VASCULAR SMOOTH MUSCLE CELLS FOR USE IN VASCULAR TISSUE ENGINEERING OBTAINED BY ENDOTHELIAL-TO-MESENCHYMAL TRANSDIFFERENTIATION (ENMT) ON COLLAGEN MATRICES

Guido Krenning*, Jan-Renier AJ Moonen*, Marja JA van Luyn and Martin C Harmsen

*Authors contributed equally

Stem Cell & Tissue Engineering Research Group, Dept. Pathology & Medical Biology, University Medical Center Groningen, University of Groningen, The Netherlands

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CHAPTER 4

ABSTRACT

The discovery of the endothelial progenitor cell (EPC) has led to an intensive research effort into progenitor cell-based tissue engineering of (small-diameter) blood vessels. Herein, EPC are differentiated to vascular endothelial cells and serve as the inner lining of bioartificial vessels. As yet, a reliable source of vascular smooth muscle progenitor cells has not been identified. Currently, smooth muscle cells (SMC) are obtained from vascular tissue biopsies and introduce new vascular pathologies to the patient. However, since SMC are mesenchymal cells, endothelial-to-mesenchymal transdifferentiation (EnMT) may be a novel source of SMC. Here we describe the differentiation of smooth muscle-like cells through EnMT. Human umbilical cord endothelial cells were cultured either under conditions favoring endothelial cell growth or under conditions favoring mesenchymal differentiation (TGFβ and PDGF-BB). Expression of smooth muscle protein 22α and α-smooth muscle actin was induced in HUVEC cultured in mesenchymal differentiation media, whereas hardly any expression of these markers was found on genuine HUVEC. Transdifferentiated endothelial cells lost the ability to prevent thrombin formation in an in vitro coagulation assay, had increased migratory capacity towards PDGF-BB and gained contractile behavior similar to genuine vascular smooth muscle cells. Furthermore, we showed that EnMT can be induced in three dimensional collagen sponges. In conclusion, we show that HUVEC can efficiently transdifferentiate into smooth muscle-like cells through endothelial-to-mesenchymal transdifferentiation. Therefore, EnMT may be used in future progenitor cell-based vascular tissue engineering approaches to obtain vascular smooth muscle cells, and circumvent a number of limitations encountered in current vascular tissue engineering strategies.
INTRODUCTION

Tissue engineering of small-diameter blood vessels aims at creating (autologous) replacement vessels for use in, amongst others, vascular replacement surgery. To date, tissue engineering strategies have been based on the combination of biomaterials and culture expanded (autologous) vascular endothelial- and smooth muscle cells obtained from biopsies [1-3]. However, isolation of autologous vascular cells is limited due to several reasons which include poor vessel quality, restricted proliferative capacity of harvested cells and technical difficulties to acquire pure populations of vascular cells. Furthermore, by using vascular biopsies, vascular tissue engineering has been a process which attempts to heal a vascular pathology by creating a new trauma.

In the past decade, the discovery of endothelial progenitor cells has paved the way for novel tissue engineering strategies, amongst which is progenitor cell-based tissue engineering (reviewed in [4;5]). Herein, circulating progenitor cells are isolated from adult autologous peripheral blood and differentiated into endothelial cells in vitro. These cultured endothelial progenitor cells are subsequently used to engineer an autologous replacement vessel [6-9]. Although promising and feasible, progenitor cell-based tissue engineering strategies have been focused on combining biomaterials and endothelial progenitor cells and thereby neglected the necessity of smooth muscle cells in the engineered vessel. As a consequence, engineered vessels comprising of only biomaterials and endothelial (progenitor) cells have a reduced contractility compared to the native vessels and may lack the strength required to withstand physiological stresses. At present, only the tissue engineering strategies relying on grafting of donor tissue (e.g. vena saphena magna) have been able to incorporate autologous smooth muscle cells in the engineered vessel and thereby retain sufficient mechanical strength in the engineered vessel to withstand physiological stresses [2-3;10].

Although some groups have described the presence of smooth muscle progenitor cells in the peripheral blood [11-14], we were unable to obtain vascular smooth muscle cell outgrowth from adult peripheral blood mononuclear cells by published protocols (unpublished data). However, transdifferentiation of endothelial (progenitor) cells into smooth muscle cells may be a novel source of functional smooth muscle cells for tissue engineering applications. Transdifferentiation of endothelial cells into smooth muscle cells (EnMT) occurs during embryogenesis where EnMT is part of heart valve formation [15-17]. We hypothesized that the transdifferentiation of endothelial cells into cells with a mesenchymal phenotype can be used to obtain smooth muscle-like cells that could serve a role in future progenitor cell-based blood vessel engineering strategies. Herein, both the vascular endothelial cells and vascular smooth muscle cells are derived from a single pool of vascular progenitor cells and may circumvent limitations encountered in current vascular tissue engineering approaches.

As proof-of-concept, we assessed the possibility to use endothelial-to-mesenchymal transdifferentiation (EnMT) as a source for mesenchymal cells with a smooth muscle-like phenotype. Human umbilical vein endothelial cells (HUVEC) were cultured under conditions that favor mesenchymal cell (trans-)differentiation. Phenotypical, as well as gene transcript analyses were used in order to characterize the mesenchymal cell differentiation. Subsequently, migration capacity and collagen gel contraction were assessed in order to show smooth muscle functionality. Furthermore, we attempted
to induce EnMT in three-dimensional collagen matrices as a prerequisite in the use of EnMT for vascular tissue engineering of blood vessels.

MATERIALS AND METHODS

CELL ISOLATION AND CULTURE

Human umbilical cord endothelial cells (HUVEC) were obtained through Prof. dr. G. Molema (Dept. Pathology and Medical Biology, UMC Groningen, The Netherlands). HUVEC were cultured in gelatin-coated culture flasks (25 cm²) in endothelial cell medium (ECM) until they reached confluence (ca. 40 000 HUVEC/cm²). ECM was comprised of RPMI 1640 (Cambrex, NJ) supplemented with 20% Fetal Bovine Serum (FBS; Gibco/Invitrogen, CA), 2 mM L-Glutamine (Sigma, MO), 1% Penicillin/Streptomycin (Sigma, MO), 5 U/mL Heparin (Leo Pharma, The Netherlands) and 5 μg/mL endothelial cell growth factors (ECGF; own isolate according to Burgess et al. [18]). Confluent HUVEC were dissociated using Accutase-solution (PAA Laboratories GmbH, Germany), and reseeded in either gelatin-coated culture plates at 35 000 HUVEC/cm² or seeded in three-dimensional collagen sponges (OptiMaix®, MatriCel GmbH, Germany). HUVEC were subsequently cultured in either endothelial cell growth medium (ECM) or in mesenchymal differentiation medium (MDM) with or without additional bFGF (10 ng/mL; PeproTech Inc., NJ). MDM was comprised of RPMI 1640 supplemented with 20% FBS, 2 mM L-Glutamine, 1% Penicillin/Streptomycin, 5 ng/mL TGFβ and 25 ng/mL PDGF-BB (both PeproTech Inc., NJ). Cells were cultured for an additional 5-21 days.

ANALYSIS OF CELL ADHESION, NUMBERS AND GROWTH

After 1, 3, 5, 14 and 21 days in culture, the number of cells in culture were counted. Non-adherent and dead cells were removed by repeated washing with PBS. Remaining adherent cells were fixed using methanol:acetone (1:1) solution at 4°C for 20 min. Subsequently, the nuclei were stained using 3 μM DAPI (4',6-diamidino-2-phenylindole; Sigma, MO). Stained nuclei were counted using a Leica DMRXA immunofluorescent microscope and Leica software (Leica Microsystems, Germany).

GENE TRANSCRIPT ANALYSIS

Total RNA was isolated from the original (day 0) HUVEC cultures and from the cells cultured in the different differentiation media cultured for 21 days. RNA isolation was performed using the RNeasy Midi Kit (Qiagen Inc., CA) according to manufacturer’s protocol. Subsequently, 1 μg of total RNA was reverse transcribed using the FirstStrand cDNA synthesis kit (Fermentas UAB, Lithuania) according to manufacturer’s instructions. One micro liter cDNA was used for amplification. RT-PCR for CD31 (sense 5'-GTGAGGGTCAACTGTTCTGT-3', antisense 5'-GTGACCACTTACCTTTGATT-3'), VE-Cadherin (sense 5'-GTTCACCTTTCTCGAGGATA-3', antisense 5'-GTAGCTGTTGTGTTCCATCT-3'), eNOS (sense 5'-CATGTTGGTGTTCCATCT-3').

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GATG-3', antisense 5'-AAAGCTCTGGTGCCGTATGC-3'), vWF (sense 5'-GGCAGCTGT TCTTATGTCTT-3', antisense 5'-TGGAACTCATTGTTTTTGG-3'), smoothelin (sense 5'-AGGGCGAGAAAGAAAGAG-3', antisense 5'-TCCTCTGTATCCAGAGCTG-3'), SM22α (sense 5'-AACAGCTGTACCCGTAGTG-3', antisense 5'-ATGACATGCTTTCCCTCCTG-3'), αSMA (sense 5'-TCATCCTCCCTGAGAAGAG-3', antisense 5'-ATGCCAGGCTACATAG GTGT-3') and GAPDH (sense 5'-CTGCGCTAGAAAACCTG-3', antisense 5'-GTCCAGGG GTCTTACTCCTT-3'; all Biolegio, The Netherlands) were performed in a final reaction volume of 25 μL under the following reaction conditions of 0.25 mM dNTP mix, 1.5 mM MgCl₂, 1 μM primer-mix, and 1U Taq DNA Polymerase. Amplification was performed on a MyCycler (Bio-Rad, CA) in 96-well plates (Greiner Bio One, the Netherlands) for 30 cycles. Amplimers were separated by gel electrophoresis in a 2% agarose gel.

PROTEIN EXPRESSION ANALYSIS

Before the start, and after 21 days of cell culture, samples were isolated for immunofluorescent staining. Cells were fixed using paraformaldehyde (2% in PBS) at room temperature for 20 min. Fixed cells were washed extensively, rehydrated with PBS and permeabilized using 0.5% Triton X-100 in PBS (Sigma, CA). Subsequently, samples were incubated with primary antibody cocktails diluted in PBS with 10% donkey serum (Jackson ImmunoResearch, UK) for 1 hour. Primary antibody cocktails consisted of either (1) mouse monoclonal antibodies to human (MaH) CD31 (1:50; Southern Biotech, AL) and rabbit polyclonal antibodies to human (RaH) von Willebrand Factor (1:200; DakoCytomation, Denmark), or (2) MaH CD144 (1:50; R&D Systems, MN) and RaH eNOS (1:50; BD Biosciences, CA), or (3) MaH Smoothelin (1:100) and RaH SM22α (1:100; both ABCAM PLC, UK), or (4) MaH α-Smooth Muscle Actin (1:100; DakoCytomation, Denmark). Samples were washed extensively and incubated in secondary antibody cocktail diluted in 3 μM DAPI/PBS with 10% human pooled serum. Secondary antibody cocktail consisted of RedX-conjugated donkey F(ab')₂ antibody fragments to mouse IgG and Fluorescein-conjugated donkey F(ab'), antibody fragments to rabbit IgG (both 1:100; Jackson ImmunoResearch, UK). Stained samples were mounted in Citifluor (Agar Scientific, UK) and visualized using a Leica DMRXA Immunofluorescent microscope and Leica software (Leica Microsystems, Germany). In some cases, cells were dissociated before the staining and analyzed by FACS analysis (BD FACScan, CA).

THROMBIN GENERATION ASSAY

To analyze the antithrombogenic properties of endothelial- and transdifferentiated smooth muscle cells, modified thrombin generation assays (HaemoScan, The Netherlands) were performed. Cells were removed from culture by accutase treatments and reseeded at a density of 35 000 cells/cm². After 24 hours, cells were incubated with fibrinogen-depleted citrate plasma for 15 min. During this period, the intrinsic coagulation cascade is activated, resulting in the generation of factor Xla and Xla. The absence of free calcium prevents further activation of the cascade. Next, 30 mM CaCl₂ and phospholipids were added and incubated at 37°C for 1 min., during which the clotting cascade be comes activated resulting in the generation of thrombin. At regular intervals, samples were drawn from the incubation mixture and added to...
ice-cold 25 mM Tris-HCl to stop any further thrombin formation. Finally, the diluted samples were incubated with 3 mM Thrombin substrate S<sub>2238</sub>. Thrombin releases p-nitroaniline chromophore from the substrate. The change in color was measured at 405 nm with 540 nm as reference wavelength in a microtiter plate reader (BioRad, VA). A calibration curve of thrombin was used to determine thrombin formation induced by the cultured cells [19].

**PDGF-BB MIGRATION ANALYSIS**

Migratory capacity towards smooth muscle cell chemoattractant PDGF-BB was measured using an AP48-chemotaxis chamber with 8 μm pore size filters (Neuro Probe, MD). A concentration of 1 nM PDGF-BB (Peprotech Inc., NJ) proved optimal for migration assays and used in further experiments. The lower chambers contained RPMI 1640 (Cambrex, NJ) supplemented with PDGF-BB or RPMI 1640 only, 25 000 cultured endothelial cells or transdifferentiated cells were placed in the upper chamber. After incubation at 37°C for 90 min, migrated cells were fixed and stained with Diff-Quik (Medion Diagnostics, Switzerland). Cells were counted manually in ten high power fields (40x objective magnification) per sample.

**COLLAGEN TYPE I GEL CONTRACTION EXPERIMENTS**

Cell culture in three-dimensional collagen gels was performed using a solution of PureCol (Inamed Biomaterials, CA). Cultured cells were dissociated using accutase-solution. Subsequently, cells were resuspended in culture medium at a concentration of 3.0×10<sup>5</sup> cells/mL. Cell suspensions were added to a solution of bovine type I collagen (PureCol®; Nutacon, The Netherlands) containing 1.5 mg/mL NaHCO<sub>3</sub> and 25 mM HEPES. Aliquots of 0.1 mL (containing 50 000 cells and 0.25 mg Collagen type I) were added to 3.5 cm dishes and allowed to solidify at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 1 hour. The collagen gels were released from the dishes with a spatula and by pipetting medium at the gel-dish interface. Released gels were imaged using a common Flatbed-scanner (ScanJet 5370C; HP, CA) and allowed to contract for an additional 24 hours. The degree of gel contraction was determined by measuring the total gel area and dividing the areas of the contracted gels by the areas of the gels before contraction.

**THREE-DIMENSIONAL COLLAGEN MATRIX EXPERIMENTS**

To assay the feasibility of EnMT within current tissue engineering strategies, HUVEC were labeled with CM-Dil (Molecular Probes, OR) according to manufacturers’ protocol and approximately 500 000 HUVEC were seeded into three-dimensional Optimaix<sup>®</sup> porcine collagen type I sponges (Ø 5 mm x 3 mm height; MatriCel, Germany). HUVEC were cultured in either ECM or MDM for 21 days. After 21 days of culturing, sponges were analyzed by transmission electron microscopy and immunofluorescent staining.
IMMUNOFLUORESCENCE MICROSCOPY

Collagen type I sponges were snap frozen in liquid nitrogen. Sections (7 μm) were cut, fixed with acetone and pre-incubated with Image-iT™ FX signal enhancer (Molecular Probes, OR) for 30 min. Next, samples were incubated with 0.1 μM Fluorescein-conjugated Phalloidin (Molecular Probes, OR) in PBS for 30 min., washed with PBS and mounted in Citifluor (Agar Scientific, UK). Stained samples were visualized using a Leica DMRXA Immunofluorescent microscope and Leica software (Leica Microsystems, Germany).

TRANSMISSION ELECTRON MICROSCOPY

Collagen type I sponges were fixed using 2% Glutar Aldehyde (Merck, Germany) in 0.1 M phosphate buffer at 4°C for 24 hours, and prepared for morphological analysis using transmission electron microscopy. In short, fixed samples were rinsed with a 6.8% sucrose solution (pH 7.4) for 30 minutes. Samples were post fixed using a 0.1M potassium hexacyanoferrate(II)trihidrate (K₄Fe(CN)₆•3 H₂O; Merck, Germany) solution containing 1% osmiumtetroxide (OsO₄) at 4°C for 45 minutes. Then, the samples were dehydrated with ethanol, embedded in EPON 812 (Serva Feinbiochemica, Germany), and polymerized at 60°C for at least 48 hours. After polymerization, ultrathin sections were cut on a Sorvall microtome (Sorvall, CT) and contrasted with uranyl acetate and lead citrate. The ultrathin sections were analyzed in a Phillips 201 transmission electron microscope (Phillips, The Netherlands) operated at 60 kV.

STATISTICAL ANALYSIS

Data is expressed as mean ± standard error of the mean. Data in Gaussian distribution was analyzed using one-way ANOVA followed by post hoc Newman-Keuls multiple comparison test or two-way ANOVA followed by Bonferroni post hoc analysis. All other data was analyzed using the Kruskal-Wallis test followed by Dunns post hoc analysis. Probabilities of P < 0.05 were considered to be statistically significant.

RESULTS

MORPHOLOGICAL ANALYSIS, CELL ADHERENCE AND GROWTH

HUVEC were seeded at a density of 35 000 cells/cm² and cultured under conditions favoring either endothelial cell growth (ECM) or mesenchymal cell differentiation (MDM) with or without added bFGF. Cell seeding efficiency, i.e. the fraction of adherent cells within 24h, was 68 – 79% for all media (data not shown). Cells cultured in endothelial cell medium (ECM) displayed proliferative behavior and reached confluence after five days of culturing. These ECM cultures were maintained under confluence for the remaining culture period of 21 days wherein no morphological changes were observed.
Figure 1. Morphological analysis and growth curves of transdifferentiating smooth muscle cells. HUVEC were cultured either in endothelial cell culture medium (B-E) or in media favoring mesenchymal cell differentiation with (F-I) or without added bFGF (J-M). Vascular smooth muscle cells (VSMC) were cultured as controls (N-Q). HUVEC cultured in MDM lost their cobblestone morphology within the first week (G and K). In the following two weeks, cells in MDM started to show signs of hypertrophy (H and L) and eventually showed some hill and valley morphology (I and M), which is characteristic for VSMC (P and Q). Islands of cells displaying the cobblestone morphology remained in the MDM culture with added bFGF (M). Analysis of cell number was performed using DAPI-labeling (R). HUVEC cultured in ECM (open squares) reached confluence after 5 days in culture and remained confluent throughout the 21-day culture period. The cells in MDM (open and closed circles) initially showed a decrease in cell number, but the cell number started to increase after 14 days in culture (S). HUVEC = Human Umbilical Vein Endothelial Cells. VSMC = vascular smooth muscle cells. ECM = endothelial cell growth medium. MDM = mesenchymal cell differentiation medium.
Cells cultured in MDM displayed a different growth pattern; no proliferation was observed during the first 14 days of culture (Figures 1F-H & J-L). In contrast, in MDM high mortality was observed resulting in a decrease in cell numbers (Figure 1S). The decrease in cell number reached its maximum at day 14 of culture where confluence-levels were between 8 – 12% for transdifferentiating cells cultured in MDM in the absence of bFGF (Figure 1H) and between 19 – 49% for transdifferentiating cells cultured in MDM in the presence of bFGF (Figure 1L). After this period, the cell numbers increased in both MDM cell cultures, reaching densities of 11 000-25 000 cells/cm² or 32-75% confluence (Figure 1S). In MDM cultures, cells lost their cobblestone morphology within the first week of culture (Figures 1G & K), started to align and started to show signs of hypertrophy (Figures 1H & L). Cobblestone-shaped areas (i.e. cells with endothelial-like morphology) disappeared completely in MDM cultures without added bFGF (Figures 1F-I), whereas in MDM cell cultures with added bFGF (Figures 1J-M) islands of cobblestone-shaped cells remained. After three weeks of culture, ECM cultures still displayed cobblestone morphology (Figure 1E), while cells in MDM cultures started to display classical hill-and-valley morphology (Figures 1I & M) which is typical for genuine vascular smooth muscle cell cultures (Figures 1N-Q).

EXPRESSION OF ENDOTHELIAL- AND SMOOTH MUSCLE CELL MARKER PROTEINS

Expression of endothelial- as well as smooth muscle cell marker molecules was assessed on gene transcript and protein expression level. Transcriptionally, vascular smooth muscle cells did not express any of the endothelial cell marker genes (Figure 2A1), whereas low passaged HUVEC (P1-2) expressed smoothelin (SMTH) and alpha-smooth muscle actin (αSMA; Figure 2A2). HUVEC cultured in media favoring mesenchymal differentiation (MDM), however, increased the gene expression of these markers and started to express smooth muscle protein 22α (SM22α) at high levels (Figure 2A2). In contrast, no differences in endothelial cell marker expression could be found in the MDM cell cultures compared to the ECM cultures. Remarkably, in ECM cultures, cells also started to express the SM22α gene, albeit at lower levels than observed in the MDM cultures.

On the level of protein expression the results were more distinct. Native HUVEC showed no protein expression of smooth muscle cell marker SM22α and only 0.1% of HUVEC had detectable protein expression levels of αSMA (data not shown). Remarkably, nearly all HUVEC stained positive for the smoothelin protein (data not shown). HUVEC cultured in ECM at 100% confluence for three weeks, started to express some of the smooth muscle cell markers, although at low levels. In these ECM cultures, protein expression of SM22α was found on 2.75 ± 0.56% of cells and expression of the αSMA protein was found on 9.19 ± 4.32% of cells. HUVEC cultured in MDM partly lost the expression of endothelial marker proteins von Willebrand Factor (p < 0.001; Figures 2B1-5), CD144 (p < 0.001), and eNOS (p < 0.01; Figures 2C1-5), while expression of smooth muscle cell marker proteins SM22α (p < 0.001; Figures 2D1-5) and αSMA (p < 0.001; Figures 2E1-4) were induced. In the MDM cultures with added bFGF, 40-60% of the HUVEC transdifferentiated into a smooth muscle cell-like phenotype. In the MDM cultures without added bFGF, transdifferentiation seemed more pronounced, however, the number of transdifferentiated cells did not differ significantly between
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A1

CD31

vWF

VE-Cadherin

eNOS

Smoothelin

A2

SM22α

αSMA

GAPDH

100 bp

HUVEC

VSMC

B1

HUVEC + ECM

B2

+ MDM (-bFGF)

B3

+ MDM (+bFGF)

C1

D1

C2

D2

C3

D3

E1

E2

E3

0.0%

HUVEC + ECM

+ MDM (-bFGF)

+ MDM (+bFGF)

VSMC

VSMC

VSMC

% Positive Cells

CD31

VE-Cadherin

Smoothelin

αSMA

Smooth Muscle Actin

vWf

Endothelial Nitric Oxide Synthase

Smooth Muscle Protein 22α

von Willebrand Factor
the two MDM cultures. Strikingly, the expression of endothelial cell marker protein CD31 and smooth muscle cell marker protein smoothelin was present in freshly isolated HUVEC as well as on all MDM cultures. Expression of these markers did not vary between cultures (Figures 2B1-4 & D1-4).

FUNCTIONAL ANALYSIS OF SMOOTH MUSCLE PHENOTYPES

Functionality of cells with endothelial- and smooth muscle cell phenotypes was assayed by analysis of factor-3 dependent thrombin generation, by migration on a PDGF-BB gradient and by collagen gel contraction assays. Factor-3 dependent thrombin generation by cells in ECM reached a maximum of 200 mU/mL, indicating that HUVEC were actively repressing the extrinsic coagulation cascade. Cells in both MDM cultures lost their ability to repress factor-3 dependent thrombin generation (p < 0.05; Figure 3A). Maximum thrombin generation in the MDM cultures reached between 350-425 mU/mL and was similar to thrombin generation by genuine vascular smooth muscle cells.

Vascular smooth muscle cells showed higher migratory rates towards a PDGF-BB gradient than did HUVEC in ECM towards that same gradient (p < 0.001; Figure 3B). Compared to ECM cultures, cells cultured in MDM also showed higher migratory activity towards PDGF-BB (p < 0.05). However, migratory activity of cells in MDM did not reach the levels of migration portrayed by genuine vascular smooth muscle cells (p < 0.001). Migration towards a PDGF-BB gradient by cells in the MDM cultures was not influenced by the presence of bFGF (p > 0.10; MDM (-bFGF) versus MDM (+bFGF)).

Contractile behavior is a key feature of vascular smooth muscle cells. Therefore, we analyzed the contractile capacity of HUVEC that had undergone transdifferentiation in a collagen gel assay. Approximately 50 000 cells were embedded in solidified collagen type 1 and the gel area was measured immediately after solidification and at 24 hours (Figures 3C1 & 2). HUVEC in ECM were unable to contract the gel area during the 24 hour period, however, HUVEC cultured in MDM significantly reduced the total gel area by more than 50% (p < 0.001; Figure 3C). Contraction of collagen type 1 gels by transdifferentiated cells (MDM) did not differ from contraction by genuine vascular

Figure 2. Gene transcript and protein expression analysis of transdifferentiating cells. After 21 days in culture, gene transcript analysis was performed for endothelial cell specific genes (A1) as well as for vascular smooth muscle cell (VSMC) specific genes (A2). Freshly isolated HUVEC express all endothelial cell specific genes as well as the smooth muscle genes SMTH and αSMA. Vascular smooth muscle cells only expressed the smooth muscle specific genes. HUVEC in MDM cultures increased expression of SMTH and αSMA and gene expression of SM22α was induced (A2). On protein level, HUVEC in ECM portrayed co-expression of endothelial cell markers CD31 (red) and vWF (green; B1) and CD144 (red) and eNOS (green; C1). Expression of smooth muscle cell markers SM22α (green; D1) and αSMA (green; E1) was almost never observed. Strikingly, all cells in ECM culture had protein expression of SMTH (red, D1). In MDM without additional bFGF, cells lost expression of endothelial cell markers CD31, vWF (red and green respectively; B2), CD144 and eNOS (red and green respectively; C2) and gained protein expression of SM22α (green, D2) and αSMA (E2). The addition of bFGF to the MDM cultures (B3, C3, D3 and E3) had no effect on protein expression levels. Protein expression was quantified by flowcytometric analysis (B4, B5, C4, C5, D4 and E4). HUVEC = Human Umbilical Vein Endothelial Cells. VSMC = vascular smooth muscle cell. SMTH = smoothelin. αSMA = alpha-smooth muscle actin. ECM = endothelial cell growth medium. MDM = mesenchymal cell differentiation medium. vWF = von Willebrand Factor. eNOS = endothelial Nitrix Oxide Synthase. ** = P < 0.01 versus ECM. *** = P < 0.001 versus ECM.
smooth muscle cells. There was no difference between the contractile properties of cells in MDM cultured in either the presence or absence of bFGF.

THREE DIMENSIONAL COLLAGEN TYPE I SPONGES

HUVECs were seeded into three-dimensional collagen type I sponges and cultured statically in endothelial cell growth medium (ECM) or in medium favoring mesenchymal cell differentiation (MDM). Before cell seeding, HUVECs were labeled with a fluorescent Dil-probe in order to visualize cells in these cultures. Cells adhered readily to the collagen type I sponges and spread throughout the sponges. There was a heterogeneous distribution of cells observed at the start of culture (not shown), which did not change throughout the culture period (Figure 4A-C).

After three weeks in culture, the sponges were fixed, sectioned and stained using f-actin binding phallotoxins. HUVECs in sponges which were cultured in ECM showed a diffuse staining pattern (Figure 4D) indicating the absence of oriented bundles of f-actin. In contrast, in the MDM samples, bundles of f-actin were clearly visible,
indicating that transdifferentiation had occurred in the collagen sponges. There was no difference found in f-actin polymerization between the MDM cultures without additional bFGF (Figure 4E) and the MDM cultures with additional bFGF (Figure 4F).

To visualize cytoplasmic structure and cell matrix interactions, transmission electron microscopy was employed. HUVEC cultured in ECM showed elongated morphology and aligned the collagen bundles (Figure 4G). In the MDM cultures, cells had an intermediate (Figure 4H) or smooth muscle cell (Figure 4I) morphology. These cells were closely associated with the collagen bundles and showed tight nuclear folds, suggestive of cellular contraction. Transdifferentiated cells with a smooth muscle cell phenotype adhered to the collagen bundles through hemidesmosomes (Figure 4J) and to other cells through tight junctions (Figure 4K). The cytoplasms of the cells were filled with small actin filaments (Figure 4L) and stress fibers (Figure 4M), all concurrent with the vascular smooth muscle phenotype.

**DISCUSSION**

In the current study, we investigated the possibility of transdifferentiating endothelial cells into smooth muscle-like cells for future use in tissue engineering of bioartificial blood vessels. We show that transdifferentiated neonatal endothelial cells (HUVEC) expressed smooth muscle markers like smooth muscle cell protein 22α (SM22α) and α-smooth muscle actin (αSMA), had an increased migratory capacity towards PDGF-BB and could contract collagen type I gels. This is all consistent with the phenotype of genuine vascular smooth muscle cells. Furthermore, we investigated the possibility to perform endothelial-to-mesenchymal transdifferentiation in 3D scaffolds. Similarly to the two-dimensional cultures, HUVEC grown in three-dimensional scaffolds underwent endothelial-to-mesenchymal transdifferentiation (EnMT) and formed smooth muscle-like cells.

Vascular tissue engineering aims at generating (small-diameter) blood vessels for replacement surgery by combining biomaterials with vascular cells. To this end, vascular endothelial cells are cultured in the lumen of a tubular scaffold to form an anticoagulant layer, while vascular smooth muscle cells are subsequently cultured in the surrounding scaffold. The smooth muscle cells provide the tissue engineered vessel with contractile behavior [2;20;21]. However, the use of autologous vascular cells is limited due to several reasons which include poor vessel quality, restricted proliferative capacity of harvested cells and technical difficulties to acquire pure populations of vascular cells. It would be desirable to employ one type of vascular progenitor cell for the differentiation of both endothelial cells and smooth muscle cells. Endothelial cells, in this respect, have been described to transdifferentiate into smooth muscle cells through a process called EnMT [22;23]. In future clinical perspective, autologous circulating endothelial progenitor cells can be isolated from patients, culture expanded and (trans)differentiated into endothelial cells and smooth muscle cells on and in tubular biomaterials respectively. This in order to tissue engineer an autologous bioartificial blood vessel.
As proof-of-concept, we show that neonatal human umbilical vein endothelial cells (HUVEC) can transdifferentiate into smooth muscle-like cells in 3D collagen matrices. Culture of HUVEC under conditions that favor mesenchymal transdifferentiation resulted in clear morphological changes, seen as the loss of cobblestone morphology, hyperplasia, and cell spreading and had the functional characteristics of genuine vascular smooth muscle cells. The expression of smooth muscle cell markers SM22α and αSMA was induced at both gene transcript and protein expression level.
Furthermore, expression of endothelial cell markers VE-Cadherin, eNOS and vWF was partly lost. Additionally, transdifferentiated cells displayed contractile properties, similar to genuine vascular smooth muscle cells.

The presence of smooth muscle cell gene transcripts already in low passaged (P1-2) HUVEC (Figure 2) indicates that HUVEC are intrinsically prone to EnMT. We initiated EnMT by replacing the VEGFa and bFGF in the endothelial growth medium by TGFβ1 and PDGF-BB. TGFβ1 stimulation caused the selection of those cells that are able to transdifferentiate into smooth muscle-like cells and thereafter acts as a mitogen on those smooth muscle(-like) cells [24]. This results in a vital and proliferating layer of smooth muscle-like cells in the third week of culture (Figure 1). In nonresponding cells, apoptosis was likely induced by TGFβ1, as was seen as a decay in cell numbers (Figure 1S) and reported previously [25;26].

On the other hand, the presence of genuine smooth muscle cells in the original HUVEC cultures could also explain the observed smooth muscle cell gene transcripts. However, FACS analysis for by HUVEC showed that only 0.1% of the cells had detectable aSMA protein expression (data not shown). Furthermore, HUVEC-derived smooth muscle-like cells did not change their CD31 protein expression, which distinguishes them from genuine vascular smooth muscle cells that lack CD31 expression. Since all the smooth muscle-like cells in our cultures displayed the CD31⁺aSMA⁺SM22α⁺ and CD144⁺vWF⁺eNOS⁺ phenotype (Figure 2), we conclude that the presence of smooth muscle cells in the original HUVEC cultures could not be the source of smooth muscle cells within our cultures.

EnMT is an intrinsic part of heart valve formation and can be initiated by multiple growth factors and cytokines. In this context, Paranya et al. reported that transdifferentiation of valve endothelial cells into smooth muscle cells occurred through TGFβ₁-mediated and non-TGFβ₁-mediated mechanisms [16]. Ishisaki et al. reported that TGFβ₁-independent EnMT occurs through increased activin A expression as a result of an absence in bFGF-signaling, which coincided with increased expression of SM22α. Expression of both activin A and SM22α were reduced upon addition of bFGF to the culture medium. In their study, the ability of bFGF to inhibit EnMT was analyzed [27]. Although the presence bFGF inhibited the SM22α expression in the studies of Ishisaki et al., the expression of smooth muscle cell markers SM22α and aSMA in the mesenchymal transdifferentiation cultures was not affected by bFGF in our study. We therefore surmise that the EnMT of HUVEC depends primarily on the effects of TGFβ₁.

Contractility of blood vessels is in large part dependent on the behavior of vascular smooth muscle cells. We showed that smooth muscle-like cells, obtained through endothelial-to-mesenchymal transdifferentiation, had similar contractile behavior as genuine vascular smooth muscle cells in an in vitro collagen gel contraction assay. Undifferentiated endothelial cells (HUVEC) did not show contractile behavior in these assays at all. Consistent with the vascular smooth muscle cell phenotype, we furthermore showed that, compared to native HUVEC, transdifferentiated HUVEC had an increased migratory capacity towards PDGF-BB and lost the ability to inhibit factor-3 dependent thrombin formation, an endothelial cell property (Figure 3). Therefore, we conclude that the transdifferentiation process caused a shift in functional behavior, changing from endothelial cell properties to smooth muscle cell properties.
Vascular tissue engineering utilizes (instructive) 3D biomaterials as temporal structural supports and to direct cell differentiation. We therefore investigated if EnMT can be induced in 3D cell cultures. For this purpose, HUVEC were seeded into 3D collagen type I sponges and cultured in media favoring either endothelial cell growth or mesenchymal cell differentiation. Consistent with the two-dimensional cell cultured, neonatal endothelial cells underwent EnMT in these 3D collagen sponges. We show that HUVEC seeded into these sponges aligned with the collagen bundles and formed strong cell-matrix interactions through the formation of hemidesmosomes. Nuclear folding and the formation of stress fibers and orientated actin bundles were both signs of cellular contraction in the 3D matrix. In contrast, HUVEC grown in endothelial

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Figure 5. Progenitor cell-based vascular tissue engineering. Schematic representation of a progenitor cell-based tissue engineering approach; (1) Autologous mononuclear (MNC) cells can be isolated from the peripheral blood of patients. (2) Cell culture of MNC in media favoring endothelial progenitor cell (EPC) outgrowth (VEGF, bFGF and HGF) causes the selection of EPC and subsequent culture expansion. (3) EPC are subsequently seeded into instructive tubular scaffolds which contain, on the luminal side growth factors that instruct endothelial cell differentiation and proliferation (VEGF, bFGF and HGF) and in the surrounding scaffold induce endothelial-to-mesenchymal transdifferentiation (TGFβ1 and PDGF-BB). (4) After maturation in a pulsatile perfusion bioreactor an autologous bioartificial tissue engineered blood vessel is produced.
growth medium did not show any of these characteristics and appeared as elongated cells aligning the collagen bundles of the 3D sponge (Figure 4). Taken together, these findings show that EnMT is possible in 3D scaffolds and can thus be used in vascular tissue engineering.

CONCLUSIONS

We here show that HUVEC have the intrinsic capacity to transdifferentiate into smooth muscle-like cells which are functionally indistinguishable from genuine vascular smooth muscle cells. Furthermore, we show that EnMT can be induced in three-dimensional scaffolds. The ability to obtain smooth muscle cells from transdifferentiated endothelial cells may have great implications in the field of vascular tissue engineering for it may solve current limitations encountered in vascular tissue engineering strategies using autologous vascular cells and pave the way for novel tissue engineering strategies \textit{i.e.} progenitor cell-based vascular tissue engineering.
REFERENCES


