adipose tissue were taken from three different regions, that is, the abdominal region and the inner or outer side of the thighs. Liposuction was performed under low negative pressure (−200 mmHg) and under high negative pressure (−700 mmHg). The ASCs were then harvested by a method originally described by Estes et al. [68], with a slight modification described in our earlier study [69].

3.1. Differentiation of ASCs toward VSMCs by the composition of the cell culture medium

In our first set of experiments, we attempted to optimize the composition of the cell culture media in a conventional static cell culture system in order to differentiate the ASCs toward VSMCs. First, three types of culture media were tested, namely a DMEM medium (Sigma-Aldrich, Cat. No. D5648) with 10% of fetal bovine serum (FBS), SmGM®-2 Smooth Muscle Growth Medium-2 BulletKit® (SMGM, Lonza, USA, Cat. No. CC-3182) and Endothelial Growth Medium-2 (EGM-2, Lonza, USA, Cat. No. 3162). These media alone, that is, without additional supplementation, did not promote the differentiation of ASCs into VSMCs. Therefore, we supplemented the media with transforming growth factor-β1 (TGF-β1; 2.5 ng/mL, Abcam) and with bone morphogenetic protein-4 (BMP-4; 2.5 ng/ml, Sigma-Aldrich) because this combination of growth factors showed greater differentiation efficiency than TGF-β1 or BMP-4 alone or in combination with angiotensin II [64]. The addition of TGF-β1 into SMGM-2 and EGM-2 media caused rapid proliferation and subsequent detachment of the ASCs. The differentiation experiments were therefore performed with DMEM +2% of FBS + TGF-β1 + BMP-4. The addition of TGF-β1 increased the proliferation of ASCs in comparison with DMEM +2% FBS without any supplement. When the ASCs were cultured with TGF-β1 and BMP-4 for three days, immunofluorescence staining revealed the formation of SM α-actin-containing filaments and an increasing number of calponin-positive cells (**Figure 3A-C**). In later culture intervals (days 14–17), cells with slight positivity for desmin and sporadic SM-MHC-positive cells were also detected. Cells cultured without the supplements only sporadically contained SM α-actin filaments or calponin (**Figure 3D** and **E**). In our experiments, we observed individual differences in proliferation and differentiation among the ASCs from various patients and also among the cells taken from the same patient but from different regions of the body.

3.2. Differentiation of ASCs toward VSMCs by the composition of the cell culture medium and by mechanical load

In our second set of experiments, we studied the differentiation of ASCs toward VSMCs by combining cell differentiation media with mechanical load. The blood pumped by the heart generates several mechanical stimuli on the arterial wall, such as the wall shear stress affecting endothelial cells, and also the pressure force and the cyclic strain stress. These types of stimuli promote or accelerate the differentiation and the phenotypic maturation of ASCs and other stem cells into VSMCs [36, 55, 66]. In order to simulate the effects of these mechanical stimuli, we have developed a unique dynamic cultivation system. This system consists of special cultivation chambers

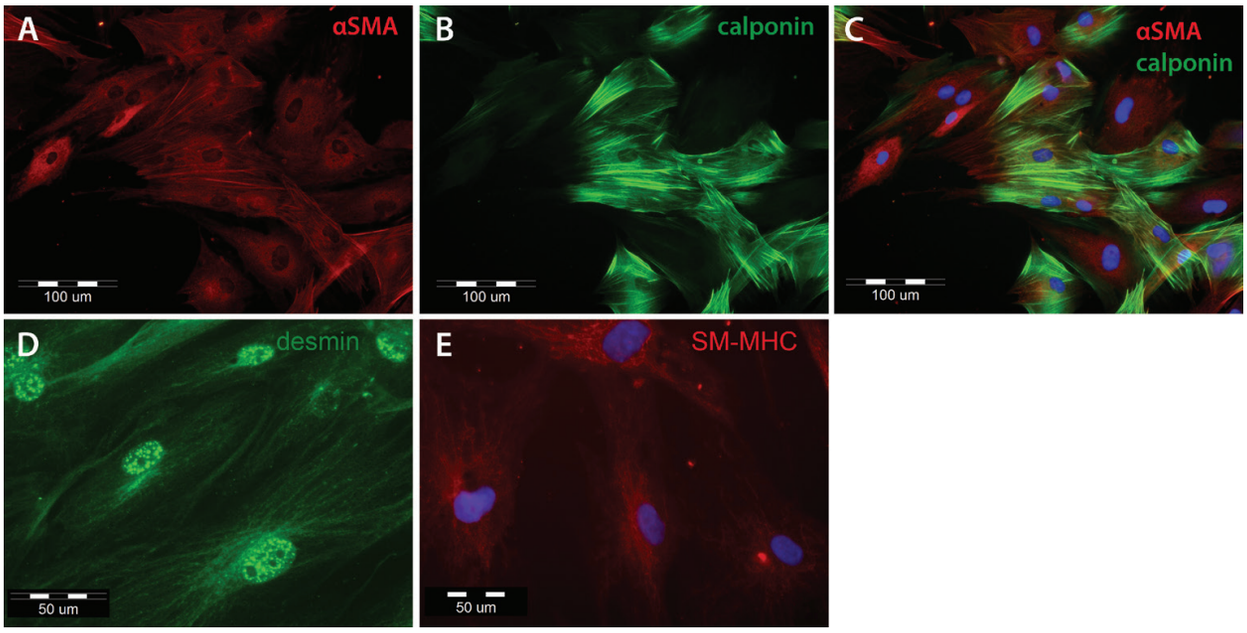

Figure 3. Immmunofluorescence staining of SM α-actin (red) and calponin (green) in ASCs on day 3 of differentiation (A–C) in a medium containing TGF-β and BMP-4. Immunofluorescence staining of desmin (D) on day 14 and of SM-MHC (E) on day 17. Cell nuclei are visualized with Hoechst #33258 (blue). Olympus IX 71 microscope, objective ×20 and ×40, scale 100 μm and 50 μm, respectively.

and a pressure generation system. The design of the chamber allows the use of rigid substrates (glass) or flexible substrates (cast silicone). A rigid substrate is used for evaluating the effect of the pressure force. A flexible substrate simulates an elastic arterial wall. The pressure force that is applied mimics the dilatation and constriction of the arterial wall by generating cyclic strain stress. To improve their hydrophilicity, the substrates are plasma treated. In addition, these substrates can be coated with collagen or fibrin gels to improve the adhesion and the initial proliferation of the cells. After cell seeding, the chamber is hermetically sealed to allow controlled stimulation (**Figure 4**). The pressure generation is maintained by a computer-controlled custom-built linear syringe pump. A pressure-based feedback-controlling algorithm is implemented to

Figure 4. Cultivation chambers used for mechanical stimulation (left). The use of transparent surfaces allows microscopic live-cell imaging (right).

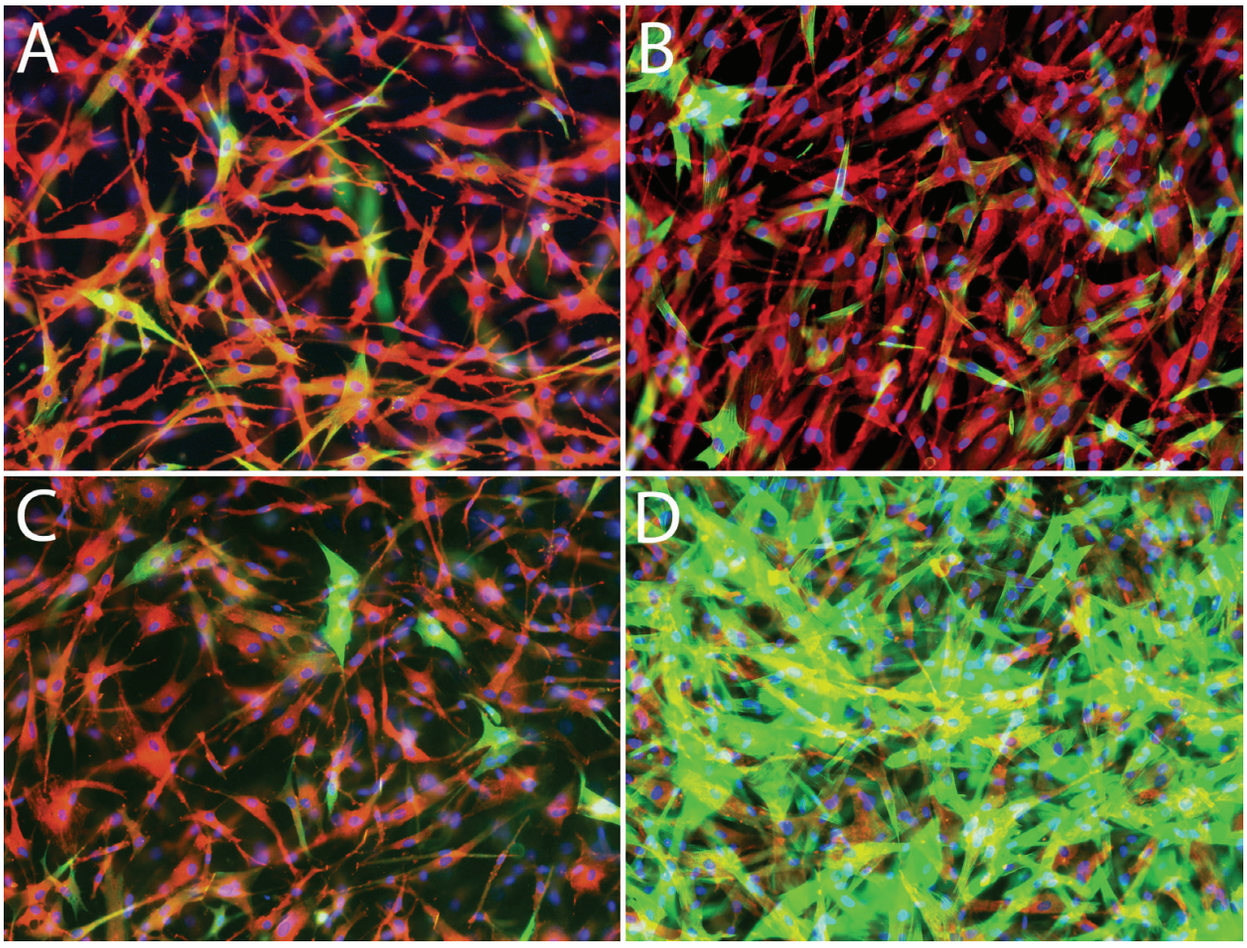

Figure 5. Immunofluorescence staining of SM α-actin (red) and calponin (green) in ASCs cultured embedded in a fibrin gel on glass under static conditions (A, C) and under dynamic conditions (B, D), using the system depicted in **Figure 4**. A, B: 3 days of cultivation; C, D: 7 days of cultivation. Cell nuclei are counterstained with Hoechst #33258. IX71 Olympus microscope, DP71 digital camera, obj. × 10.

maintain stable conditions. Pressure pulses are generated between two set points that simulate systolic and diastolic pressure. Maximum pressure can be set up to 300 mmHg (40 kPa), with a maximum pulse rate of up to 180 beats per minute (3 Hz).

Our experimental results, obtained in the dynamic culture system described above, indicated positive effects of pressure stimulation on the differentiation of ASCs toward VSMCs. ASCs in low passages 2–4, with initial density of approx. 70,000 cells/cm^2, were cultured in high glucose DMEM medium (Sigma-Aldrich, Cat. No. D5648), supplemented with 2% FBS, TGF-β1 (2.5 ng/mL, Abcam) and BMP-4 (2.5 ng/ml, Sigma-Aldrich) for 3 or 7 days under static culture conditions or under dynamic pulse pressure stimulation. This stimulation was set to physiological 120/80 mmHg (15.9/10.6 kPa) and pulse rate simulating 60 beats per minute (1 Hz). The cell culture medium was replaced after 3 days. The ASCs were stained for SM α-actin (Sigma-Aldrich, Cat. No. S2547), an early marker of VSMC differentiation, and for calponin (Abcam, Cat. No. ab46794), an intermediate marker of VSMC differentiation, and the cell nuclei were counterstained with Hoechst #33258. Pressure loading supported ASC proliferation after 3 days (**Figure 5B**) and after 7 days (**Figure 5D**). This was manifested by a higher cell population

density than in the static culture (**Figure 5A** and **C**). The ASCs were positively stained for SM α-actin on all samples. Moreover, increased numbers of cells positively stained for calponin were found in ASCs cultured in fibrin gel under pulse pressure on day 7 (**Figure 5D**). This suggests that the differentiation of ASCs into VSMCs in the presence of TGF-β1 and BMP-4 was significantly enhanced by dynamic pressure loading.

3.3. Differentiation of ASCs in co-culture with vascular endothelial cells

In our third set of experiments, we studied the behavior of ASCs in co-culture with vascular endothelial cells. ASCs are known to possess the ability to stimulate endothelial cells to form capillaries. In a co-culture model of ASCs and endothelial cells, the ASCs in close contact with endothelial cells differentiated after 7 days into pericyte-like cells, which stained positively for SM α-actin and stabilized the wall of newly formed capillaries (**Figure 6**). Similar results were achieved in a study by Rohringer et al. [70], who co-cultured ASCs

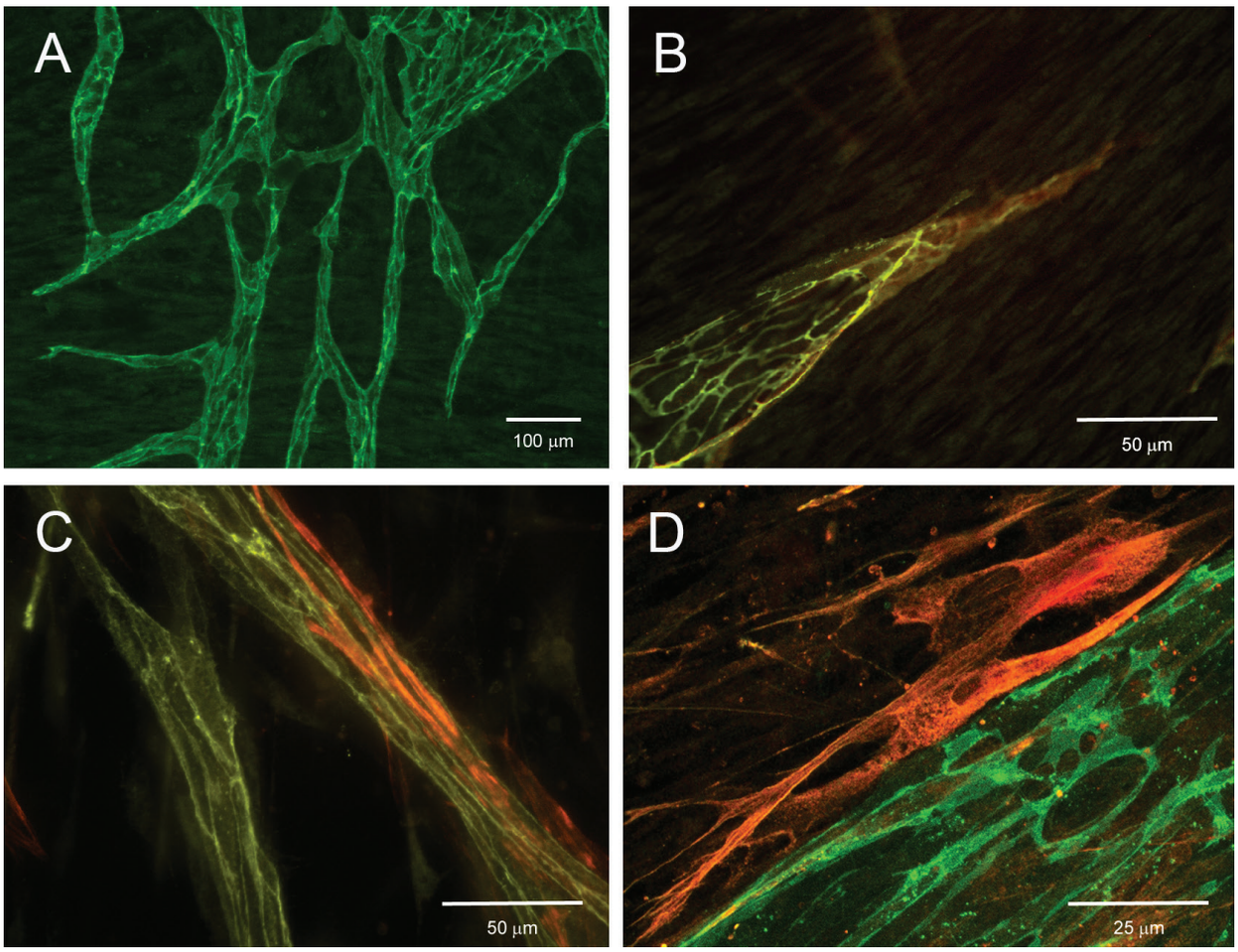

Figure 6. Capillary formation in the co-culture model of ASCs with endothelial cells. A: Vascular network formed by endothelial cells in the co-culture model, endothelial cells stained against VE-cadherin. B: Detail of vascular sprouting; VE-cadherin in green, CD146 (a marker of pericytes) in red. C: Stabilization of capillaries by perivascular cells after 7 days of co-culture; VE-cadherin in green, SM α-actin in red. D: Detail of the close contact between perivascular cells and endothelial cells; VE-cadherin in green, SM α-actin in red. Nikon Ti-E inverted fluorescence microscope with a CARV II confocal scanner.

and vascular endothelial cells in a fibrin gel and demonstrated that the proximity of ASCs and endothelial cells stimulated the formation of tubular structures by endothelial cells, which were stabilized by ASCs developing the characteristics of pericytes. Recent studies have documented similarities between mesenchymal stem cells and pericytes. Pericytes are contractile cells that are in close contact with endothelial cells in capillaries and serve to control the blood flow. When grown in vitro, pericytes express similar surface antigens as ASCs (CD73, CD90 or CD105) [71] and lack hematopoietic markers (CD45) and endothelial markers (CD31, von Willebrand factor, VE-cadherin). Pericytes are also capable of multipotential differentiation, for example, adipogenic, osteogenic, chondrogenic and myogenic differentiation [72]. However, the level of CD146, which is considered to be a marker of pericytes, differs greatly among different isolations of ASCs (in our experience from 0.5–90%). CD146 (also known as MCAM, S-endo-1, MUC18 or P1H12) is not expressed solely in pericytes. It is also considered to be a marker of endothelial progenitor cells and endothelial cells. It was recently shown that CD146 acts as a receptor for Wnt5a and regulates cell migration [73] or that it is involved in controlling the formation of the blood–brain barrier, where it ensures communication between endothelial cells and pericytes [74]. In the co-culture model of endothelial cells with ASCs, it remains elusive whether every ASC that is in close contact with an endothelial cell can act as a pericyte, or whether pericytes form a subpopulation of the heterogeneous population of ASCs with a specific, irreplaceable function.

4. Conclusion

Vascular smooth muscle cells (VSMCs) are the most numerous component of the arterial and venous wall, and they ensure the physiological functions of blood vessels. Under pathological conditions, however, VSMCs lose their differentiation markers, which is accompanied by activation of migration and proliferation of these cells. This can lead to stenosis or obliteration of the injured blood vessels. For this reason, VSMCs were not included in the early generations of vascular replacements, which were either cell-free or pre-endothelialized in vitro. Reconstruction of *the tunica media* containing VSMCs remains at the experimental level. The *tunica media* can be reconstructed with the use of differentiated VSMCs taken from blood vessels (usually subcutaneous veins), isolated surgically or with the use of stem cells, which is a more advanced approach. Various types of stem cells have been used for differentiation into VSMCs and for constructing vascular replacements, including embryonic stem cells, induced pluripotent stem cells, stem cells from extrafetal tissues and stem cells isolated from adult tissues, such as bone marrow, skeletal muscle, epidermis and adipose tissue. Adipose-tissue derived stem cells (ASCs) seem to be the most promising source of VSMCs because they can be isolated in relatively large quantities, by relatively non-invasive methods (liposuction) and in autologous form. Differentiation of ASCs into VSMCs can be induced by appropriate scaffolds (preferably three-dimensional and compliant) by appropriate composition of the cell culture media (e.g., a low-serum medium supplemented with TGF-β1 and BMP-4) and particularly by mechanical stimulation in dynamic cell culture systems generating pulsatile stress, cyclic strain and pressure stress. In co-cultures with endothelial cell forming tubular structures, ASCs form pericyte-like cells.

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Conflict of interest

The authors declare no conflict of interest.

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