Research Article

Human Vascular Model with Defined Stimulation Medium – A Characterization Study

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Summary

The formation of blood vessels is a vital process in embryonic development and in normal physiology. Current vascular modelling is mainly based on animal biology leading to species-to-species variation when extrapolating the results to humans. Although there are a few human cell based vascular models available, these assays are insufficiently characterized in terms of culture conditions and developmental stage of vascular structures. Therefore, well characterized vascular models with human relevance are needed for basic research, embryotoxicity testing, development of therapeutic strategies and for tissue engineering.

We have previously shown that the *in vitro* vascular model based on co-culture of human adipose stromal cells (hASC) and human umbilical vein endothelial cells (HUVEC) is able to induce an extensive vascular-like network with high reproducibility. In this work we developed a defined serum-free vascular stimulation medium (VSM) and performed further characterization in terms of cell identity, maturation and structure to obtain a thoroughly characterized *in vitro* vascular model to replace or reduce corresponding animal experiments.

The results showed that the novel vascular stimulation medium induced an intact and evenly distributed vascular-like network with morphology of mature vessels. Electron microscopic analysis assured the three-dimensional microstructure of the network containing lumen. Additionally, elevated expression levels of the main human angiogenesis-related genes were detected.

In conclusion, with the newly defined medium the vascular model can be utilized as a characterized test system for chemical testing as well as in creating vascularized tissue models.

Keywords: serum-free media, angiogenesis, mesenchymal stromal cells, coculture techniques

1 Introduction[#]

The formation of the blood vessel network is a vital process in growth and organ development (Carmeliet and Jain, 2011; Carmeliet, 2005). In the embryo, endothelial precursor cells form new vessels that differentiate into a primitive vascular network (vasculogenesis) (Carmeliet and Jain, 2011). Subsequent vessel sprouting (angiogenesis) creates a network of arteries and veins as well as capillaries that facilitate the exchange of gases and metabolites (Carmeliet and Jain, 2011; Adams and Alitalo, 2007). To reach this level of complex organization, the immature vascular network must mature at the level of the vessel wall

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[#]Abbreviations

AA, ascorbic acid; APC, allophycocyanin; BSA, bovine serum albumin; CD144, vascular endothelial cadherin; EGF, epidermal growth factor; EGM-2, endothelial cell growth medium-2; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FGF-2, basic fibroblast growth factor; hASC, human adipose stromal cells; HE, heparin sodium salt; HS, human serum; HUVEC, human umbilical vein endothelial cells; HY, hydrocortisone (cortisol); IGF-I, insulin-like growth factor I; PBS, phosphate buffered saline; RT, room temperature; PDGFRβ, platelet derived growth factor beta; PE, phycoerythrin; PE-CY7, phycoerythrin-cyanine; SFM, basal serum-free medium; αSMA, alpha smooth muscle actin; TRITC, tetramethyl rhodamine isothiocyanate; VEGF, vascular endothelial growth factor; VSM, vascular stimulation medium; vWf, von Willebrand factor

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morphology and as a whole network. Morphological maturation involves recruitment of mural cells, deposition of extracellular matrix and organ-specific specialization of cells, such as inter-endothelial junctions and surface receptors. Maturation of the network involves branching and expanding the network to meet local, tissue-specific demands (Jain, 2003).

Since the formation and maintenance of the vascular network is a complex process, problems related to its regulation are common (Ucuzian and Greisler, 2007). Inadequate vessel maintenance or growth causes ischemia in myocardial infarction and neurodegenerative or obesity-associated disorders, whereas excessive vascular growth or abnormal remodeling promotes cancer, inflammatory disorders and eye diseases (Potente et al., 2011). Moreover, genetic studies have shown that perturbing embryonic vascular development can have adverse consequences from benign vascular malformation to embryolethality and congenital defects (Knudsen and Kleinstreuer, 2011).

Angiogenesis models are important tools for studying the mechanisms of angiogenesis and the therapeutic strategies to modulate neovascularization (Ucuzian and Greisler, 2007). Due to an increasing amount of compounds affecting the vascular system, accurate vasculogenesis and angiogenesis models are needed for chemical safety testing and for drug development (Sarkanen et al., 2011; Bishop et al., 1999). Currently, preclinical animal models are dominantly used for angiogenesis testing although they are not considered optimal in efficacy or relevance to humans. The most commonly used *in vivo* angiogenesis assays include the chick chorioallantoic membrane (CAM) assay, Matrigel plug assay, zebrafish embryo system, corneal micropocket assay, rat/mouse hind limb ischemia model and rat aortic ring assay (Norrby, 2006; Auerbach et al., 2003). Despite the advantage of providing more information on complex cellular interactions compared to *in vitro* models, animal models are burdened by several disadvantages, such as variability, animal-specificity and ethical concerns (Norrby, 2006). Human cell based models have the potential to be valuable tools in predicting effects in man. However, the human relevance of these *in vitro* models needs to be confirmed in terms of cell identity, physiological architecture and functionality (Bale et al., 2014; Hartung, 2011). In addition, the developmental stage of the model system and a defined medium composition are critical, especially when toxicological applications are considered. Serum-free medium with xeno-free and defined supplements is considered essential for *in vitro* models to decrease the variation between experiments due to unknown components in the medium, and further, unknown binding properties of these components (Shen et al., 2013; Brunner et al., 2010; van der Valk et al., 2010). Currently, culture media are still commonly supplemented with serum, although it has a highly uncharacterized composition, including various cytokines and growth factors, as well as a lot-to-lot variability (Lindroos et al., 2011; Brunner et al., 2010).

We and others have shown that adipose stromal cells and umbilical vein endothelial cells are capable of self-assembling into a dense, three-dimensional vascular-like network (Sarkanen et al., 2012; Merfeld-Clauss et al., 2010; Verseijden et al., 2010). While adipose stromal cells secrete factors that induce endothelial cell (EC) sprouting and lumen formation (Rubina et al., 2009; Traktuev et al., 2008; Rehman et al., 2004; Kilroy et al., 2007; Bishop et al., 1999), the supporting stromal cells also enhance vascular basement membrane and lumen formation (Merfeld-Clauss et al., 2010; Newman et al., 2013; Stratman et al., 2009).

The aim of this study was to develop a defined medium and further characterize the *in vitro* vascular model developed by us, which is composed of human adipose stromal cells (hASC) and human umbilical vein endothelial cells (HUVEC) (Sarkanen et al., 2012). Our results showed that the new vascular stimulation media (VSM) developed in this study produces an extensive vascular-like network with mature properties and provides a valid alternative to commercial EGM-2 medium when comparing vascular-like network formation capacity. This vascular model has the potential to be used in the safety and efficacy assessment of angiogenic compounds. In addition, the vascular-like network combined with target cells, such as cardiomyocytes (Vuorenpää et al., 2014), can be used as a tissue engineering platform to create vascularized tissue models.

2 Materials and methods

This study conforms to the principles outlined in the Declaration of Helsinki. The human adipose tissue samples were obtained from surgical operations and human umbilical cords were received from caesarean sections with individual written informed consent at Tampere University Hospital, Tampere, Finland. The use of hASC and HUVEC were approved by the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland with permit numbers R03058 and R08028, respectively.

Isolation and culture of human adipose stromal cells

hASC were isolated from human adipose tissue by using a mechanical and enzymatic procedure described previously (Sarkanen et al., 2012). Briefly, human adipose tissue specimens were mechanically cut into small pieces and enzymatically digested with 0.15% collagenase I (Invitrogen, Paisley, Scotland, UK) in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 (DMEM/F12, Gibco, Invitrogen, Carlsbad, CA, USA). hASC were cultured in DMEM/F12 supplemented with 10% human serum (HS, Lonza Group Ltd, Basel, Switzerland) and 1% L-Glutamine (Gibco). The cells were tested for mycoplasma contamination (MycoAlert® Mycoplasma Detection Kit, Lonza Group Ltd) before experimental use.

Isolation and culture of human umbilical vein endothelial cells

HUVEC were isolated from human umbilical cord veins using 0.05% collagenase I as described previously (Sarkanen et al., 2011). The cells were cultured in EGM™-2 Endothelial Cell Growth Medium-2 (EGM-2, Lonza Group Ltd). Before use the cells were tested for mycoplasma contamination (MycoAlert® Mycoplasma Detection Kit, Lonza).

Establishment of the co-culture forming the vascular-like network

Co-culture of hASC and HUVEC was established as described previously (Sarkanen et al., 2012). Briefly, hASC were seeded in

Tab. 1: Stimulation media tested in vascular model

Acronym	Basal medium	Serum	Growth factors	Supplementation
EGM-2	EBM-2	2% FBS	VEGF, FGF-2, IGF-I, epidermal growth factor	hydrocortisone, ascorbic acid, heparin, GA-1000
EGM-2/HS	EBM-2	2% HS	VEGF, FGF-2, IGF-I, epidermal growth factor	hydrocortisone, ascorbic acid, heparin, GA-1000
basal SFM	DMEM/F12	–		ITS, BSA, NaP, L-glutamine, T3,
VSM	DMEM/F12	–	VEGF, FGF-2	ITS, BSA, NaP, L-glutamine, T3, hydrocortisone, ascorbic acid, heparin sodium salt

0.1% ITS (insulin-transferrin-sodium selenite media supplement), 1.28 mM L-glutamine, 1% BSA (bovine serum albumin), 2.8 mM NaP (sodium pyruvate), 100 IU/ml P/0.1 mg/ml S, 0.1 nM T3 (3,3',5-triiodo-L-thyronine sodium salt), HS (human serum), FBS (fetal bovine serum), VEGF (vascular endothelial growth factor A), FGF-2 (fibroblast growth factor 2), IGF-I (insulin-like growth factor I), GA-1000 (30 μg/ml gentamicin and 15 ng/ml amphotericin), VSM (vascular stimulation medium), SFM (serum free medium)

EGM-2 (Lonza) at 20,000 cells/cm^2. After 1-3 h HUVEC were seeded on top of hASC at 4,000 cells/cm^2. The hASC were used at passage 2 and HUVEC at passage 4 in the co-culture (passage number increased at seeding). The day after plating, the stimulation media were applied to the co-culture (see Tab. 1): 1) EGM-2 containing epidermal growth factor, vascular endothelial growth factor A (VEGF), fibroblast growth factor 2 (FGF-2), insulin-like growth factor I (IGF-I), ascorbic acid (AA), heparin, hydrocortisone (HY), antibiotic mix: 30 μg/ml gentamicin and 15 ng/ml amphotericin and 2% fetal bovine serum (FBS); 2) EGM-2/HS, where 2% FBS was replaced with 2% HS; 3) serum free basal medium (SFM); 4) Vascular stimulation medium (VSM).

In this first media comparison study, the AA, HY and heparin in VSM were taken from the EGM-2 kit and used according to the manufacturer's protocol (concentrations of these are not publicly available). After that, concentrations of AA, heparin sodium salt from bovine intestinal mucosa (HE) and HY purchased from Sigma were optimized for VSM. In the concentration optimization study the tested concentrations of AA were 0, 50, 100, 200, 500, 1000 and 2000 μg/ml; of HY 0, 20, 200, 1000, and 2000 ng/ml and of HE 0, 50, 500, 10,000 and 50,000 ng/ml. The concentrations of 10 ng/ml VEGF and 1 ng/ml FGF-2, used for VSM, had been optimized in our previous study (Sarkanen et al., 2012). The co-cultures were grown for 6 days prior to immunocytochemistry or quantitative real-time PCR (qPCR) processing. Stimulation medium was changed once during the 6 day culture.

Quantitative real-time PCR

Genes activated in co-culture of hASC and HUVEC in EGM-2 medium versus co-culture in VSM were analyzed by qPCR. Total RNA was extracted at day 6 using the RNAeasy minikit (Qiagen) following the manufacturer's protocol. A step to eliminate genomic DNA contamination was included in the isolation and performed with RNase-free DNase set (Qiagen). Reverse transcription of total RNA to cDNA was performed using RT^2 First Strand Kit (Qiagen) following the manufacturer's instructions.

Human Angiogenesis RT^2 *Profiler™* PCR Array (Qiagen, Valencia, California, USA) was used to profile the expression of 84 key angiogenesis-related genes (the comprehensive list of genes included in the array can be found in http://www.sabiosciences.com). The array was performed according to the manufacturer's protocol using BioRad CFX96 Real Time System (BioRad Laboratories, USA). The array contained five housekeeping genes and controls, including genomic DNA control, Reverse Transcription Control and Positive PCR controls. The co-culture of hASC and HUVEC grown in EGM-2 medium was used as a control. Three independent experiments with the array were performed using the same cells.

Immunocytochemistry

To analyze the vascular-like network formation and different cell types present in hASC and HUVEC co-culture, immunocytochemical staining was performed. In primary antibody staining, endothelial cell specific antibody for rabbit anti-human von Willebrand factor IgG (anti-VWF, 1:100, F3520, Sigma) with common pericytic marker α-human smooth muscle actin (monoclonal anti-SMA clone 1A4, 1:200, M0851, DAKO), vascular smooth muscle cell marker smooth muscle myosin heavy chain (anti-SMMHC, clone hSM-V, 1:800, M7786, Sigma), contractile smooth muscle cell marker calponin (anti-calponin, clone hCP, 1:800, C2687, Sigma), pericytic and smooth muscle cell progenitor marker platelet derived growth factor receptor-β (anti-PDGFRβ, clone PDGFR-B2, 1:800, P7679, Sigma), vascular endothelial cadherin (CD144,Clone 55-7H1, 1:50, 555661, BD Pharmingen), monoclonal occludin (clone 1G7, 1:300, WH0004950M1, Sigma) or basement membrane marker collagen IV (anti-COLIV, clone COL-94, 1:500, C1926, Sigma), all anti-human and all produced in mouse, except vWf, were used. Co-culture was fixed with 70% ethanol at day 6. After fixation, the cells were permeabilized with 0.5% Triton-X100 (MP Biochemicals, Ohio, USA) and non-specific binding sites were blocked with 10% bovine serum albumin (BSA, Roche Diagnostics Corporation, Indianapolis, USA). Primary antibody in 1% BSA was applied to the cells. Secondary antibodies used were polyclonal goat anti-rabbit IgG tetramethylrhodamine (TRITC, 1:50, T6778 Sigma), anti-rabbit IgG A568 (1: 400, A11011, Invitrogen) and anti-mouse IgG fluorescein isothiocyanate (FITC, 1:100, F4143, Sigma). After immunocytochemical staining the

vascular-like network was analyzed and photographed with Nikon Eclipse TS100 inverted fluorescence microscope (Nikon, Tokyo, Japan) and Nikon digital sight DS-U2 camera (Nikon). Images were further processed with NIS Elements (Nikon, Tokyo, Japan) and Adobe Photoshop CS3-software (Adobe Systems Incorporated, San Jose, CA, United States).

Flow cytometric surface marker expression analysis of HUVEC and hASC

hASC were cultured in hASC medium (passage 1) for 6-7 days and HUVEC were cultured in EGM-2 medium (passage 3) for 3 days prior to surface marker expression analysis using a BD FACSCanto II flow cytometer (BD Biosciences, Erembodegem, Belgium). For the flow cytometry analysis cells were divided into 5 ml polystyrene round bottom FACS tubes (BD, New Jersey, USA) at 250,000 cells per tube. The cells were washed once with warm staining buffer (1% BSA in PBS) and centrifuged at 131 x g for 5 min, after which they were stained either for surface markers or for intracellular markers.

Fixation and permeabilization were only performed for staining of intracellular markers. The fixation was conducted by incubating the samples for 30 min in 2% paraformaldehyde in PBS at room temperature (RT). The cells were then centrifuged at 500 x g for 5 min. Permeabilization of the cells was performed by 10 min incubation in 0.1% Triton-X100 in PBS at RT after which the cells were centrifuged at 500 x g for 5 min and washed once or twice with staining buffer before addition of antibodies.

The labelled mouse anti-human antibodies used were intracellular vWf-A2-allophycocyanin IgG2b (APC, #IC27641A) and eNOS- phycoerythrin IgG1 (PE, #560103), and surface markers CD144-FITC IgG1 (#560411), CD73-Phycoerythrin-Cyanine IgG1 (PE-CY7, #561258), CD309-PE IgG1 (#560872), CD68-FITC IgG2b (#562117), NG2-PE IgG1 (#FAB2585P), CD90-FITC IgG1 (#561969), CD105-V450 IgG1 (#561447), CD34-APC IgG1 (#561209), CD140b-PE IgG2a (#558821), CD31-V450 IgG1 (#561653), CD45-PE IgG1 (#560975), CD14-FITC IgG1 (#561712). Isotype controls mouse IgG2b-APC (#IC0041A), Mouse IgG1-PE-CY7 (#557872), mouse IgG1-PE (#559320), mouse IgG1-FITC (#555748), mouse IgG2b-FITC (#556655), mouse IgG1-V450-(#642268), mouse IgG1-APC (#550854), mouse IgG2a-PE (#551438). All antibodies and their corresponding isotype controls used in the flow cytometry analysis were purchased from BD except NG2, vWf and IgG2b-APC, which were purchased from R&D Systems.

Labelled antibodies were added into cell suspension in cold staining buffer and incubated on ice for 30 min in the dark. After incubation the cells were washed once with staining buffer and twice with PBS. Surface marker stained cells were centrifuged at 200 x g for 5 min and intracellular marker stained cells were centrifuged for 5 min at 500 x g.

Flow cytometry analysis was performed with cells suspended in ice cold PBS and 5,000 events were analyzed per sample. Compensation was done with compensation particles, i.e., BD™ CompBeads (BD) according to the manufacturer's instructions. The results were analyzed with BD FACSDiva™ Software (BD). The positive expression was obtained by gating 98% of the events isotype control results and then inverting the gate to obtain a percentage of positively stained cells in the samples. The results were calculated as percentages with SD.

Electron microscopy

hASC and HUVEC co-culture was performed in 24-well UpCell plates (ThermoFisher) for transmission electron microscopy (TEM) and on glass cover slips coated with 0.1% gelatin for scanning electron microscopy (SEM). Co-culture was maintained for 6 days and washed twice with PBS prior to fixation.

SEM specimens were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer after which the SEM samples were dehydrated in alcohol and dried in Baltec critical point dryer (Baltec, CPD030, Balzers, Liechtenstein). A layer of platinum was sputtered onto the specimens with a sputter coater (Agar Scientific, Stansted, England). Specimens were examined in a Zeiss Ultra Plus scanning electron microscope, (Carl Zeiss MT – Nanotechnology System Division, Carl Zeiss NTS GmbH, Oberkochen, Germany) using 5 kV as an accelerating voltage.

TEM specimens were detached from the temperature sensitive 24-well UpCell plate and transferred to fixative with 1% glutaraldehyde, 4% formaldehyde mixture in 0.1 M phosphate buffer for 10 min. The cell sheet was immersed in 2% agarose in distilled water and postfixed in 1% osmiumtetroxide, dehydrated in acetone and embedded in Epon LX 112 (Ladd Research Industries, Vermont, USA). Thin sections were cut with Leica Ultracut UCT ultramicrotome, stained in uranyl acetate and lead citrate and examined in a Philips CM100 transmission electron microscope. Images were captured by a Morada CCD camera (Olympus Soft Imaging Solutions GMBH, Munster Germany).

Quantitative analysis of vascular-like network formation

Vascular-like networks, i.e., vWf-positive tubule structures formed in different stimulation media, were imaged using CellIQ (Chipman tech., Tampere, Finland) with 10x objective and 5x5 grid. The quantitation of the area of the vascular-like network was performed using ImageJ software (National institutes of health, NIH, Maryland, USA) for the image analysis. Images were first converted to 8-bit gray scale, then background was subtracted and finally the binary threshold function was adjusted to obtain the best contrast of the vascular-like network against the background. With these settings, the total area of vascular-like network was calculated as the total number of pixels in images with set threshold.

Statistical analysis

Statistical analyses were performed and graphs processed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). The results concerning vascular-like network formation were subjected to one-way ANOVA followed by Dunnett's post-test when applicable. The results were reported as total area ± SD and differences were considered significant when $p < 0.05$*, $p < 0.01$** and $p < 0.001$***.

PCR results were analyzed with the PCR Array Data Analysis Web Portal (http://www.SABiosciences.com/pcrarraydataanalysis.php). The following formula was used to calculate the relative amount of the transcripts of the co-culture of hASC and

Tab. 2: Phenotypic characterization of human adipose stromal cells (hASC p1) and human umbilical vein endothelial cells (HUVEC p3)
The expression of surface and intracellular markers was analysed by flow cytometry (5000 events per sample). Results are shown as percentage of positive cells.

Cells	Protein	Antigen	Positive cells %	SD	*n*
hASC	vWf	von Willebrand factor	7.5	4.3	11
	CD144	vascular endothelial cadherin	1.4	2.9	9
	eNOS	endothelial nitric oxide synthase	0.7	1.1	8
	CD140b	PDGF Receptor b	52.8	16.0	6
	CD45	leukocyte common antigen	16.4	14.9	6
	CD14	expressed on monocytes/macrophages	0.7	0.8	7
	CD68	expressed on macrophages and monocytes	1.1	1.4	8
	CD309	VEGF receptor 2	1.2	3.5	6
	CD31	platelet endothelial cell adhesion molecule	0.9	3.1	6
	NG2	chondroitin proteoglycan	31.8	29.1	8
	CD90	Thy-1	82.9	15.5	10
	CD105	endoglin	64.1	27.1	7
	CD73	ecto-5'-nucleotidase	88.8	6.7	12
	CD34	sialomucin-like adhesion molecule	40.9	34.3	10
HUVEC	vWf	von Willebrand Factor	45.6	13.2	13
	CD144	vascular endothelial cadherin	70.6	14.7	17
	CD309	VEGF receptor2	31.4	17.0	10
	CD73	ecto-5'-nucleotidase	68.7	17.1	14
	CD68	expressed on macrophages and monocytes	1.9	10.1	11
	eNOS	endothelial nitric oxide synthase	48.4	18.4	13
	CD105	endoglin	57.4	26.6	9
	CD34	sialomucin-like adhesion molecule	43.1	18.6	9
	CD31	platelet endothelial cell adhesion molecule	81.3	13.7	8
	CD140b	PDGF Receptor b	7.2	8.7	8
	NG2	chondroitin proteoglycan	2.0	1.1	7

HUVEC in the VSM compared to transcripts of the co-culture in EGM-2 medium: $\Delta\Delta CT = \Delta CT$ (VSM) – ΔCT (EGM-2). A two-fold change compared to housekeeping gene *GAPDH* was considered significant.

3 Results

3.1 Phenotypic characterization

Phenotypic characterization of the cells used in the vascular model was performed by analyzing the surface and intracellular markers of hASC and HUVEC separately (Tab. 2). The mesenchymal stem cell markers CD73, CD90 and CD105 were highly expressed (> 64%) in hASC. Also, pericyte marker PDGFR-β (CD140b) was strongly expressed (> 52%) in the hASC population. Hematopoietic marker CD34 was moderately expressed (< 40%), whereas the expression levels of endothelial markers CD144 and CD31 and macrophage/monocyte markers CD68 and CD14 were very low (< 1.4%).

HUVEC were shown to express endothelial marker CD31 and a specialized vascular endothelial marker CD144 at a high level (> 70%). Angiogenic endothelial marker CD105 and cell surface enzyme CD73 were found in the HUVEC population (57-68%). Markers for macrophages/monocytes (CD68) and mural cells (NG2) were low (≤ 2%).

3.2 VSM induced optimal vascular-like network formation

Of the different stimulation media (Tab. 1), VSM induced an optimal vascular-like network formation (Fig. 1). The imaging analysis showed morphological differences in the vascular-like network in VSM compared to EGM-2 medium. VSM produced a uniformly distributed vascular-like network with connected branches and fewer cell aggregates. Low serum (2%) and se-

rum-free medium enabled stable cell attachment and induced a dense, connected vascular-like network with intact tubule walls. Human serum was found to induce a denser and more connected vascular-like network than FBS (Fig. 1).

In the supplement optimization several different concentrations of ascorbic acid, heparin sodium salt and hydrocortisone were tested and compared to vascular-like network formed in EGM-2 medium. An optimal vascular-like network formation was obtained with the AA concentration 100 μg/ml (Fig. 2A), HE concentration 0-500 ng/ml (Fig. 2B) and HY concentration 0.2 μg/ml (Fig. 2C). The results showed that a vascular-like network was formed in the absence of HE supplement with minor morphological differences. However, low concentrations of HE (50-500 ng/ml) increased the total area of the vascular-like network significantly compared to EGM-2 medium (Fig. 2B).

3.3 Expression of angiogenesis-related genes in VSM compared to EMG-2

Expression of 84 angiogenesis-related genes was analyzed from a vascular-like network formed in co-culture of hASC and HUVEC in EGM-2 medium (control) and in VSM. Moderate differences in the expression of angiogenesis-related genes were detected between EGM-2 and VSM. In VSM, nine genes were up-regulated (*Angpt1, F3, FIGF, IGF-I, LEP, MDK, MMP2, MMP9, PGF*) and nine (*CCL11, CXCL9, FN1, IL6, IL8, SERPINE1, TGFB2, THBS2, TIMP1*) were down-regulated in comparison to EGM-2 medium. Fold changes and statistical significances of the up- and down-regulated angiogenesis-related genes are shown in Table 3.

3.4 Maturation stage, extracellular matrix production and 3D properties of the vascular-like network formed in VSM

Immunocytochemical staining of the vascular-like network formed in VSM showed the presence of platelet derived growth factor receptor-β (PDGFRβ) positive cells (Fig. 3D). Also, smooth muscle actin (SMA), smooth muscle myosin heavy chain (SMMHC) and contractile smooth muscle calponin positive cells surrounded the tubule structures (Fig. 3A-C). CD144 and occludin positive staining indicated the presence of adherence junctions between endothelial cells (Fig. 3E-F).

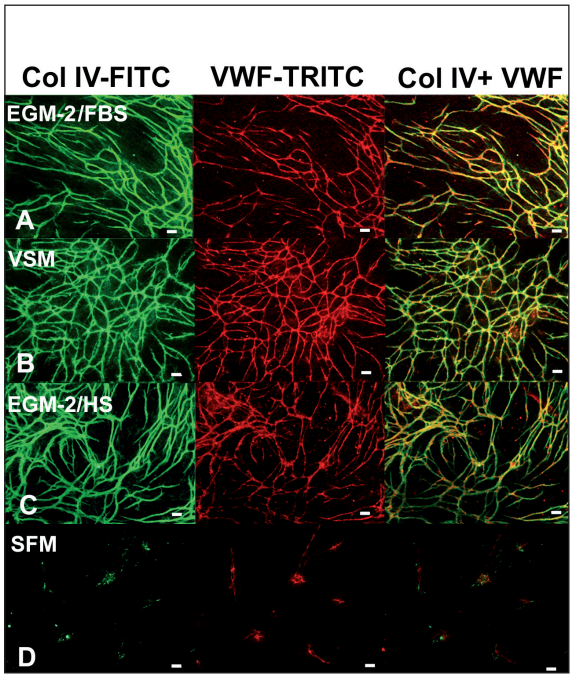

Fig. 1: Morphology of vascular-like network in different stimulation media with different serum concentrations

Vascular-like networks were stained against Col IV (FITC, green) and vWf (TRITC, red). Co-localization is shown in the merged image. Vascular-like network formed in (A) commercial EGM-2 medium with 2% FBS, (B) serum-free VSM, (C) EGM-2 with 2% HS and (D) basal SFM. Scale bars 100 μm in each figure. Col IV= collagen type IV, VWF= von Willebrand factor, VSM= vascular stimulation medium, FBS= fetal bovine serum, HS= human serum, SFM=basal serum free medium.

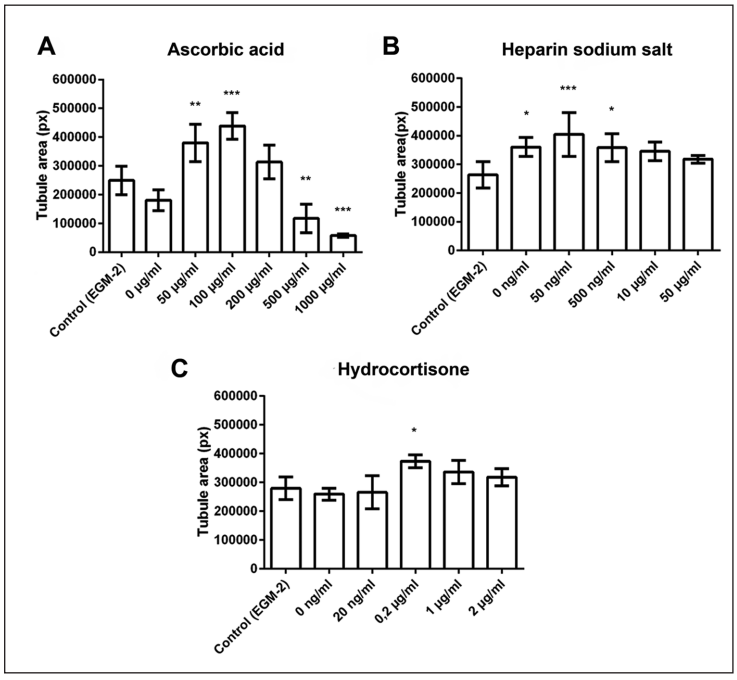

Fig. 2: Vascular-like network formation (area in pixels) in different concentrations of (A) ascorbic acid, (B) heparin sodium salt and (C) hydrocortisone in VSM

Vascular-like network formed in VSM supplemented with different concentrations of ascorbic acid, heparin sodium salt or hydrocortisone were compared to EGM-2 medium and differences presented as follows: $p < 0.05$*, $p < 0.01$** and $p < 0.001$***. An optimal vascular-like network formation was obtained with 100 μg/ml ascorbic acid and 0.2 μg/ml hydrocortisone. Heparin sodium salt could be used in concentrations 0-500ng/ml. Results are depicted as total area with standard deviations, n = 5 in each supplement and concentration.

Tab. 3: Fold changes and statistical significance of expression of angiogenesis-related genes in vascular-like network formed in VSM compared to EGM-2 medium

Genes	Target	Fold change 2^(- ΔΔCT)	Statistical significance
Up-regulated genes			
ANGPT1	angiopoietin 1	2.964	*
F3	coagulation factor III	3.255	*
FIGF	vascular endothelial growth factor D	3.043	ns
IGF-I	insulin-like growth factor I	16.555	ns
LEP	leptin	2.932	ns
MDK	neurite growth-promoting factor 2	2.06	ns
MMP2	matrix metallopeptidase 2	2.011	*
MMP9	matrix metallopeptidase 9	3.919	*
PGF	placental growth factor	2.319	*
ANGPT2	angiopoietin 2	1.769	**
Down-regulated genes			
CCL11	chemokine ligand 11	2.613	ns
CXCL9	chemokine ligand 9	2.197	ns
FN1	fibronectin 1	2.295	*
IL6	interleukin 6	2.331	ns
IL8	interleukin 8	2.546	ns
SERPINE1	serpin peptidase inhibitor	3.465	**
TGFB2	transforming growth factor beta 2	2.080	ns
THBS2	thrombospondin 2	2.085	**
TIMP1	TIMP metallopeptidase inhibitor 1	5.01	**
VEFG-A	vascular endothelial growth factor A	1.804	ns
TGFβ1	transforming growth factor beta 1	1.807	*
FGF-2	fibroblast growth factor 2	1.917	ns

Electron microscopic analysis confirmed the microstructure of the tubules formed in VSM. Tubules were shown to have a lumen, basement membrane and junctions (Fig. 4A-C). The three dimensional structure of the vascular-like network and shape of the tubules can be seen in the close-up image Figure 4D.

4 Discussion

Present chemical safety testing and non-clinical drug development rely mainly on animal biology although relevant safety testing data should be of human origin. Further, *in vitro* test systems should be thoroughly characterized in their human relevance and functionality. In this study, we developed a novel vascular stimulation medium (VSM) and further characterized the *in vitro* human vascular model developed by us (Sarkanen et al., 2012). This defined *in vitro* vascular model has the potential to be used for safety assessment of compounds and for tissue engineering applications.

The phenotypic analysis of the building blocks of the vascular model, i.e., the hASC and HUVEC, was performed using flow cytometry. HUVEC showed a strong expression of CD105 (endoglin), suggesting an active rather than quiescent state of the cells (Bernabeu et al., 2009). Interestingly, a recent study reported a minor role of endoglin in vasculogenesis whereas VEGF-induced angiogenesis was severely impaired in an *endoglin* deficient mouse embryonic stem cell model (Liu et al., 2014). The high expression of CD144 and CD31 supports the active status of HUVEC in the formation of intercellular junctions. The level of CD73, a 5'-ectonucleotidase that normally suppresses pro-inflammatory responses in human endothelial cells (Grunewald and Ridley, 2010) was high in our HUVEC analysis. Pericyte markers NG2 and CD140b (PDGFRβ) were low, indicating the absence of mural cells in the HUVEC population.

This study confirms the earlier results shown by us (Sarkanen et al., 2012) and others (Lindroos et al., 2010; Traktuev et al., 2008) that the stromal-vascular fraction extracted from adipose tissue is heterogenic. hASC expressed mesenchymal stem cell markers CD90, CD105 and CD73 at high levels, showing the

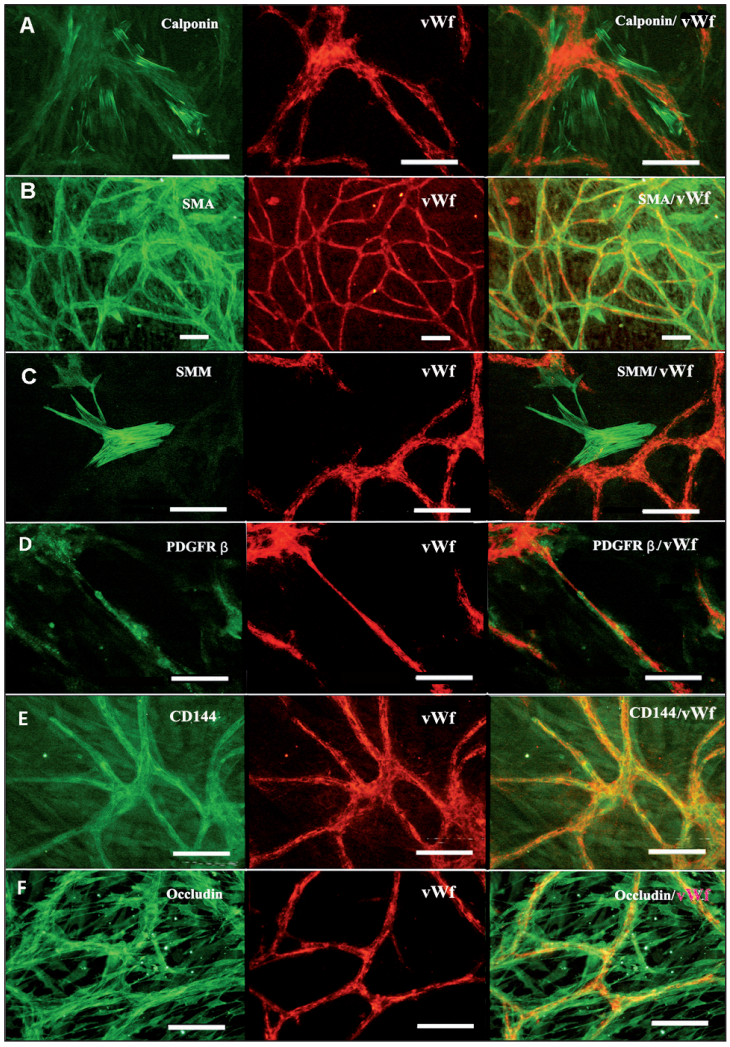

Fig. 3: Vascular-like network formed in serum-free VSM shows vascular maturation markers at day 6

α-vWf-staining is shown in red in all figures. (A) Calponin-, (B) SMA- and (C) SMM-positive cells surrounding the vascular-like network. (D) α-PDGFRβ stained pericytes are located along the tubules. (E) CD144- and (F) occludin-positive junctions can be found in the tubule structures. Scale bar 100 µm in each image. VSM= vascular stimulation medium, vWf = von Willebrand factor, SMA = smooth muscle actin, SMM = smooth muscle myosin heavy chain, PDGFRβ = platelet derived growth factor receptor β, CD144 = vascular endothelial cadherin.

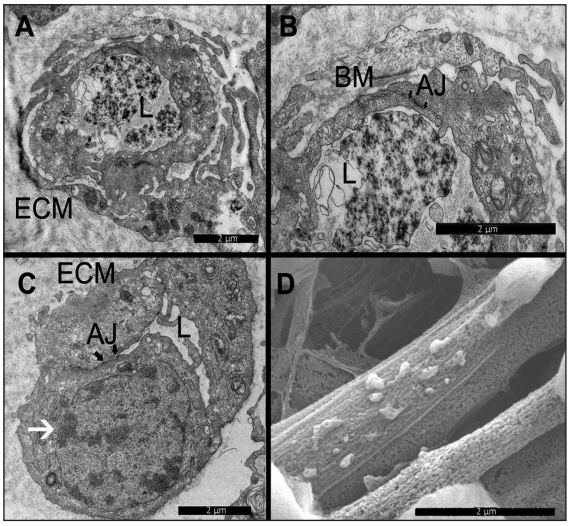

Fig. 4: Transmission (A, B, C) and scanning (D) electron microscopy images of the vascular-like network cultured in VSM

(A) Mature tubule with lumen = L and extracellular matrix = ECM. The lumen is filled with debris from the apoptotic cells. Scale bar 2 *µ*m. (B) Close-up image with basement membrane = BM, adherence junction = AJ and lumen of the vascular-like tubules. Scale bar 2 *µ*m. (C) Tubule in earlier stage of vascular development showing endothelial cell with large nucleus (white arrow), lumen, adherence junctions and ECM. Scale bar 2 *µ*m. D) Scanning electron microscopy image shows the 3D structure and the tubule shape of the vascular-like network. Scale bar 2 *µ*m. VSM = vascular stimulation medium.

presence of a cell population with multilineage differentiation potential. Pericyte markers CD140b (PDGFRβ) and NG2 were expressed at high to moderate level, supporting the finding that pericytes from the hASC population are lining the vascular-like network in our model.

We also analyzed whether the population of endothelial progenitor cells capable of vasculogenesis could be found in hASC as suggested by some earlier reports (Sarkanen et al., 2012; Miranville et al., 2004; Heydarkhan-Hagvall et al., 2008). The results showed that, in addition to the high expression of CD90, there is a moderate expression of CD34; these are markers for mesenchymal stem cells and endothelial progenitors. A hASC population of CD90+/CD34+ cells was shown to be capable of differentiating into endothelial cells and form capillary-like structures (De Francesco et al., 2009).

The expression of mature endothelial cell markers CD31 and CD144 in the hASC population was very low. Interestingly, there is a report suggesting that adipose stromal cells may enhance endothelial differentiation of progenitor cells (Rubina et al., 2009). However, another essential marker for endothelial progenitor cells, CD309 (VEGFR2) (Yoder, 2012), showed very low expression in the six hASC lines that were analyzed in this study. Therefore, the presence of an endothelial progenitor cell population in the stromal-vascular fraction needs to be studied further.

Serum-free VSM was shown to produce a reproducible, extensive mature vascular-like network and provide a suitable alternative to the commercial EGM-2 medium (previously used in the model, containing 2% FBS). Serum had a strong correlation with vascular-like network formation. At higher serum (10%) concentrations the network was shorter with broken tubule walls, and random detachment of the cell layer occurred (data not shown). In low- or serum free (0-2%) environment,

network formation increased, tubules were highly branched and tubule walls remained intact. In serum-free conditions we detected thinner and more branched tubules as also reported by Yang and Xiong (Yang and Xiong, 2012). Serum-free medium is ideal for use in drug development, since serum is a complex mixture of components with unknown composition and protein binding affinities (Shen et al., 2013). Furthermore, a tissue construct aimed for clinical therapy should be cultured in human serum or, preferably, in a serum-free environment to avoid the risk of infection and severe immune reactions in the recipient (Patrikoski et al., 2013; Lindroos et al., 2011, 2010; Holm et al., 2010).

Fetal bovine serum (FBS), although being the most widely used growth supplement, holds ambiguous, unknown effects for cell culture and also raises ethical concerns due to the number of bovine fetuses needed for serum production (Gottipamula et al., 2013; Brunner et al., 2010). In this study, microscopical analysis showed that HS was more inductive for vascular-like network formation than FBS. Replacement of FBS with HS has been reported to support equal or higher proliferation rates and differentiation capacity of adipose stromal cells (Lindroos et al., 2011; Brunner et al., 2010). Furthermore, human mesenchymal stem cells have been shown to maintain their immunophenotype and multilineage potential in serum-free medium (Patrikoski et al., 2013; Mark et al., 2013).

In this study we developed a defined vascular stimulation medium since commercial media producing companies do not necessarily publish the specific composition of their media. This might complicate the use of commercial media in toxicological studies as medium components may interact with test compounds. In addition to commonly used VEGF and FGF-2 defined by us earlier (Sarkanen et al., 2011), we found that the network formation can be further improved by addition of AA, HE and HY. Ascorbic acid (vitamin C) is an essential nutrient for human endothelial cells necessary for their effective migration and for the synthesis of collagen type IV, an important component of basement membrane (Telang et al., 2007). In this study, the formation of basement membrane around the tubules was impaired in the absence of AA (data not shown). However, AA inhibited angiogenesis at high concentrations (1000-2000 μg/ml) as reported previously (Mikirova et al., 2008). Therefore, AA is essential for collagen IV formation in basement membrane at low concentrations.

Although HE is needed for the attachment of some growth factors to their cell surface receptors (Ashikari-Hada et al., 2005) and was found to be beneficial for the network formation, it did not induce a significant advantage to the morphology or branching of the tubules. Since HE is an animal derived substance it should rather be avoided where clinical applications are concerned. The lack of need for added HE can be explained by the secretion of perlecan by HUVEC (Murikipudi et al., 2013; Schlessinger et al., 2000). Our results correlate with earlier studies (Khorana et al., 2003; Jung et al., 2001) showing that HE inhibits vascular-like network formation at high concentrations.

Hydrocortisone, although not pro-angiogenic itself, has a beneficial effect on angiogenesis (Goding, 2009). In this study, hydrocortisone increased the number of branches in the vascular-like network. However, it produced cell aggregates at high concentrations whereas a lack of hydrocortisone induced a sparse vascular-like network. Our results suggest that hydrocortisone acts as a mitogen in the vascular model. Other supplements of the VSM included ITS (insulin, transferrin, selenic acid medium supplement), BSA, sodium pyruvate, L-glutamine and T3 (3,3',5-triiodo-L-thyronine). The concentrations of these supplements were reported earlier by us (Vuorenpää et al., 2014) and were used in this study with minor modifications.

Lumen formation is critical for the transformation of cords into a perfusable vascular system (Charpentier and Conlon, 2014). Electron microscopic analysis assured the formation of 3D vascular microstructure including lumen in the novel VSM. Tubules at different stages of lumen development were detected, indicating the ongoing process of network formation at day 6. The initiation of lumen development is triggered by apical-basal polarity of the endothelial cells in which CD144 plays a critical role in promoting the localization of polarity markers (Charpentier and Conlon, 2014). The electron microscopic analysis as well as immunocytochemistry results of this study showed that hASC and HUVEC co-culture actively produces extracellular matrix (ECM) components, including fibrillins, thus creating natural 3D scaffold around them. The reciprocal interaction between ECM stroma and vascular network is important in directing vessel growth (Hoying et al., 2014; Du et al., 2014). The hASC and HUVEC co-culture gives mechanical support for other target cells, e.g., cardiomyocytes, and, additionally, the microenvironment formed by the co-culture enhances target cell viability as reported previously (Vuorenpää et al., 2014). This 3D vascular model provides a more *in vivo*-like test system without an artificial scaffold that may interfere with the cell-cell interactions or affect the toxicological applications of the model.

In the genotypic analysis 84 human angiogenesis-related genes were studied. Nine genes were down-regulated and nine genes up-regulated in VSM compared to EGM-2 medium. The expression of *angiopoietin 1*, a marker for mature tubules, was significantly higher in VSM compared to EGM-2 medium, whereas *angiopoietin 2*, a marker for the early stage, i.e., sprouting angiogenesis, was slightly upregulated in VSM. *VEGF-A* and *FGF-2*, indicators of early stage angiogenesis, were moderately yet not significantly down-regulated in VSM compared to EGM-2. Since both media included VEGF and FGF-2, the expression of these growth factors was apparently unnecessary for the cells. On the contrary, *placental growth factor* (PGF) showed modest up-regulation in VSM. PGF and its receptor VEGFR-1 are minimally expressed in adult quiescent vasculature, but are markedly up-regulated during pathological conditions (Carmeliet et al., 2001). *TGFβ1*, a marker for tubule maturation, was slightly down-regulated in VSM. Vessel maturation relies partly on TGF-β signaling and TGF-β stimulates mural cell proliferation and migration and production of ECM (Potente et al., 2011). The gene expression analysis confirms the finding that VSM enhances the maturation of the vascular-like network.

Interestingly, the expression of *insulin like growth factor* (IGF-I) showed the highest up-regulation in VSM. IGF-I treatment in myocardial infarction has been shown to increase circulating angiogenic growth factors, thus providing protection

against myocardial ischemia in rats (Mathews et al., 2011). *In vitro* IGF-I stimulates migration and tube-forming activity of endothelial cells (Shigematsu et al., 1999; Nakao-Hayashi et al., 1992). Also leptin, a hormone secreted by adipocytes, was found to be up-regulated. Leptin signaling acts as a link between adipocytes and the vasculature (Sierra-Honigmann et al., 1998). It also increases the production of VEGF and enhances the expression of *MMP-2* and *MMP-9* (matrix metallopeptidases) in HUVEC (Park et al., 2001). This was seen as an up-regulation of *MMP-2* and *MMP-9* enzymes that are involved in degradation of the ECM at the early stages of angiogenesis (Kasper et al., 2007; Cornelius et al., 1998).

In vivo studies in mice have revealed that the adult quiescent vasculature becomes less dependent on VEGF for its maintenance (Gerber et al., 1999). However, during pathological conditions – such as ischemia, inflammation or malignancy – angiogenic endothelial cells are stimulated by increased VEGF levels (Carmeliet et al., 2001). The VEGF signaling pathways have been conclusively identified as central for the processes of vasculogenesis, angiogenesis and lymphangiogenesis. VEGF-D induces sprouting lymphangiogenesis when overexpressed in transgenic mice and also in various tumor models (Lohela et al., 2009). The up-regulation of *vascular endothelial growth factor D (FIGF)* as a marker for lymphangiogenesis suggests the versatile modifications of our vascular model. In future applications, it may be possible to replace HUVEC with lymphatic endothelial cells to form a lymphangiogenesis model.

5 Conclusion

The vascular model characterized in this study forms a vascular-like network with mature properties. The developed novel medium provides a valid alternative to commercial EGM-2 medium and benefits the use of the model in toxicological studies as well as in efficacy studies in drug development. This characterized vascular model is currently under intra-laboratory validation that is performed according to OECD guidance document No. 34 (OECD, 2005) to verify the reliability and human relevance of the test system with known reference chemicals. The validated vascular model will be used in toxicity testing and can be combined with other target cell types from different tissues to create complex, vascularized tissue models.

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Conflict of interest statement

None of the authors have any conflicts of interest.

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