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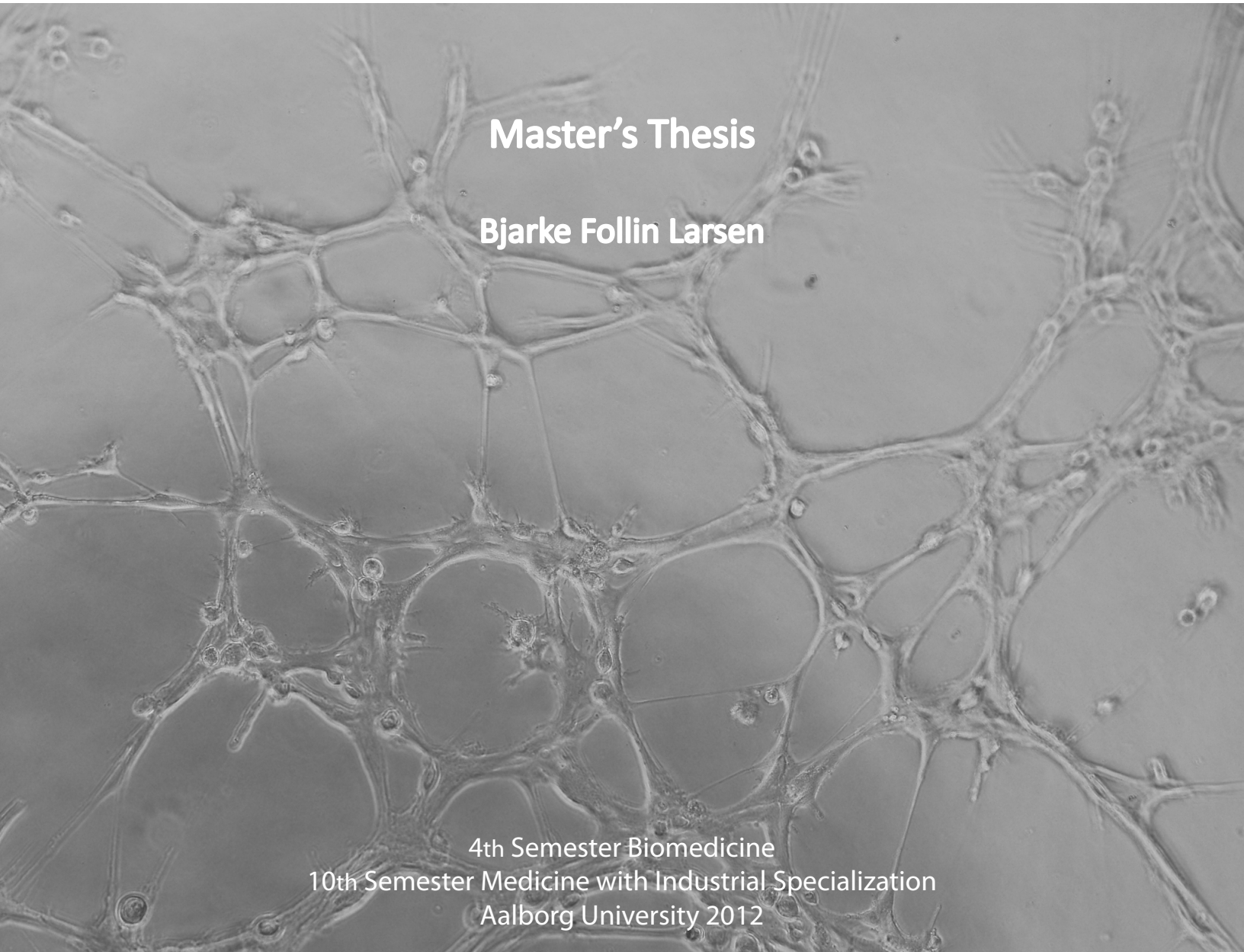
The Effect of Stimulation with Vascular Endothelial Growth Factor on Phenotype and Function of Adipose-derived Stromal Cells from Patients with Ischemic Heart Disease



Master's Thesis

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List of Abbreviations

ADSC	Adipose-derived stem cell
BM-MNC	Bone-marrow mononuclear cells
BrdU	Bromodeoxyuridine
CD	Cluster of Differentiation
EPC	Endothelial progenitor cell
FACS	fluorescence activated cell sorting
FBS	Fetal bovine serum
FC	Flowcytometry
FOXF1	Forkhead box protein F1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HUVEC	Human umbilical vascular endothelial cell
ICC	Immunocytochemistry
IHD	Ischemic heart disease
ISCT	International Society for Cell Therapy
MNC	Mononuclear cells
MSC	Bone-marrow derived stem/stromal cell
PBS	Phosphate buffered saline
PDGFRβ	Platelet-derived growth factor β
PPIA	Peptidylpropyl isomerase A
RT-qPCR	Reverse transcriptase quantitative real-time polymerase chain reaction
Tie-2	TEK tyrosine kinase endothelial-2
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR1 and 2	VEGF receptor 1 and 2
vWF	von Willebrand factor

1. Abstract

Objectives: Adipose-derived stem cells (ADSCs) are currently used in a clinical study as treatment for ischemic heart disease (IHD). The cells are stimulated with vascular endothelial growth factor (VEGF) to differentiate them towards endothelial lineage prior to injection. The aim of this study was to investigate the effect of VEGF treatment on ADSCs from IHD patients, in terms of differentiation towards endothelial lineage and mesenchymal stem cell characteristics.

Methods: The ADSCs were stimulated with human recombinant VEGF-A_{165} for 1, 2, and 3 weeks, with controls receiving serum-deprived medium and complete medium. The expression of VEGF receptors and endothelial and stem cell markers was measured at mRNA level using RT-qPCR and at protein level using immunocytochemistry (ICC) and flowcytometry (FC). Functionally, *in vitro* angiogenesis potential was evaluated on ECMatrix®. Furthermore, mesenchymal stem cell characteristic markers were investigated using FC.

Results: ADSCs treated with VEGF and serum-deprived medium significantly increased the expression of FOXF1 and PDGFβ compared with ADSCs in complete medium culture. A non-significant trend of increased vWF, VEGFR1 and VEGFR2 expression was observed. Endothelial markers were sporadically positive for all media types on ICC, but not on FC. *In vitro* angiogenesis only occurred with ADSCs stimulated with VEGF and serum-deprived medium. Mesenchymal stem cell characteristics were equal across media types.

Conclusion: We found evidence of predisposition for ADSC differentiation towards endothelial lineage when cultured with serum-deprivation, with no additional effect of VEGF. The effect was subtle, with significant increase of only the earliest marker of endothelial differentiation on mRNA level. The serum-deprivation rendered the cells prone to form tubules on ECMatrix®, and did not affect the expression profile of mesenchymal stem cells markers. VEGF stimulation seems to predispose ADSCs for differentiation towards endothelial lineage, but the cells need additional stimuli to complete the process.

2. Introduction

Ischemic heart disease (IHD) was the leading cause of death worldwide in 2008, with 7.25 million associated deaths. The incidence is estimated to increase due to the ageing population and the current obesity pandemic [1, 2]. The prevalence is highest in developed countries, and approximately 200,000 people lived with IHD in Denmark in 2011 [3]. IHD is insufficient supply of blood to the myocardium, primarily caused by narrowing of the coronary arteries due to atherosclerosis. This results in local ischemia in the myocardium supplied by the affected vessels. If sufficient blood supply is not reestablished, these ischemic areas develops into infarctions, which is death of the affected cardiac tissue, and may lead to heart attack [4]. A considerable part of the IHD patient group demonstrates insufficient response to current available therapies [5]. A plausible approach to reestablish the blood supply to the ischemic areas and prevent deterioration of heart function is the use of stem cells [6].

2.1 Stem Cells

Stem cells are unspecialized cells characterized by their ability to self-renew, to differentiate into various tissue-specific cells, and to preserve the potential for differentiation and self-renewal through multiple cell divisions [7]. These abilities renders them suitable for regenerative purposes[8]. Stem cells can be divided into

embryonic stem cells, derived from the inner cell mass of a blastocyst, and mesenchymal stem cells which exist in niches in many different types of tissue in the body. In terms of clinical cell therapy trials, mesenchymal stem cells are used more frequently than embryonic stem cells due to safety and ethical reasons [7]. Mesenchymal stem cells are defined by certain criteria, established by the International Society for Cellular Therapy (ISCT), which are commonly referred to as the ISCT criteria. According to these criteria, mesenchymal stem cells are plastic adherent, exhibit *in vitro* multipotent differentiation, and present with a certain surface marker profile [9]. This profile consists of positive (≥ 95 % of population) stem cell markers; cluster of differentiation (CD)105, CD90, and CD73, and negative (≤ 2 % of population) markers for leukocytes; CD45, endothelial progenitor cells; CD34, B-cells; 19, monocytes; CD14, and immune stimulation; human leukocyte antigen-DR (HLA-DR). The mesenchymal stem cells comprise bone-marrow derived stem/stromal cells (MSCs) and adipose-derived stem/stromal cells (ADSCs), amongst others. MSCs are the most extensively investigated cell type of the two. However, ADSCs possess comparative abilities, can be easily harvested through liposuction, produce higher yield of stem cells per harvest, and proliferate faster during *ex vivo* expansion[10-14]. This makes the ADSCs very suitable for therapeutic applications, which has increased the interest for ADSCs in recent years [15].

2.2 Stem Cell Therapy against Cardiac Diseases

The regenerative and angiogenic properties of stem cells, combined with the nature of IHD, the extensive and growing patient group, and the lack of regenerative treatment for this disease has resulted in numerous clinical trials attempting to use stem cells as treatment. Treatment with MSCs has been rendered safe and patients have shown improvement of several different parameters of cardiac function. Treatment with ADSCs seems to have similar effects, which has been demonstrated in animal models [14, 16]. The mechanisms of the stem cell therapy against cardiac diseases are not yet fully understood, but evidence points towards three main factors [15]: First, Stem cell transplantation has been shown to stimulate angiogenesis demonstrated by increased capillary proliferation [17]. Second, transplants have been shown to inhibit inflammatory cytokines associated with cardiomyocyte toxicity and inhibition of cardiac contraction [18]. Third, stem cell therapy has a regulatory effect on cardiac fibroblast proliferation and collagen expression, countering pathological cardiac remodeling [17, 19].These mechanisms are hypothesized to be responsible for the observed effect of cardiac treatment utilizing MSCs. Hence, in order to increase the effect of stem cell treatment, it could prove beneficial to precondition the stem cells, to improve these abilities, prior to injection.

2.2.1 Differentiation Towards Endothelial Lineage

A great part of the effect of stem cell therapy is attributed to the angiogenic abilities of the stem cells, and in part by their potential for differentiation into endothelial cells. This was indicated in an animal study using transplanted bone marrow mononuclear cells (BM-MNC). Two weeks after transplantation, BM-MNCs committed to endothelial lineage were actively eliminated, which resulted in significant deterioration of left ventricular function. [20] MSCs can be differentiated towards endothelial lineage by stimulation with vascular endothelial growth factor (VEGF), an angiogenic cytokine which promotes endothelial cell proliferation, migration, and vessel permeability, and is thought to have a key role in the angiogenic effect of stem cells [21, 22]. There is evidence of the baseline expression level of VEGF genes in the

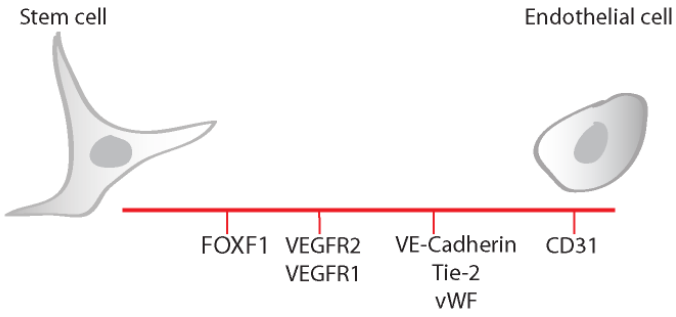


Figure 1: Temporal gene expression of endothelial markers. FOXF-1 is a transcription factor with VEGFR2, currently the earliest extracellular marker of endothelial differentiation, as a downstream target. VE-Cadherin, Tie-2, vWF and CD31 are commonly used markers of endothelial differentiation. FOXF1:, VEGFR2: Vascular endothelial growth factor receptor 2, VE-Cadherin: Vascular endothelial-Cadherin, Tie-2: TEK tyrosine kinase endothelial-2, vWF: von Willebran factor, CD31: Cluster of differentiation 31. Based on *Oswald et al., Cao et al., Furgeson et al.,* and *Astorga et al.*

chronic ischemic myocardium being similar to normal conditions. This could mean that there is no additional VEGF to stimulate differentiation towards endothelial lineage. Therefore our group has previously conducted a clinical study using MSCs preconditioned with VEGF to stimulate this differentiation before injection into IHD patients. [16, 23] The treatment was not compared with un-stimulated MSCs, but the procedure was rendered safe, and significant improvement of several disease parameters was observed and sustained through at least one year [16]. Before using cells for clinical therapy, they must first be *ex vivo* expanded to reach a sufficient number [21] . The proliferation rate of MSCs resulted in very long expansion time to reach this number. The long expansion time is not advantageous for the patients, and therefore a study is currently being performed by our group, using ADSCs instead of MSCs. As mentioned earlier, concentration of stem cells is 200 times higher in adipose tissue compared to bone marrow and ADSCs have a higher proliferation rate than MSCs, results in decreased culture expansion time. [11] Since MSCs and ADSCs are comparable in many aspects, VEGF treatment is hypothesized to stimulate the ADSCs into endothelial lineage, However, this remains to be investigated. A way to measure differentiation towards endothelial lineage, is to tests for the presence of lineage specific markers. These markers are the early VEGF receptors 1 and 2 (VEGFR1 and VEGFR2), the intermediate von Willebrand Factor (vWF), Vascular endothelial cadherin (VE-Cadherin), and TEK tyrosine kinase endothelial-2 (Tie-2), and the late marker CD31 [24-26]. In addition to these traditional endothelial markers, a transcription factor with VEGFR2 as a downstream target, Forkhead box protein F1 (FOXF1), could be the earliest marker of endothelial differentiation [27]. An estimation of the temporal expression of endothelial markers during differentiation is shown in Figure 1.

The mentioned preconditioning is performed in an attempt to improve the effect of the cell therapy. Other factors which could affect the outcome significantly are cell retention in the affected area or the choice of cell donor [28].

2.3 Autologous versus Allogenic Transplantation

Most previous and ongoing trials are using autologous stem cells. However it is still not clear whether the use of autologous transplant is superior to allogenic transplant. In other forms of transplantation, autologous is preferred due to the risk of rejection. ADSCs have been shown to avoid the initiation of an immune response and to possess immunosuppressive abilities, and are even capable of controlling graft-versus-host-disease *in vivo* [29, 30]. The non-immunogenic and immunosuppressive abilities of ADSCs are

conserved through *in vitro* expansion; which means that ready-for-use allogenic cells should function without rejection [31]. Another argument for using allogenic stem cells is the functionality of autologous cells, which could be affected by various diseases and age of the donor.

2.3.1 Effect of Donor Age and Health

Whether it is the stem cells that are affected by aging tissue or tissue deterioration resulting from aging of stem cells is not clear, but changes in stem cells from aged donors have been observed at multiple levels. These observations generally point towards cell functionality declining with age[32]. Evidence suggests the same trend for ADSCs, though there are conflicting results. In studies using ADSCs from mice, one group found the proliferative potential and telomere length decreased in ADSCs from aged mice, together with increased frequency of apoptosis. An age-dependant difference in expression of pro-angiogenic factors was observed, with decreased expression of most factors in ADSCs from aged mice. In continuation of this, human umbilical vascular endothelial cells (HUVECs) cultured with conditioned medium from aged mice resulted in decreased tube formation on Matrigel, a semi-solid culture substrate, compared to culturing with conditioned medium from ADSCs from young mice [33]. For human ADSCs, decreased ADSC cell number, population doublings and increased markers of senescence was observed with increasing donor age [34]. However, a recent study found ADSCs from aged and osteoporotic donors to exhibit proliferation rate and osteogenic differentiation comparable to controls, and furthermore showed that ADSCs are not as affected by age as MSCs. Besides age, various diseases, like heart disease, could also affect the stem cells. [35]

Stem cells are greatly influenced by their environment, so if the stem cell niches in the body are affected by disease it is plausible that the abilities of the stem cells are also affected. The only study found to investigate the abilities of ADSCs from donors with heart disease, showed that endothelial differentiation was feasible despite the disease, but no comparison with healthy controls was performed. Hence no evidence regarding potential differences between ADSCs from patients with heart disease and healthy donors could be found [36]. However, both endothelial progenitor cells (EPCs) and BM-MNCs from patients with chronic ischemic cardiomyopathy had decreased colony-forming capacity, and decreased migratory response to VEGF compared with controls. EPCs from IHD patients had shorter telomeres, decreased telomerase activity and increased oxidative stress compared with controls [37]. Furthermore, transplantation of patients BM-MNCs led to lower limb blood flow compared to transplant from healthy donors, in an animal ligation model [38, 39].

Since both age and some diseases can interfere with the functionality of the autologously transplanted stem cells, this could explain the only moderate results obtained from clinical studies using stem cell treatment for cardiac diseases. This makes it crucial for future study designs of clinical studies using ADSCs to investigate the effect of donors on cell ability. A recent study from our group found only minor differences between MSCs from young healthy donors and IHD patients in response to VEGF treatment, but no studies have investigated neither the effect of VEGF treatment on ADSCs, nor potential differences in cell ability between ADSCs from IHD patients and healthy donors [40].

2.4 Study aim

The aim of the study is to investigate the effect of VEGF treatment on ADSCs from IHD patients. The cells are stimulated in a manner similar to the preconditioning for the ongoing clinical trial using

ADSCs with human recombinant VEGF-A_{165} 50 ng/mL for one week, and for two additional weeks. The effect of the treatment is measured on both mRNA level and on protein level with expression of various endothelial markers, stem cell markers, and VEGF receptors, together with a functional *in vitro* angiogenesis assay. A parallel study, using ADSCs from healthy donors, has been performed by a fellow student in our group; Josefine Tratwal Pedersen. This makes it possible to assess potential differences in treatment response for ADSCs from IHD patients and healthy donors. In addition, proliferation is compared between the two groups of donors.

Our hypothesis is that VEGF treatment would differentiate ADSCs towards endothelial lineage, shown by an increase in both the gene and protein expression of endothelial markers, and increased tubulogenesis in a functional *in vitro* angiogenesis assay. Furthermore, it is hypothesized that the treatment affect the gene expression of relevant VEGF receptors, and that the effects of the VEGF treatment is more pronounced with increased stimulation time. The treatment is hypothesized not to alter the stem cell characteristic markers from the ISCT criteria, compared with cells cultured in compete medium. In addition, when comparing with the parallel study using ADSCs from healthy donors, it is hypothesized that both groups would respond equal to the treatment, but that the proliferation of ADSCs from healthy donors is increased.

3. Methods

For all methods detailed protocols can be found in the appendix B-K.

Donors. For this study, ADSCs from seven patients with IHD were used. The IHD patients who donated ADSCs were enrolled in the placebo group in the ongoing first-in-man clinical trial using ADSCs, MyStromalCell. The protocol for the study is in line with the declaration of Helsinki, and approved by the National Ethical Committee (02-268856) and the Danish Medicines Agency (2612-2867). The inclusion and exclusion criteria for the study can be found in Appendix A, or in the original paper [41]. The IHD patient donors were seven males in the age 65.7 ± 6.9 years.

Isolation and culture. Approximately 100mL lipoaspirate was obtained from liposuctions of subcutaneous abdominal fat. The lipoaspirate was washed twice with phosphate buffered saline (PBS) pH 7.4 (Gibco cat. no. 10010-015) to remove residual blood. The adipose tissue was digested by incubation with Collagenase NB (0.6 PZ U/mL, Serva GmbH, Heidelberg, Germany dissolved in HBSS (+$CaCl_2$ +$MgCl_2$) at 37°C for 45min while being turned. The Collagenase was neutralized with complete medium (Dulbecco's Modified Eagle Medium, low glucose (1 g/l) supplemented with 25 mM HEPES and L-Glutamin, 10 % Fetal Bovine Serum farma grade (FBS), and 1 % Penicillin/Streptomycin) and filtered through a 100 µL mesh (Cell Strainer, BD Falcon, cat. no. 352360), separating the adipocytes from the remaining mononuclear cells (MNCs). The MNCs were centrifuged at 1200g for 10 min at room temperature, re-suspended, and counted using nucleocounter® NC-100™ (Chemometec, Allerød, Denmark) according to manufacturer's instructions. The cells were seeded in T75 flasks (nunc, Thermo Scientific, cat. no.: 156494) in a density of 4.5 x 10^6 cells/flask and incubated at standard conditions, with 37°C and 5 % CO_2 humidified air in HERA Cell 150 Incubator. The medium was removed after two days, and the flasks were washed with PBS to remove leukocytes, before continuing the culture. After approximately a week of culture, the cells were washed with PBS, detached with TrypLE® (TrypLE® Select / Gibco 12563-029), centrifuged at 300g for 5min at room temperature. After this, the cells were counted

on nucleocounter®, and frozen at passage 1 in 1mL FBS with 5 % dimethyl sulfoxide (WAK-Chemie Medical GmbH, cat. no. WAK-DMSO-10) in -80°C, with transfer to liquid nitrogen the following day.

Thawing and passaging. When starting op a culture, two-six vials containing 1 x 10^6 cells each, were rapidly thawed using a 37°C water bath. The dimethyl sulfoxide cell suspensions were added 1 mL of complete medium before being transferred to one T75 flasks per vial. The media were changed the following day. When the culture reached a confluence level of approximately 90 %, the cells were washed with PBS and detached with TrypLE®. The cells were centrifuged at 300g for 5min at room temperature, counted on nucleocounter®, and passaged or seeded for the individual setups. For experimental setups, cells were seeded in T75 flasks in a density of 3 x 10^5 cells/flask. All experiments were performed with cells in passage 2-3.

As control for endothelial markers for immunocytochemistry (ICC), quantitative polymerase chain reaction (qPCR) and flowcytometry (FC), HUVECs were used. These were thawed as described above and cultured in endothelial growth medium-2 (EGM-2 Bulletkit, Lonza, cat. no. CC3162), with change of media every two-three days. All cells were incubated at standard conditions, with 37°C and 5 % CO_2 humidified air.

Stimulation with VEGF. ADSCs were seeded in T75 flasks with a density of 3 x 10^5 cells/flask in complete medium. After reaching a confluence level of approximately 80 %, the cells were stimulated for 1, 2, and 3 weeks with different media. The media types were: 1) Complete medium, containing 10 % FBS as described above. 2) Serum-deprived medium, similar to complete medium, but only containing 2 % FBS (Dulbecco's Modified Eagle Medium added 2 % FBS, 1 % penicillin/streptomycin). 3) VEGF stimulation medium, identical to serum-deprived medium, but with the addition of VEGF (50 ng/mL recombinant human VEGF-A_{165} (R&D Systems, Minneapolis, MN, USA)). The media in all flasks were changed every 2-3 days until the cells were used for further processing.

Reverse transcription quantitative real-time PCR. To assess the effect of VEGF treatment and potential differences between the groups on gene expression level, reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR) was performed on selected genes of interest.

Nucleic acid extraction: The cells were washed with PBS, detached with TrypLE®, centrifuged for

Target	Forward primer	Reverse primer
CD105	AACACCATCGAGCCGGG	GAACTCGGAGACGGATGGG
VEGFR1	ATGCTGGATTGCTGGCACA	TCAAACATGGAGGTGGCATT
VEGFR2	CAGCAGGATGGCAAAGACTACA	GGCAGAGAGAGTCCAGAATCCTC
PDGFRβ	AGGACAACCGTACCTTGGGTGACT	CAGTTCTGACACGTACCGGGTCTC
FOXF1	CACTCCCTGGAGCAGCCGTATG	AAGGCTTGATGTCTTGGTAGGT
vWF	CGGCTTGCACCATTCAGC	CCATCCTGGAGCGTCTCATC
CD31	ACAGCCTTCAACAGAGCCAAC	GAAAGAATGACTCTGACTGTCAGTATT
PPIA	TCCTGGCATCTTGTCCATG	CCATCCAACCACTCAGTCTTG
GAPDH	CAACGGATTTGGTCGTATTGG	GCAACAATATCCACTTTACCAGAGTTAA

Table 1: Primers used for the RT-qPCR analysis. The target of the primers can be divided into stem cell marker CD105, VEGF receptors; VEGFR1, VEGFR2, and PDGFβ, endothelial markers FOXF1, vWF, and CD31, together with reference genes; PPIA and GAPDH. CD: Cluster of differentiation, VEGFR1 and 2: Vascular endothelial growth factor receptor 1 and 2, PDGFβ: Platelet-derived growth factor β, FOXF1: Forkhead box protein F1, vWF: von Willebran factor, PPIA: Peptidylprolyl isomerase A, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

5min at 300g at room temperature, and collected as cell pellets. Total RNA was isolated with Qiagen RNeasy® Mini Kit (Qiagen cat. no. 74106), according to manufacturer's instructions, with the addition of DNase (Qiagen RNase-free DNase Set., cat. no. 79254). The RNA concentration and purity was assessed using spectrophotometry (Nanodrop; Thermo Science, Wilmington, DE, USA). The RNA purity was validated by observing A260/A280 ratios for protein contamination and A260/A230 ratios for ethanol contamination. RNA integrity of two random samples from each extraction was tested using Agilent Bioanalyzer 2100 using RNA Nano Chips (Agilent 5067-1521) according to manufacturer's instructions. The RNA samples were stored at -80°C. Representative results from the Bioanalyzer are depicted in Appendix L.

cDNA synthesis: The reverse transcription reactions were prepared using Affiniscript (AffinityScript QPCR cDNA Synthesis Kit, Stratagene cat. no. 600559) according to manufacturer's instructions. The total reaction volumes were 20µL, consisting of 0,5 µg RNA, 10 µL first strand master mix, 3 µL Oligo (dT) primer, 1 µL Affinity transcript reverse transcriptase, and RNAase-DNAse free water. The reactions were performed with an initial stage of 24°C for 5 minutes, 42°C for 45 minutes and 95°C for 5 minutes on Veriti 96 well fast thermal cycler (Applied Biosystems model no. 9901). Following synthesis, the cDNA was stored at -20°C.

Quantitative real-time PCR: Brilliant II QPCR master mix with low ROX (Agilent technologies cat. no. 600806) was used with a total reaction volume of 25µL in 96-well optical reaction plates (Agilent Technologies, cat. no. 401333) with 5 µL of diluted cDNA. Non-template-controls were included for each primer mastermix. The plate was sealed with optical plastic caps (Agilent Technologies, cat. no. 401425). qPCR was performed using Mx3000 (Stratagene, AH-diagnostics, Aarhus, Denmark) and acquisition was performed by Mx3000 version 4.0 software for Windows (Stratagene, AH-diagnostics, Aarhus, Denmark). The reaction was initiated by heating to 95°C for 10 min, followed by 40 cycles elongation at 60°C for 1 min and denaturation at 95 °C for 30 sec. The products were verified by performing a gel electrophoresis on the products from the wells, on random samples.

Target and reference genes: The genes of interest were stem cell marker CD105, endothelial markers FOXF1, vWF, CD31, and VEGF receptors VEGFR2, VEGFR1, and platelet-derived growth factor β (PDGFRβ), as described in Table 1. VEGFR1 and 2 are also endothelial markers. To check the efficiency of each run, a reference curve was run on each plate, consisting of pooled cDNA diluted to fit a logarithmic curve. Peptidylprolyl isomerase A (PPIA) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were selected as reference genes from a larger panel, based on stability both in terms of donor variation and expression through the treatment. These comparisons were made by GenEx (Multid Analysis AB, Sweden). The special reference gene software subprogram geNorm found PPIA and Beta-glucoronidase to be the best choice of reference gene pair, while the other subprogram, Normfinder, suggested PPIA and GAPDH as the most suitable and stable reference genes. This selection is according to MIQE guidelines for good qPCR practice [42]. The fold changes in gene expression, in relation to 1) the geometric mean of PPIA and GAPDH 2) the expression in complete medium controls, were calculated using the ΔΔCt method, with $2^{\Delta\Delta Ct}$ as the fold change. Sequences for all primers can be found in Table 1, and results from GenEx are shown in Appendix M.

Surface marker expression measured by flowcytochemistry. To quantitatively investigate certain surface markers of the VEGF stimulated

Antibody	Conjugate	Supplier	Cat. No.
CD105	PE	R&D System	FAB10971P
CD90	FITC	Beckman Coulter	PN IM1839U
CD73	PE	BD Pharming	555749
CD13	ECD	Beckman Coulter	A33097
CD45	PC7	Beckman Coulter	IM3548
CD34	APC	Beckman Coulter	IM2472U
CD19	ECD	Beckman Coulter	A07770
CD14	PC7	Beckman Coulter	A22331
HLA-DR	FITC	Beckman Coulter	IM0463U
VEGFR2	APC	R&D System	FAB357A
Tie-2	PE	R&D System	FAB3131P
VE-cadherin	APC	R&D System	FAB9381A
CD31	PE	BD Pharming	555446

Table 2: The antibodies used for flourescent activated cell sorting. The antibodies are targeting stem cell markers; CD105, CD90, CD73, and CD13, blood cell markers; CD45, CD34, CD19, and CD14, the immunological marker; HLA-DR, and endothelial markers; VEGFR2, Tie-2, VE-Cadherin, and CD31. The antibodies were conjugated with (FITC) flourescein isothicyanate, (PE) phycoerythrin, (ECD) phycoerythrin and Texas Red, (APC) allophycocyanin or (PC7) phycoerythrin-cyanin. CD: Cluster of Differentiation, HLA-DR: Human Leukocyte Antigen-DR, VEGFR2: Vascular Endothelial Growth Factor 2, VE-Cadherin: Vascular Endothelial Cadherin.

and un-stimulated ADSCs, FC was performed. The relevant surface markers were those for stem cells CD105, CD90, CD73, and CD13, leukocytes; CD45, endothelial progenitor cells; CD34, B-cells; 19, monocytes; CD14, immune stimulation; HLA-DR, and endothelial markers VEGFR2, Tie-2, VE-Cadherin, and CD31. Details are shown in Table 2. ADSCs from three donors were stimulated as described above. After 1, 2, and 3 weeks of stimulation, the cells were detached using Accutase (PAA Laboratories, Austria), centrifuged, counted, and frozen as described above. On the day of analysis, the cells from all weeks and media types for one donor were thawed and added a fluorescence activated cell sorting (FACS)-PBS solution (FACS-PBS (Hospital pharmacy, Copenhagen, Denmark), 10 % EDTA (Hospital pharmacy, Copenhagen, Denmark), and 1 % new born calf serum (GIBCO, Invitrogen, Taarstrup, Denmark)), centrifuged at 300g for 5 min, and counted as described above. The cells were resuspended in FACS-PBS and added to tubes with or without antibodies for 15min. The antibodies used are described in table 3 together with conjugates. Viability was determined by adding 1 µL of SYTOX to stain dead cells (SYTOX®, Invitrogen, cat. no. MP34857). HUVECs were used as positive controls for staining with endothelial markers. Samples were run on a Navios Flow Cytometer (Beckman Coulter) and analyzed in Navios software and Kaluza (Beckman Coulter).

Surface marker expression visualized by immunocytochemistry. To qualitatively assess the expression of certain stem cell markers and markers of endothelial differentiation, immunocytochemistry (ICC) was performed. The stem cell markers were CD73 and CD90, while the markers for endothelial differentiation were FOXF1, vWF, VE-Cadherin, VEGFR2, and CD31. Details can be found in Table 3. ADSCs from 1 donor were seeded on 4-chamber chamberslides of Permanox™ (Lab-Tek™ Chamber Slides, Thermo Scientific, cat. no. 177437) in a density of 3000 cells/cm2. The cells were stimulated as described above. After 1, 2, and 3 weeks of stimulation, chamberslides were washed with PBS, fixated in 4 % Paraformaldehyde in PBS pH 7.4 (Hospital pharmacy, Copenhagen, Denmark) for 10 min, and incubated with primary antibodies overnight at 4°C. The following day the chamberslides were washed with PBS, and incubated with secondary antibodies for one hour at room temperature. The chambers were removed, and cover slips (Menzel-Glaser, 25x60mm, cat. no. BB025060A1) were mounted using mounting medium (ProLong® Gold Antifade Reagent with DAPI, Molecular Probes®, cat. no. P36931). Visualization was performed using an Olympus IX51 microscope equipped with

Target	Manufacturer	Cat. no.
CD73	Abcam	ab71322
CD90	Stemgent	09-0029
vWF	DAKO	IR527
Tie-2	Santa Cruz	sc-9026
VEGFR2	R&D Systems	AF357
VE-Cadherin	Nordic BioSite	LS-B2138
CD31	DAKO	M0823
FOXF1	Abcam	ab23194

Table 3: The antibodies used immunocytochemistry. The antibodies are targeting stem cell markers; CD90 and CD73, and endothelial markers; FOXF1, vWF, Tie-2, VEGFR2, VE-Cadherin, and CD31. CD: Cluster of differentiation, FOXF1: Forkhead box protein F1, VEGFR2: Vascular endothelial growth factor receptor 2, VE-Cadherin: Vascular endothelial cadherin, Tie-2: TEK tyrosine kinase endothelial-2, vWF: von Willebran factor.

Olympus U-RFL-T fluorescence system. Each antibody was incubated in two wells for each media type. Control for secondary antibody specificity was performed by incubation with secondary antibody with no primary antibody.

***In vitro* angiogenesis assay.** The impact of cell source and media type at functional level was assessed by performing an *in vitro* angiogenesis assay (*in vitro* angiogenesis Assay Kit, Millipore, cat. no.ECM625) to test the ability of the ADSCs to form tubules. ADSCs for each media group from 3 donors were stimulated as described above. After 1, 2, and 3 weeks of stimulation, the cells were washed with PBS, detached using Accutase, and counted. They were seeded with a density of 10,000 cells/well in duplets in a 96 well plate on ECMatrix® prepared according to manufacturer's instructions. The media-type used was similar to the treatment the cells had received, i.e. the ADSCs cultured in complete medium received complete medium on the ECMatrix®. For each well, three pictures were

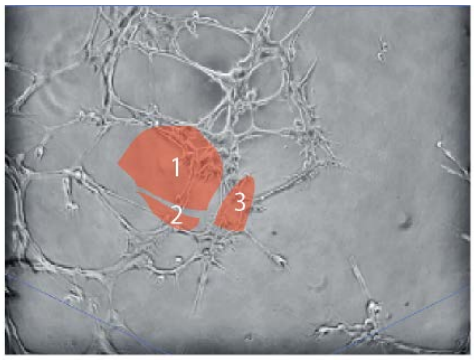


Figure 2: Counting of polygons on pictures from in vitro angiogenesis assay. Only completely defined polygons, defined by surrounding tubules, were counted. Examples of 3 counted polygons are painted red.

captured every four hours with phase contrast microscopy using 10X objective on Olympus IX51 microscope equipped with an Olympus TL4 halogen light source. For quantification, the numbers of polygons created, by gathering of tube-like ADSCs, were counted. This was done manually after obscuring the picture name, thereby blinding the person performing the counting.

Proliferation assay. Proliferation was assessed using Bromodeoxyuridine (BrdU) cell proliferation ELISA Kit (Roche®, cat. no. 11647229001). ADSCs from three donors were seeded in 96-well plates with a density of 1,000 cells/well in complete medium. After adhering overnight, BrdU was added to each well to reach a concentration of 10 µM. Media and BrdU labeling was changed after 48 hours, and additional BrdU was added to the media after 72 hours. The BrdU reaction was stopped by tapping the media from the wells and drying them. Fixation and addition of antibodies, washing solution and substrate solution was performed according to manufacturer's instructions. After stopping the substrate reaction with 25µL 1M H_2SO_4 (vWR, cat. no-30149291), the plate was shaken on IKA VIBRAX VXR basic with 500 rpm for 20sec, and the absorbance was measured using Bio-Rad micro plate reader Model680, with absorption

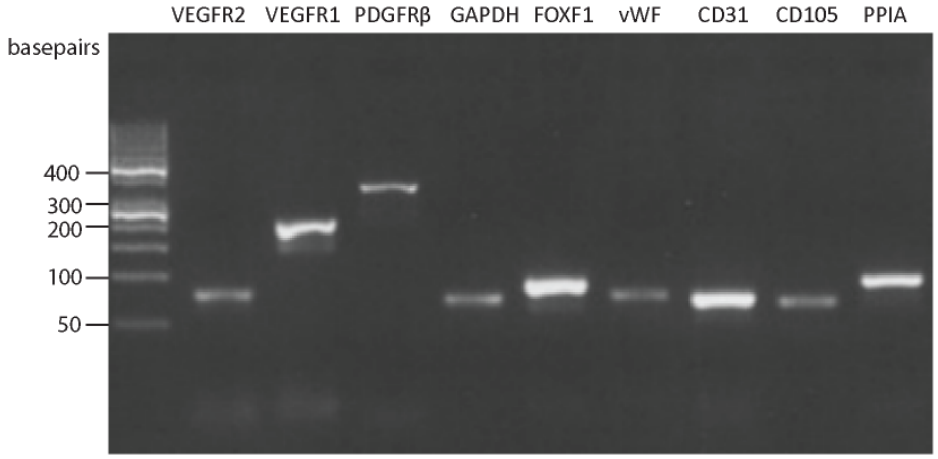


Figure 3: Representative gel electrophoresis of products of RT-qPCR reaction. Cluster of differentiation, VEGFR1 and 2: Vascular endothelial growth factor receptor 1 and 2, PDGFβ: Platelet-derived growth factor β, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, FOXF1: Forkhead box protein F1, vWF: von Willebran factor, PPIA: Peptidylprolyl isomerase A.

measured at 450nm and background absorption at 655nm.

Statistics. The sample sizes for the BrdU, ECMatrix®, and FC experiments were too small to perform proper statistical analysis, and the results from these experiments are therefore estimated from the figures. When comparing the effect of culture medium, a paired t-test was performed between the different types of medium for each week and each gene. When data was not normally distributed, tested using Kolmogorov-Smirnov and Shapiro-Wilk tests for normality, the non-parametric Wilcoxon signed rank test was performed. The same tests were used to compare the effect of culture time for each donor for each gene. When comparing ADSCs from IHD patients with those from healthy donors, an independent samples t-test, or the non-parametric Mann-Whitney U-test, was performed. For all comparisons, $p < 0.05$ was considered significant.

Comparison with healthy donors. A parallel study was performed in our group, using ADSCs from healthy donors. The donors were two male and five female and in the age 38 ± 12.3 years. The results of these are compared in results and discussion.

4. Results

Verification of RT-qPCR product. The products from random RT-qPCR runs were loaded for gel electrophoresis. As shown in Figure 3, product was obtained from all genes of interest and reference genes.

Effect of media-type and stimulation time on mRNA level. When comparing the effect of the different types of media, significant differences were only found when comparing with ADSCs cultured in complete medium, represented by the baseline on the graphs in Figure. For endothelial markers, the expression of FOXF1 was significantly increased in ADSCs for all weeks cultured in serum-deprived medium and VEGF stimulation medium compared with ADSCs cultured in complete medium ($p < 0.05$). Expression of vWF was significantly increased in ADSCs stimulated with VEGF for 2 weeks, and no increase was observed for CD31. For VEGF receptors, expression of PDGFRβ was significantly increased in ADSCs cultured in serum-deprived medium and VEGF stimulation media ($p < 0.05$),

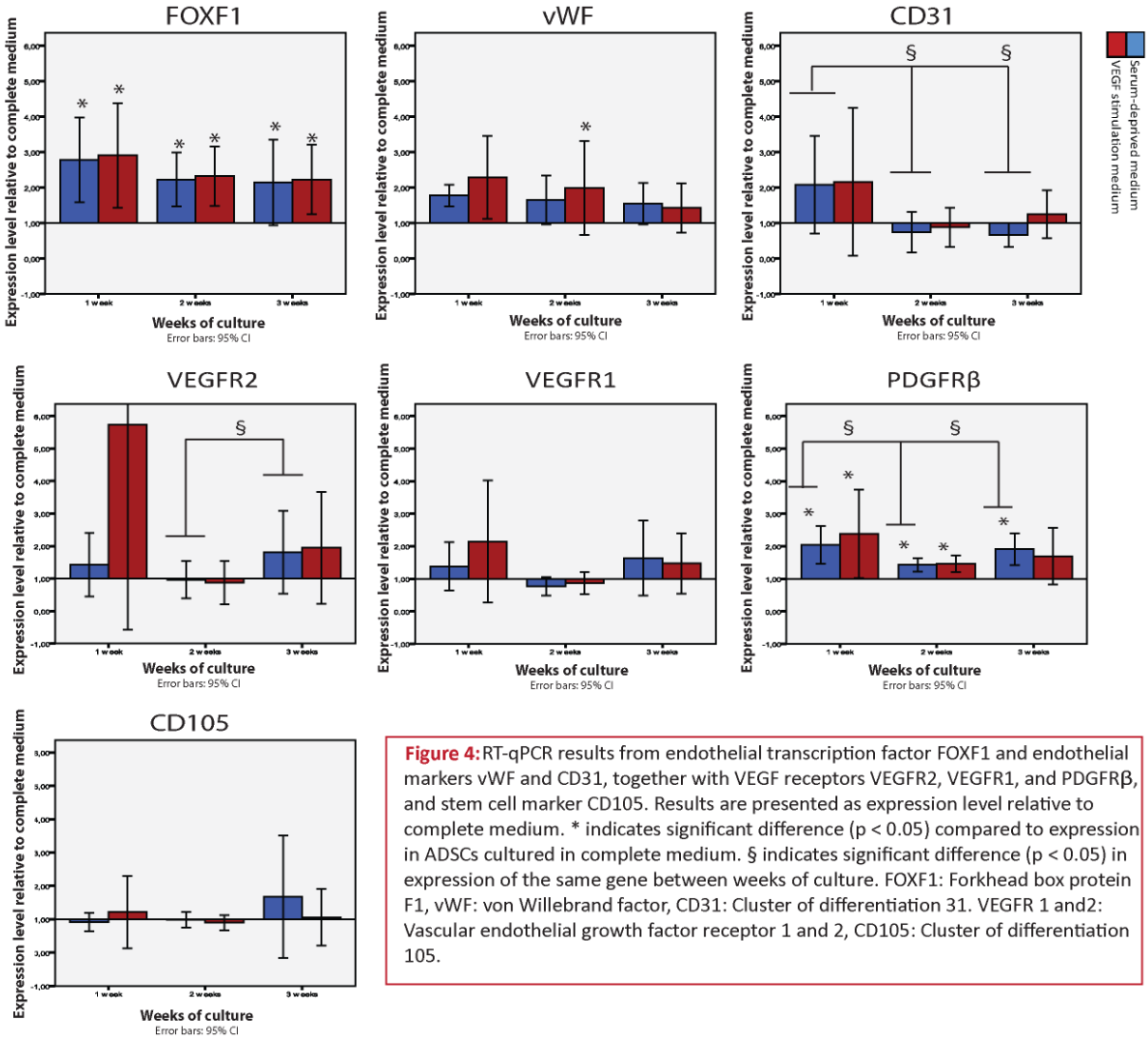


Figure 4: RT-qPCR results from endothelial transcription factor FOXF1 and endothelial markers vWF and CD31, together with VEGF receptors VEGFR2, VEGFR1, and PDGFRβ, and stem cell marker CD105. Results are presented as expression level relative to complete medium. * indicates significant difference ($p < 0.05$) compared to expression in ADSCs cultured in complete medium. § indicates significant difference ($p < 0.05$) in expression of the same gene between weeks of culture. FOXF1: Forkhead box protein F1, vWF: von Willebrand factor, CD31: Cluster of differentiation 31. VEGFR 1 and2: Vascular endothelial growth factor receptor 1 and 2, CD105: Cluster of differentiation 105.

compared with ADSCs cultured in complete medium. This is significant for all weeks of culture except for ADSCs stimulated with VEGF for 3 weeks. The expression of VEGFR2, VEGFR1, and CD105 was not significantly increased, as shown on Figure 4. All markers, except CD105, seemed to be increased in ADSCs cultured in serum-deprived medium and VEGF stimulation medium compared to ADSCs cultured in complete medium, but this difference was not significant for most comparisons of vWF and all for CD31, VEGFR2 and VEGFR1. Comparing the effect of culture time on the expression of before mentioned markers, only ADSCs cultured in serum-deprived medium exhibited significant differences. The expression of CD31 decreased from week 1 to week 2 and 3. In addition, expression of VEGFR2 and PDGFRβ was increased from week 2 to week 3. Mean Cq-values for ADSCs and HUVEC controls are shown in table 4.

	FOXF1	VEGFR2	vWF	CD31
ADSC Week 1	31.2 ± 1.3	34.4 ± 1.2	30.8 ± 1.1	32.7 ± 2.2
ADSC Week 2	30.6 ± 0.9	34.8 ± 1.6	29.9 ± 0.8	32.3 ± 1.2
ADSC Week 3	30.8 ± 0.8	35.1 ± 0.9	30.4 ± 0.7	33.7 ± 1.2
HUVEC	32.9 ± 0.1	28.4 ± 0.9	18.6 ± 0.0	23.8 ± 0.6

Table 4: Cq-values for endothelial markers from RT-qPCR reactions for ADSCs and HUVEC. Cq-values are means of ADSCs cultured in VEGF stimulation medium and serum-deprived medium, with the same seven donors in each group. Cq-values for HUVEC were collected from one RT-qPCR control reaction. ADSC: Adipose-derived stem cell, HUVEC: Human umbelical vascular endothelial cells, Cq: Quantification cycle.

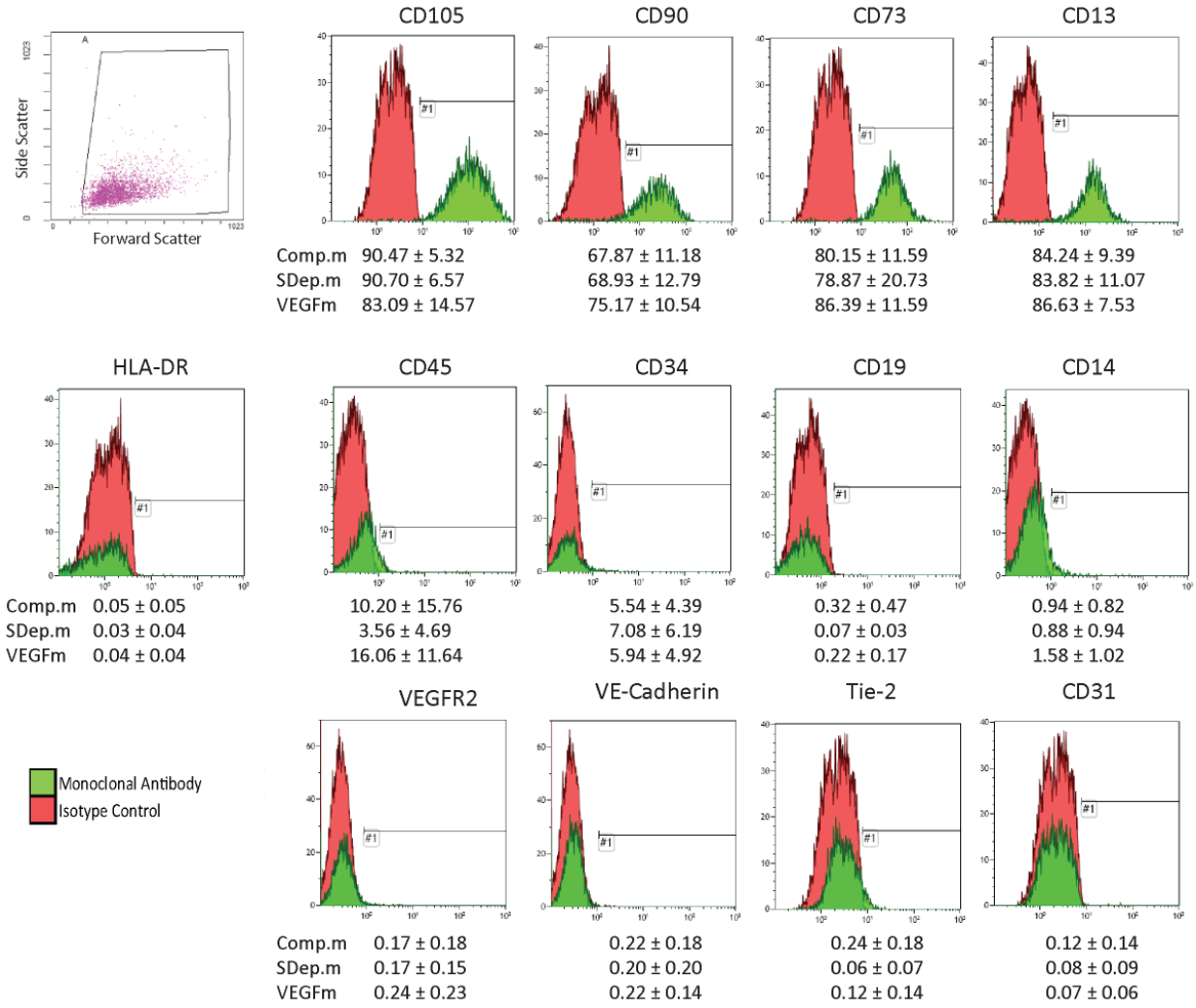


Figure 5 : Flowcytometric analysis of ADSCs cultured in different types of media. For cells cultured in serum-deprived medium are shown representative scatterplot and histograms for stem cell markers CD105, CD90, CD73, and CD13, immune stimulation marker HLA-DR, leukocyte marker, CD45, endothelial progenitor marker CD34, B-cell marker CD19, monocyte marker CD14, and endothelial markers from a representative donor. The percentage of positive cell staining for each media type is indicated below each figure. 10 %: Complete medium, 2 %: 2 % control medium, VEGF: VEGF stimulation medium. Positive cell staining is indicated by green, while isotype controls are indicated by red. CD: Cluster of Differentiation, HLA-DR: Human Leukocyte Antigen-DR, VEGFR2: Vascular Endothelial Growth Factor 2, VE-Cadherin: Vascular Endothelial Cadherin, Tie-2: TEK tyrosine kinase endothelial-2. Comp.m: Complete medium, SDep.m: Serum-deprived medium, VEGFm: vascular endothelial growth factorstimulation medium.

Effect of stimulation on stem cell characteristics and endothelial markers on FC. For all markers, the expression seemed to be equal across all weeks of stimulation. As a consequence of this, the summarized mean percentage expression values were calculated using data from all weeks for each media type. These are shown in Figure 5, together with scatterplot and histograms for each marker, from a representative sample. From the means, it seems the percentage of CD90+ cells is increased for VEGF stimulated ADSCs, while CD45+ cells are less abundant for ADSCs in serum-deprived medium. The sample size was too small for proper statistical analysis. Generally, the expression of stem cell markers CD105, CD90, CD73 and CD13 was high, while expression of HLA-DR, CD19 and CD14 was close to zero. Similarly, endothelial markers VEGFR2, Tie-2, vWF and CD31 were close to zero, while CD45 and CD34 were expressed in low percentages. Percentages for all measurements are shown in a table in Appendix O.

Expression of endothelial markers visualized by ICC. The expression of endothelial markers FOXF1, VEGFR2, vWF, Tie-2, VE-Cadherin and CD31, together with stem cell markers CD90 and CD73, were assessed using ICC. The stem cell markers were expressed equally regardless of culture medium and weeks of culture. FOXF1 could be detected on cultured in complete medium for 3 weeks, and VEGF stimulation medium for 1 and 3 weeks. VEGFR2 was positive on ADSCs stimulated with VEGF for 1 week. Tie-2 was detected on ADSCs stimulated with VEGF through 1, 2, and 3 weeks, and cells in complete medium through 1 and 3 weeks. vWF was positive on cells stimulated 1 week with VEGF and 2 weeks with complete medium. VE-Cadherin was positive on ADSCs stimulated with VEGF or cultured in complete medium for 1 week. CD31 was not positive on any cells. Representative pictures of positive marker staining are illustrated in Figure 6, and all pictures can be found in Appendix P.

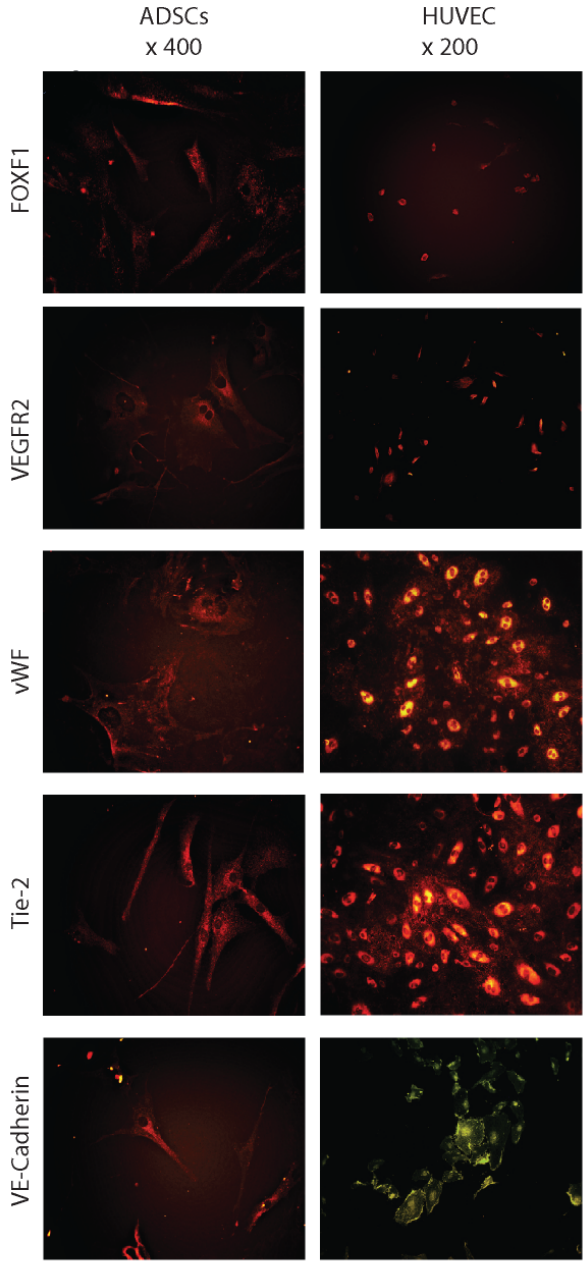
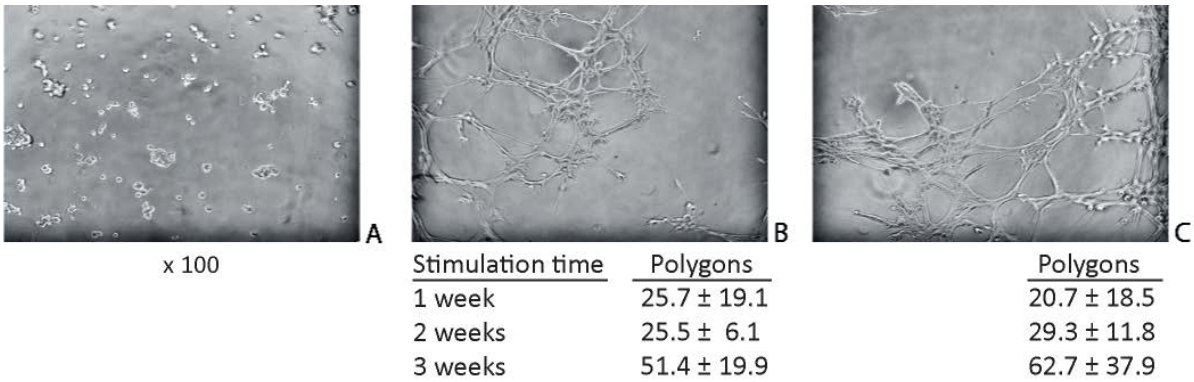


Figure 6: Representative images of ADSCs stained positive for stem cell and endothelial markers on immunocytochemistry. ADSC: Adipose-derived stem cells, HUVEC: Human umbilical vascular endothelial cells, FOXF1:, VEGFR2: Vascular endothelial growth factor receptor 2, VE-Cadherin: Vascular endothelial cadherin, Tie-2: TEK tyrosine kinase endothelial-2, vWF: von Willebrand factor, CD31: Cluster of differentiation 31.

Effect of media-type and stimulation time on *in vitro* angiogenesis. The ability of the ADSCs to form tubules was assessed by performing an *in vitro* angiogenesis assay, using ADSCs from three donors. There was almost no tubulogenesis for the cells stimulated with complete medium, containing 10 % FBS. The cells would gather in spheres rather than tubes, and polygons were seldom observed. A representative example of this is shown in Figure 8. Since the tubulogenesis for these cells were regarded as close to zero, with few exceptions, the counting for ADSCs with complete medium was excluded for two thirds of the experiment. The number of donors was not sufficient for proper statistical analysis. The largest mean number of polygons was at four hours, and these numbers are presented for each week together with pictures for each media type in Figure 8. The means do not suggest a difference in number of polygons between ADSCs in serum-deprived medium and VEGF stimulation medium. However, it seems that ADSCs culture time affects their ability to assemble into polygons, with longer culture time resulting in increased number of polygons. A graph for all polygon counts is in Appendix Q.



Stimulation time	Polygons	Polygons
1 week	25.7 ± 19.1	20.7 ± 18.5
2 weeks	25.5 ± 6.1	29.3 ± 11.8
3 weeks	51.4 ± 19.9	62.7 ± 37.9

Figure 7: Representative pictures of tubulogenesis of ADSCs on ECMatrix® after 4 hours, together with mean number of polygons from each week after 4 hours. ADSCs cultured in complete medium (A), serum-deprived medium (B), and VEGF stimulation medium (C). ADSC: Adipose-derived stem cell.

Decreased proliferation spurt for ADSCs from IHD patients. The proliferation rate was measured with BrdU incorporation over 96 hours for ADSCs from three IHD patient and three healthy donors. The number of donors was not sufficient for proper statistical analysis. However, the mean proliferation curve, depicted in Figure 8, suggests a decreased initial growth spurt for ADSCs from IHD patients compared to healthy donors. Furthermore, the proliferation of ADSCs from both groups seemed to increase until day three after labeling, equal to day four after seeding.

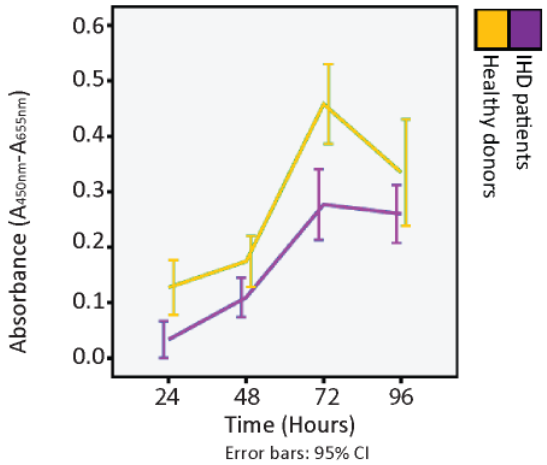


Figure 8: ADSC proliferation measured by absorbance at 450nm – absorbance at 655nm using a colorimetric BrdU assay. ADSCs from IHD patients were compared to ADSCs from healthy donors. BrdU: Bromodeoxyuridine, IHD: Ischemic heart disease, ADSC: Adipose-derived stem cell.

Effect of VEGF treatment on healthy donors. The data from the parallel study using ADSCs from healthy donors, produce similar results as those mentioned above. The same trends were present for comparison of media and culture time for each assay. FOXF1 exhibited decreased expression at mRNA level in ADSCs from healthy donors compared to ADSCs from IHD patients, when cultured for 2 weeks in serum-deprived medium. This was the only significant difference between the two groups, and no clear tendencies were observed in this aspect. Figures for results from healthy donors can be found in Appendix N-Q.

5. Discussion

The purpose of this study was to investigate the effect of VEGF treatment on ADSCs from IHD patients, in terms of endothelial marker expression, tubulogenic abilities, and preservation of stem cell characteristics. On mRNA level, the expression of FOXF1, a transcription factor for endothelial markers, and PDGFRβ were significantly increased. In addition, a non-significant trend of increase in expression of the other endothelial markers was observed. The increase was only observed when comparing

with ADSC cultured in complete medium, while ADSCs in serum-deprived medium exhibited similar changes. On protein level, endothelial markers were sporadically visualized, but the percentages were too low for detection with FC. On the functional level, *in vitro* angiogenesis was only observed for ADSCs cultured in VEGF stimulation medium and serum-deprived medium, with equal tubulogenesis for the two groups. Finally, the ADSCs preserved their stem cell characteristic markers throughout the treatment, with no variation between the stimulation groups. Generally, there was a difference between VEGF treatment and culture with complete medium. However, culture with serum-deprived medium produced similar effects on the ADSCs.

Predisposition towards endothelial lineage in response to VEGF treatment and serum-deprivation. We expected the expression of endothelial markers of endothelial differentiation to be increased on all levels in ADSCs stimulated with VEGF compared to culture in complete medium. However, in contrast to our expectations, the treatment with VEGF did not up-regulate endothelial markers when compared with serum-deprived medium. Hence, the observed effects are due to serum-deprivation rather than the VEGF treatment.

Response on mRNA level. Serum deprivation significantly increased the expression of the endothelial lineage-associated transcription factor FOXF1 and the VEGF receptor PDGFRβ. It was expected that FOXF1 would be the first marker to be expressed, since it is a transcription factor with VEGFR2 as a downstream target [27]. The additional endothelial markers exhibited a tendency towards increased expression, but this tendency was not significant. When comparing the expression levels of FOXF1 and PDGFRβ to the other endothelial markers, except CD31, the means of the relative fold increase in expression of PDGFRβ and FOXF1 is not due to higher values, but seems to be due to a lower variation of data. Hence, it could seem that effect of serum-deprivation on ADSCs doubles the expression of endothelial markers, but the effect is too small and the variance too large for these findings to be significant. Comparing Cq-values, the traditional endothelial markers were detected relatively late in the RT-qPCR reaction compared with HUVEC controls, indicating a huge difference in expression. HUVECs were not expected to express higher amounts of FOXF1, since this transcription factor is active during development [27]. The detection of the markers was close to the limit of detection. However, all of the products from the RT-qPCR reaction were verified using gel electrophoresis, except for VEGFR2 for some samples, indicating very low levels of VEGFR2. This is consistent with *Ball et al.* who found no VEGFR2 and VEGFR1 on MSCs. Furthermore, a study by *Ball et al.*, found the proliferative and migratory effect of VEGF on MSCs to be mediated by PDGFRβ, which we found to be significantly increased in serum-deprived ADSCs. This could be a cellular response initiated to accommodate the need for additional VEGF for migration and proliferation. Regarding the effect of prolonged culture, there was no clear tendency for the expression of FOXF1 or PDGFRβ to increase with culture time. The lack of increased expression during culture, could indicate that the serum-deprivation prepare the ADSCs for differentiation towards endothelial lineage, but is not sufficient to start the final process.

Response on protein level. Endothelial markers expression on protein level was only detected by ICC, and not by FC. ICC is qualitative, and positive signal only indicates that the epitope is present in the culture, and not to which extent. Since the FC results were negative (< 1 %) for Tie-2, VE-Cadherin, and VEGFR2, which are visible by ICC,

the markers could be expressed in very low quantity. This could be due to heterogeneity of the population, with some cells being affected more by the treatment than others.

Response on functional in vitro angiogenesis. The most obvious effect of serum-deprivation was found on *in vitro* angiogenesis potential, with an indication of serum having an inhibitory effect in this aspect. There seemed to be an increase in number of polygons with increasing culture time for both the ADSCs in the VEGF stimulation medium and serum-deprived medium. This trend could also be due to an effect of the complete medium used for counting the cells prior to seeding on ECMatrix®, since the cell count was lower for the first weeks of culture. If this is the case, it indicates that the tubulogenesis of the ADSCs is not due to the preconditioning of the cells, but rather a consequence of the media in which they are seeded on the ECMatrix®.

Predisposition of ADSCs towards endothelial lineage as result of serum-deprivation. As mentioned above, the serum-deprivation resulted in a significant increase in FOXF1 expression and non-significant tendencies of a two-fold increase in the other endothelial markers on mRNA level. Together with the sporadic traces of endothelial markers on protein level, and the quick tubulogenesis response, it points towards a predisposition of the ADSCs towards endothelial lineage, as a result of serum-deprivation. The fact that the ADSCs readily formed tubules when seeded on ECMatrix® indicates that they are fully capable of differentiating into endothelial morphology, but needs other stimuli in addition to VEGF treatment. This is supported when reviewing the literature for endothelial differentiation of ADSCs. Several other groups have successfully differentiated ADSCs towards endothelial lineage with the use of VEGF treatment, but always in combination with additional stimuli. *Fischer et al.*, used endothelial cell growth supplement and shear force to differentiate ADSCs [43]. *Miranville et al.* and *Zhang et al.* both found expression of CD31 and vWF after stimulating ADSCs with VEGF as supplement for endothelial growth medium together with insulin-like growth factor and endothelial growth medium-2, respectively [36, 44]. These media contain many different growth factors, which could assist VEGF in driving the cells through differentiation. In addition to growth factors, several studies have seeded ADSCs on a substrate, such as ECMatrix® or Matrigel, providing mechanical stimuli for differentiation [26, 45].

This limited response to VEGF of the ADSCs was not expected, since *Haack-Sorensen et al.* successfully induced the expression of VEGFR2, vWF, and CD31 on both mRNA and protein level in MSCs with only one week of similar stimulation with VEGF [21]. In addition, the same amounts of VEGF have been used by *Nourse et al.* to differentiate embryonic stem cells towards endothelial lineage, suggesting a general response of stem cells to the VEGF stimulation [46]. *Haack-Sorensen et al.* used similar methods as in this study to differentiate MSCs towards endothelial lineage [21]. This indicates that ADSCs does not exhibit a response to VEGF on the same scale as MSCs, in terms of differentiation towards endothelial lineage.

Retained stem cell characteristics during treatment. We expected that the ADSCs maintained their stem cell characteristics, based on markers from the ISCT criteria, compared with the controls cultured in complete medium. We found no difference in expression of stem cell markers on mRNA level using RT-qPCR and on protein level using ICC for any of the media groups. The percentage of the other markers, closely resembles the percentages presented in the ISCT criteria, despite low sample size, low cell count and severe cell death. The percentages of

the stem cell markers were not quite met, and the percentages of CD45 and CD34 positive cells were higher than anticipated. However, The ISCT criteria are defined on the basis of MSCs and not ADSCs, and though these are comparable, their protein expression profiles are not identical [9, 13]. *Noel et al.* made this comparison, and found their ADSC population to be CD34 positive. CD34 positivity indicates the presence of endothelial progenitor cells, which could explain the sporadic presence of endothelial markers on ICC. The high percentage of CD45+ cells correlated with a high number of dead cells, as defined by SYTOX uptake. The remaining dead cells do not seem to interfere with the results from the other markers. This could indicate unspecific binding of the CD45 antibody to intracellular components, since staining with SYTOX is an indication of increased cell membrane permeability. This would be a plausible explanation for high CD45 signal after several weeks of culture, during which the CD45+ leukocytes would normally have been washed away. When taking this into consideration, the ADSCs from both IHD patient and healthy donors seem to retain their mesenchymal stem cell characteristic markers, as defined by ISCT, during the culture with the different types of media, including treatment with VEGF. To fully fulfill the ISCT criteria, differentiation into adipogenic, osteogenic, and chondrogenic lineage would have to be performed for ADSCS cultured in each media type. This could also provide additional evidence of serum-deprivation resulting in endothelial predisposition, if the ADSCs from these media groups were more reluctant to differentiate into adipocytes, osteocytes and chondrocytes.

Comparison with ADSCs from healthy donors. We hypothesized, that there would not be any change in the effect of VEGF treatment between ADSCs from IHD patients and healthy donors, but that there would be a decreased proliferation rate of ADSCs from IHD patients. In terms of differentiation potential, ADSCs isolated from IHD patients and healthy donors exhibited equal response to VEGF treatment and equal tubulogenesis on ECMatrix®. This is similar to the results on MSCs by *Friis et al* [40]. However, since the treatment did not affect the ADSCs as much as MSCs, it is difficult to conclude on differences in this aspect. *Alt et al.* observed decreased osteogenic and adipogenic differentiation potential of ADSCs from aged persons, but no comparisons have been made with regard to endothelial differentiation. We found evidence of a decreased initial growth spurt, but not in general proliferation rate, of ADSCs from healthy donors compared to ADSCs from IHD patients. This result is consistent with *Chen et al.*, showing slightly decreased clonogenic capacity of ADSCs from elderly donors [35]. The same study observed less effect of age and osteoporosis on ADSCs compared to MSCs. This is in contrast with our proliferation results, when combined with *Friis et al*, who found no difference in proliferation between two donor groups similar to those used in this study [40]. The decreased proliferation was not as pronounced as expected, since heart disease has been shown to affect the proliferation potential of both BM-MNCs and EPCs [38, 39]. No study so far has investigated the effect of proliferation of ADSCs from age-matched healthy donors and donors with heart disease. As discussed by *Friis et al.*, it would be interesting to perform the same study on cells isolated from IHD patients without co-morbidities, to observe the isolated effect of IHD on ADSCs [40]. However, the patient group receiving stem cell therapy includes many different co-morbidities, and this setup is therefore the most clinical relevant.

6. Conclusion

This study found evidence of ADSC differentiation towards endothelial lineage when cultured in serum-deprivation, with no additional effect of VEGF. The effect was subtle, with significant increase of only the earliest marker of endothelial differentiation on mRNA level. The serum-deprivation rendered the cells prone to readily form tube-like structures on ECMatrix®, and did not affect the expression of traditional stem cells markers. VEGF stimulation seems to predispose ADSCs for differentiation towards endothelial lineage, but the cells need additional stimuli to complete the process.

7. Perspectives

The study was performed as *in vitro* investigation of methods being applied in an ongoing clinical trial. The fact that the ADSCs did not differentiate *in vitro*, does not mean they will not do it *in vivo*. Many additional stimulants are present *in vivo*, such as additional growth factors and altered substrate rigidity, as with the ECMatrix® which seemingly resulted in fast differentiation. If results of the clinical studies show an effect of the VEGF treatment prior to transplantation, additional studies should be conducted in order to further elucidate the difference between VEGF treatment and serum-deprivation. If serum-deprivation is sufficient to achieve similar results, there would be no need for the VEGF supplement. There could however be another effect of VEGF treatment, such as differences in paracrine profile, which we did not investigate. A recent study by *Yan et al.*, found that VEGF stimulated ADSCs secreted increased amount of VEGF themselves [47]. Generally, there has been a change of paradigm in the field of stem cells, towards secreted factors being their main effect instead of differentiation [48]. This has been confirmed in several experiment showing equal effects of transplantation and administration of conditioned medium from MSCs [49]. Furthermore, when comparing our results with *Haack-Sorensen et al.,* it is evident that MSCs differentiate more readily towards endothelial lineage than ADSCs, when treating with VEGF [21]. Further investigation is needed to elucidate if MSCs are generally more prone to perform endothelial differentiation than ADSCs. This could affect the future development of vascular grafts.

8. Acknowledgements

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Appendix A: Inclusion and exclusion criteria for IHD patients

Inclusion criteria

- Age between 30-80 years
- Moderate-to-severe angina or angina-equivalent dyspnea despite optimal medical therapy
- Significant vessel decrease documented by coronary angiography within 12 months of screening
- Not eligible for other revascularization procedures
- Left ventricular ejection fraction > 40 % (measured by echocardiography, SPECT, CT or MRI)
- 2-10 min duration of bicycle exercise tolerance test
- CABG or PCI within 6 months of entry and ruled out re-stenosis by angiography.
- Ventricular wall thickness of treatments zone > 7mm (measured by echocardiography, CT or MRI).

Exclusion criteria

- Pregnant or lactating women
- Clinically significant anemia, leucopenia, leukocytosis or thrombocytopenia
- Conditions other than angina limiting exercise test
- Immunocompromised patients
- Valvular heart disease requiring surgical treatment
- Acute coronary syndrome less than6 weeks prior to screening
- Suspicion of current malignancy or history of malignancy < 5 years prior to screening
- Other experimental medications within 4 weeks prior to exercise test.

The screening was performed by at least two experienced interventional cardiologists.

Appendix B: Protocol for isolation of ADSCs

Materials:

- DMEM Low glucose 1g/L with glutamine and 25 mM HEPES (PAA E15-808)
- Penicillin-streptomycin (1,000 units/mL penicillin Gsodium, 10,000 µg/mL streptomycin sulfate (Gibco 15140-122)
- Fetal Bovine Serum, Pharma Grade Gamma Irradiated AUS Origin (PAA A15-512)
- HBSS 10 x (14065, Gibco, Invitrogen, 13mM $CaCl_2$)
- Collagenase NB (lot: 070827) approximately 0.12 U/mg (SERVA electrophoresis GmbH, Heidelberg, Germany)
    - Diluted with to working solution at 0.6 PZ U/mL with HBSS
- PBS pH 7.4 (Gibco, cat. no. 10010-015)
- Serres suction system
- 50 mL conical centrifuge tube Falcon
- Cell scraper (TPP, vWR, cat. no. 99002)
- Mini incubator (Labnet, in vitro)
- 100 µm filter (Cell Strainer, BD Falcon, cat. no. 352360)
- Nucleocounter® NC-100™ (Chemometec, Allerød, Denmark)

Method:

1. Note patient ID, Lab no., date and initials
2. Control coherence between name and personal security number on the received adipose tissue and etiquette
3. Suck the liquid phase away
4. Note volume of total lipoaspirate
5. Mix 1:1 volume of PBS with lipoaspirate in a bottle and shake it
6. Allow phase separation for 2min
7. Remove liquid phase by suction by Serres suction system
8. The adipose tissue is transferred to 50 mL centrifuge tubes and is centrifuged for 5min at 300g and room temperature.
9. Transfer 25 mL adipose tissue to 50 mL centrifuge tubes
10. Add Collagenase working solution 1:1 and incubate approximately 45min while rotating in mini incubator
11. Divide 25 mL solution to centrifuge tubes and neutralize Collagenase by adding 1:1 complete medium
12. Filter through 100µm filter into centrifuge tubes
13. Centrifuge for 10min at 1200g at room temperature and discard supernatant
14. Re-suspend cell pellet in 5mL complete medium, collect all pellets in one suspension
15. Perform cell count on the nucleocounter by collecting 120 µL of the cell suspension in an eppendorph tube
    a. Use the nucleocounter cassette to count dead cells from this suspension
    b. Take 35 µL of the remaining cell suspension into a new vial. Add 35 µL of reagent A and mix well before adding 35 µL of reagent B and use a nucleocounter cassette to perform a total cell count
16. Plate the mono nuclear cells in a density of 300,000 cells/T75 flask in 20mL complete medium and incubate at 37°C and 5 % CO_2.
17. Change medium the following day, using Serres suction system, to remove hematopoitic cells.
18. Wash with 10mL PBS and add 15mL complete medium
19. Change medium twice a week
20. When a confluence level of 80 % is reached

Appendix C: Protocol for thawing, stimulation, and freezing of ADSCs

Materials:

- DMEM Low glucose 1g/L with glutamine and 25 mM HEPES (PAA E15-808)
- Penicillin-streptomycin (Gibco 15140-122)
- Fetal Bovine Serum, Pharma Grade Gamma Irradiated AUS Origin (PAA A15-512)
- PBS pH 7.4 (Gibco, cat. no. 10010-015)
- Serres suction system
- TrypLE Select (Gibco 12563-029)
- 50 mL conical centrifuge tube Falcon
- T75 flask
- VEGF

Methods:

Thawing

1. Prepare a T75 flask with 15mL complete medium
2. Quickly thaw vial in 37°C water bath until ~90 % of the cell suspension is liquid
3. Transfer cell suspension to a T75 flask, by first adding 1mL complete medium, re-suspending, and transferring 1mL suspension
    a. Add 1mL to the remaining cell suspension before transfer
    b. Be gentle when re-suspending
4. Change medium the following day

Stimulation

1. When a culture reaches a confluence sufficient for the experimental setup, remove the medium using Serres suction system
2. Wash with PBS
3. Add 3mL TrypLE and incubate until cells have detached
4. Inactivate TrypLE by adding 7mL complete medium to the flask
5. Transfer the suspension to a centrifuge tube and centrifuge at 300g for 5min
6. Discard the supernatant and re-suspend in 2-3mL complete medium
7. Count the cells using nucleocounter®, according to manufacturer's instructions
8. Seed 300.000 cells/flask in 15mL complete medium, incubate, and change medium twice a week
1. When cells reach 80 % confluence, remove the medium using Serres suction system and wash with PBS
2. Mix a medium like the complete medium, but with only 2 % FBS
3. Calculate how much stimulation medium is needed, pour this amount into a bottle, and add 50µL VEGF for each 100mL 2 % medium
4. Use 15mL stimulation medium for each T75 flask

Freezing

1. Wash cells with PBS
2. Add 3mL Accutase and incubate until cells have detached
3. Inactivate with complete medium, centrifuge at 300g for 5 min and discard supernatant
4. Count on nucleocounter
5. Add 1mL freezing medium (FBS and 5% DMSO)
6. Freeze in -80°C for 24 hours and freeze in liquid nitrogen afterwards

Appendix D: Protocol for RNA isolation and purification

Materials:

- Complete medium
  - DMEM Low glucose 1g/L with glutamine and 25 mM HEPES (PAA E15-808) with 10 % FBS and 1 % penicillin/streptomycin
- RNeasy Mini Kit (Qiagen, cat. no. 74106)
- Ethanol 70% (Merck cat. no. 1388543747)
- RNase-free Dnase set (Qiagen, cat. no. 79254)
- Microcentrifuge (Spectrafuge 24D, series no. D612990)
- TrypLE Select (Gibco 12563-029)
- PBS 1x pH 7,4 (Gibco, cat. no. 10010)
- Syringe 1 mL (B.Braun Melsunger AG, Omnifix-F 1mL, cat. no. 161406V)
- Needle 25G 0,5 mm x 16 mm (BD Microlance, cat. no. 300600)
- Rnase AWAY (Molecular Bioproducts Inc., cat. no. 7000)
- DNase (Qiagen RNase-free DNase Set., cat. no. 79254)

Methods:

1. Wash the cells with PBS
2. Detatch the cells using TrypLe for approximately 10min at 37^{o}C
3. Neutralize TrypLe with 7mL complete medium
4. Centrifuge for 5min at 300g and room temperature and discard supernatant
5. Add 350 µL RLT-buffer to pellet
6. Pass the lysate 10 times through needle
7. Add 350 µL of 70 % ethanol and mix
8. Transfer 700 µL sample to Rneasy MinElute spin/spin column and centrifuge at 10.000 rpm for 20sec and discard flowthrough
9. Add 350 µL RW1 buffer and centrifuge at 10.000 rpm for 20sec and discard flowthrough
10. Mix 10 µL DNase 1 stock solution with 70 µL RDD buffer and mix gently
11. Add DNase solution (80 µL) directly on the membrane of the column and let it work for 15min at room temperature
12. Add 350 µL RW1 buffer to the column and centrifuge at 10.000 rpm for 20sec and discard flowthrough
13. Add 500 µL RPE buffer and centrifuge at 10.000 rpm for 20sec and discard flowthrough
14. Add 500 µL RPE buffer and centrifuge at 10.000 rpm for 2min and discard flowthrough
15. Place the column in a new collecting tube and centrifuge with open lit at max rpm for 5min and discard flowthrough
16. Place the column in a 1.5 mL eppendorf tube (Rnase-free) and add 30 µl RNase-free water to the middle of the membrane. Let the column be at room temperature for 10min, then centrifuge at 10.000 rpm for 1min
17. Place the eluate on ice
18. Measure the RNA concentration and purity on the the Nanodrop 1000

a. The purity of the RNA is assessed by the A260/A280 ratio, which must be in the range of 1.7-2.1
b. In addition, the A260/A230 ratio must be approximately 2.0

19. Print copy and save backup on the computer (as full-report)
20. Freeze the eluate at -80^{o}C

Appendix E: Protocol for cDNA synthesis

Materials for each RNA sample:

- strips
- caps
- 0.5 µL RNA
- Assay materials
  - X µL RNase-free water
  - 10 µL cDNA synthesis mastermix
  - 3 µL Oligo (dT) primer
  - 1 µL AffinityScript RT RNase block enzyme mixture

Methods:

1. Insert values for RNA concentration (from Nanodrop during RNA purification) into the cDNA Excel calculation table to calculate the needed amount of RNA for the reaction
2. Clean workspace with DNase-away
3. Mix the different reagents in the …
4. Insert the .. in the ABI's Veriti 96 well fast thermal cycler and run a program with following settings:
   a. Stage 1: 25°C for 5min
   b. Stage 2: 42 °C for 45min
   c. Stage 3: 95 °C for 5min
   d. Stage 4: 4 °C
5. The product is stored at -20 °C

Appendix F: Protocol for qPCR

Materials:

- 96-well optical reaction plates (Agilent technologies, cat. no. 401333)
- Plastic caps (Thermo Scientific flat cap strip, cat. no. AB-0783, natural)
- Forward and Reverse primers
- Brilliant II SYBR® Green with low ROX (Agilent technologies cat. no. 600806)
- DNase-free water
- Diluted cDNA (1:5 in ½ EDTA and 1:5 in DNase-free water)

Methods:

1. Turn on thermocycler and wait 1min before turning on computer
2. Enter the program "Quantitative PCR 4.0 Brilliant II QPCR" and chose SYBR Green (with Dissociation curve) and set the program for 60°C and normal 2-step
3. Choose ROX and SYBR Green in the menu "Collect fluorescence data" and choose ROX in the menu "reference dye"
4. In the menu "Thermal Profile Setup", change the settings to:
    a. 95°C for 10min
    b. 40 cycles of 95 °C for 30sec and 60 °C for 1min
    c. 1 cycle of 95 °C for 1min, 55 °C for 30sec and 95 °C for 30sec
5. For each gene of interest, insert the number of wanted reactions at the correct primer concentration in the PCR Excel calculation table, to calculate the needed amount of reagents for the mastermix, without the cDNA
    a. The number of reactions is 13 for the gene chosen for the reference curve (using duplets)
6. Add 20 µL of the mastermix without cDNA to each well with the relevant primer
7. Add 5 µL of cDNA without producing bobbles
8. Seal plate with caps
9. Centrifuge for 1min at 300g.
10. Insert plate into thermocycler, check settings, save experiment name and pres start

Appendix G: Protocol for gel electrophoresis

Materials:

- 50x TAE buffer (Qiagen cat. no. 129237)
- NuSieve 3-1 agarose (Lonza cat. no. 50094)
- GelStar Nucleic Acid Gel Stain (Lonza cat. no. 50353)
- Gelpilot 5x loading dye (Qiagen cat. no. 239901)
- Gelpilot 50 bp ladder (100) (Qiagen cat. no. 239025)
- Small container
- Casting form
- Comb
- Power supply (Biorad RH nr. 217436)
- UV table (Uvtec RH no. 212801)
- 8µL sample and 2 µl Gelpilot 5x loading dye

Methods:

1. Let Gelstar Nucleic Acid Gel stain thaw for 30min protected from light at room temperature
    a. Vortex and microcentgrifuge before use
2. Weigh 3.0 g NuSieve 3-1 agarose in a 500mL beaker , do not use spoon
3. Add 100mL TAE 1x buffer to the agarose
4. Melt agarose in microwave oven for 4-5min at max effect
5. Cool down by placing the beaker in a 60^{o}C water bath
6. 10µL Gelstar nucleic acid stain 10.000x concentrate in DMSO is added for each 100mL agarose gel
7. Strap the small gel casting form and place the wanted comb into the form
8. Pour the hot agarose into the form until it is covered
9. Push bubbles to the bottom of the gel using a pipette tip and let the gel solidify for ~60min
10. Fill half of the gel container with 1x TAE buffer
11. Place the solid gel carefully in the gel container without removing the comb, and fill the container with 1x TAE buffer
12. Load 2 µl Gelpilot 50 bp ladder in the first or second well
13. Load 10µL product in each well
14. Place a larger ladder on the other side of the gel
15. Run the gel at 100V for 60min.
16. Visualize on UV table with camera

Appendix H: Protocol for FACS

Materials:

- FACS-PBS (Hospital pharmacy, Copenhagen, Denmark)
- Centrifuge tubes
- Flow tubes
- SYTOX (SYTOX®, Invitrogen, cat. no. MP34857)

Methods:

1. Quickly thaw vial in 37°C water bath until ~90 % of the cell suspension is liquid
    a. Cells must have been detached with accutase prior to freezing
2. Transfer cell suspension to a centrifuge tube, by first adding 1mL FACS-PBS, re-suspending, and transferring 1mL suspension
    a. Add 1mL FACS-PBS to the remaining cell suspension before transfer
3. Add FACS-PBS to a total volume of 10mL
4. Centrifuge at 300g for 5 min and discard supernatant
5. Re-suspend in 2mL FACS-PBS and count using nucleocounter
6. Add FACS-PBS to reach a cell concentration of 150.000 cells/100µL
7. Incubate with antibodies for 15min protected from light
8. Wash with FACS-PBS and centrifuge at 300g for 5min
9. Re-suspend in 500µL PBS
10. Add 1µL SYTOX blue dead cell stain 7min before each run

Appendix I: Protocol for immunocytochemistry

Materials:

- 4-chamber chamber slides in permanox (NUNC, cat no 177937)
- Lillis formalin buffer (4% paraformaldehyde in PBS pH 7.4) (Lab 00220, Bie & Berentsen)
- PBS wo ca++ (cat no 10010, Gibco, Invitrogen)
- Bovine Serum Albumin (# A4503-50G, Sigma Aldrich)
- ProLong Gold Antifade reagent with DAPI (P36931, MolecularProbes, Invitrogen)
- Coverslips 25 x 60 mm (Menzel-Glaser, 25x60mm, cat. no. BB025060A1)

Methods:

1. Wash the chambers 3 times with 500 µL PBS
2. Fixate each chamber in 400 µL Lillis formalin buffer (in the fume hood) for 10 min
    a. Discard fixative as H-trash in special container in the fume hood
3. Wash the chambers 3 times with 500 µL PBS
4. Block unspecific background by incubating each chamber with 200 µL 2 % BSA in PBS for 20min at room temperature
5. Primary antibodies are diluted according to their titration in 1 % BSA and 200 µL are added to the relevant chambers and incubated overnight at 4^{o}C or 1 hour at room temperature
    a. Remember one chamber with 1 % BSA without primary antibody for each secondary antibody used, to control for unspecific binding
6. Wash the chambers 3 times with 500 µL PBS
7. Take ProLong Gold Antifade out of the freezer to allow it to thaw
8. Secondary antibodies are diluted 1:250 in 1 % BSA and 200 µL are added to the relevant chambers and incubated 1 hour at room temperature protected from light
9. Wash the chambers 3 times with 500 µL PBS
10. Remove chambers and gently dry the remaining slide
11. Add a drop of 20 µL ProLong Gold Antifade reagent with DAPI between each former chamber without producing bubbles
12. Mount coverslip and press air bubbles out using a pipette tip
13. Let the mounted preparation harden overnight in room temperature and protected from light
14. Visualize using the microscope and store at 4^{o}C protected from light

Appendix J: Protocol for *in vitro* angiogenesis assay

Materials:

- 96-well plate (nunc)
- Complete medium (DMEM Low glucose 1g/L with glutamine and 25 mM HEPES (PAA E15-808) with 10 % FBS and 1 % penicillin/streptomycin)
- 2 % control medium (DMEM Low glucose 1g/L with glutamine and 25 mM HEPES (PAA E15-808) with 2 % FBS and 1 % penicillin/streptomycin)
- Stimulation medium (DMEM Low glucose 1g/L with glutamine and 25 mM HEPES (PAA E15-808) with 2 % FBS and 1 % penicillin/streptomycin and 50pg/mL human recombinant VEGF-A_{165})
- Accutase Assay materials
    - ECMatrix™ solution
    - Diluent Buffer

Methods:

1. Thaw ECMatrix and diluent buffer overnight at 4°C or in 0 °C water
2. Work in cooled environment or keep solutions on ice to avoid polymerization
3. Add 100 µL diluents solution to 900 µL ECMatrix solution
4. Transfer 50 µL of diluted ECMatrix solution to each well and incubate at 37 °C for at least one hour for polymerization to occur
5. Remove medium from cell flasks and wash with PBS
6. Add Accutase (1mL for T25, 3 mL for T75) and incubate at 37 °C until cells are almost detached, then gently tap flasks to loosen cells
7. Inactivate Accutase with complete medium (4mL for T25, 7mL for T75), collect in conical centrifuge tubes and centrifuge for 5min at 300g and room temperature
8. Re-suspend cells in 1.5mL of appropriate medium (stimulation, complete or 2% control) and count on nucleocounter
9. Calculate the volume of cell suspension needed for seeding of 10.000 cells for each well, subtract this number from 150 µL = x µL. Add x µL to the wells for this cell line.
10. Add the appropriate volume (this differs between cell lines and conditions) of cell suspension to the relevant wells of the ECMatrix coated plate and incubate in 37°C and 5 % CO_2
11. Capture 3 pictures from each well every 4th hour for 20 hours

Appendix K: Protocol for BrdU proliferation assay

Materials:

- 96 well plate (nunc)
- Complete medium
  - DMEM Low glucose 1g/L with glutamine and 25 mM HEPES (PAA E15-808) with 10 % FBS and 1 % penicillin/streptomycin
- PBS pH 7.4 (Gibco, cat. no. 10010-015)
- H_2SO_2 (vWR, cat. no. 30149291)
- Assay materials
  - BrdU labeling
    - Dilute to 1:100 for working solution with complete medium
  - FixDenat
  - Anti-BrdU-POD
    - Dilute 1:100 in Anti-BrdU dilution solution
  - Substrate solution

Methods:

1. Seed 1,000 cells/well in a 96 well plate in complete medium and incubate overnight at 37°C
2. Add 10 µL BrdU working solution to each "label" well (see figure) and continue incubation
3. When stopping a plate, remove medium by inverting it and blow-dry for 5min
   a. Can be stored at 4°C for a week after this
4. Add 200 µL FixDenat to all wells and incubate for 30min at room temperature
5. Invert and tap plate to remove FixDenat, and add 100 µL Anti-BrdU-POD working solution and incubate for 90min at room temperature
   a. Add to two blank wells to make Anti-BrdU-POD controls
6. Wash 3 times with 200 µL FBS
7. Add 100 µL substrate solution and incubate for 10min at room temperature protected from light
8. Stop reaction by adding 25µL H_2SO_2
9. Gently shake at ~700rpm for 1min and immediately read absorption on plate reader

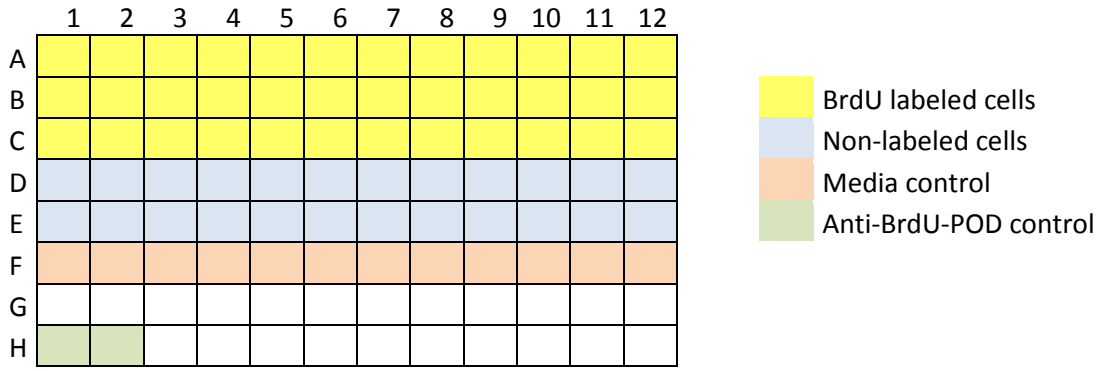
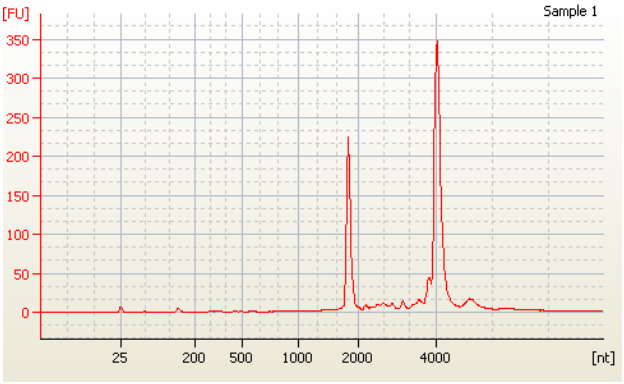
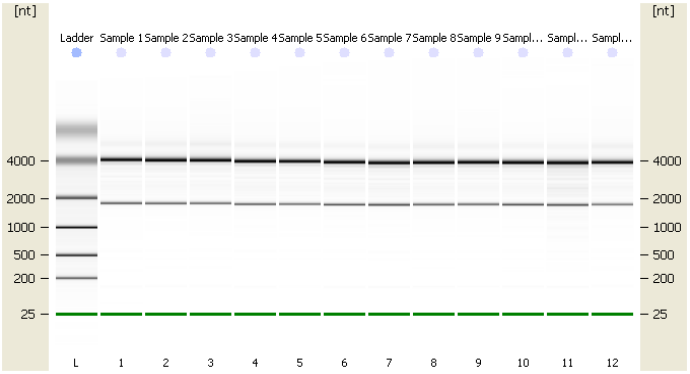


Table: One column is sufficient for one cell line.

Appendix L: Representative Bioanalyser

RNA integrity measurements





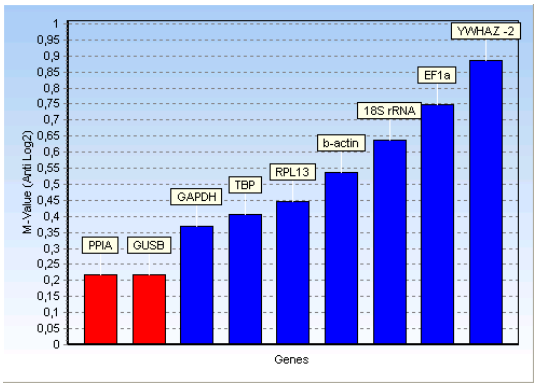
RIN values: 10

Appendix M: Selection of reference genes

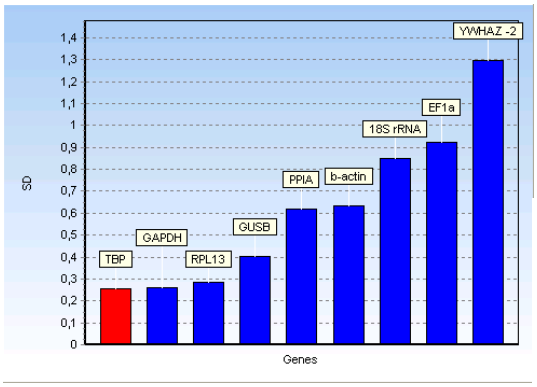
Donor variation

Non-stimulated

GeNorm

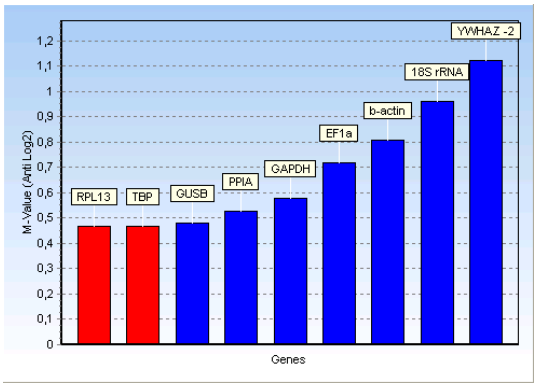


Normfinder

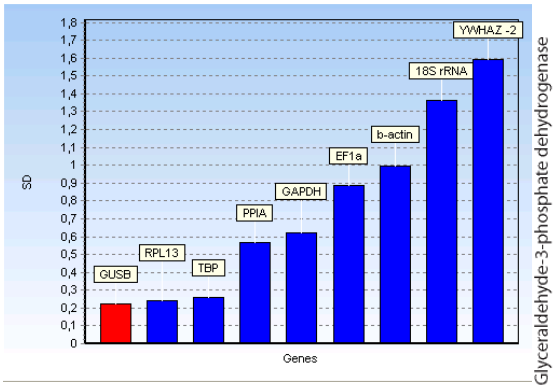


VEGF stimulated

GeNorm



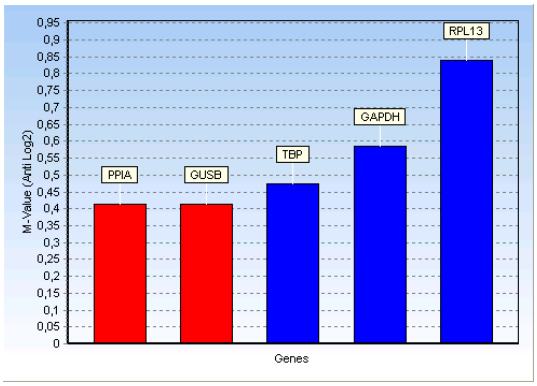
Normfinder



GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GUSB Glucoronidase beta
TBP TATA box binding protein
RPL13 Ribosomal protein L13
PPIA Peptidylprolyl isomerase A
EF1a Elongation factor 1-alpha
b-actin beta actin
18S rRNA EMG1 nuclear protein homolog
YWHAZ Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

Treatment variation

GeNorm



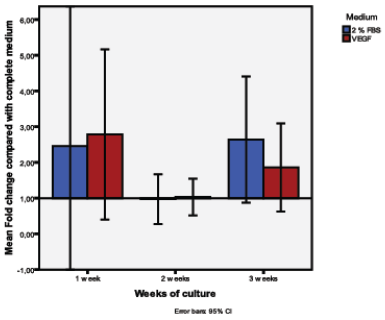
Normfinder

	Intergroup variation	Variability
• GAPDH	-0.0594	0.2751
• GUSB	0.3309	0.4612
• TBP	0.3351	0.4271
• RPL13	-0.7738	0.9764
• PPIA	0.1671	0.3303

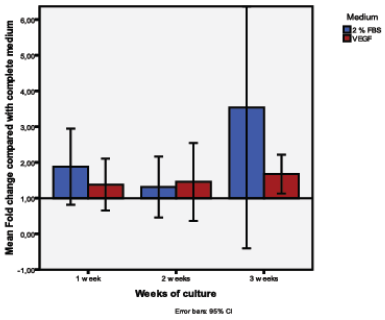
- Variability: 0.2751
- Variability for best combination of two genes: 0.2088
- Best gene: GAPDH
- Best combination of two genes: GAPDH and PPIA

Appendix N: RT-qPCR results for ADSCs from healthy donors

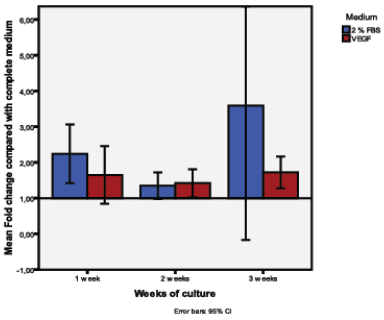
VGEFR2



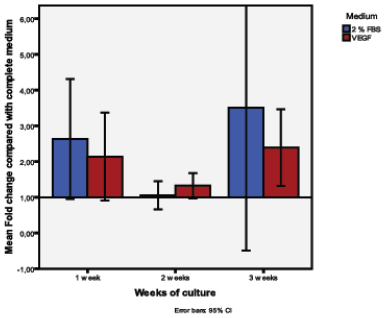
VEGFR1



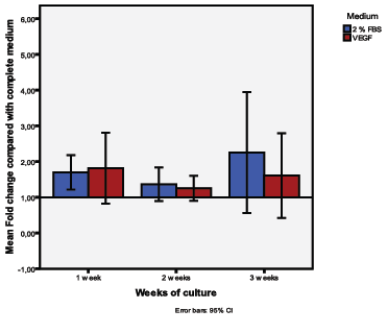
PDGFB



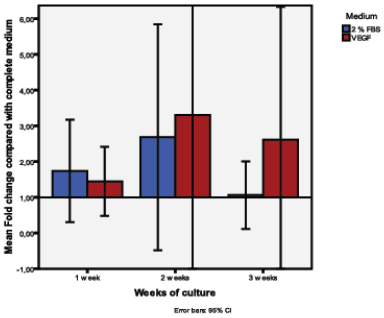
FOXF1



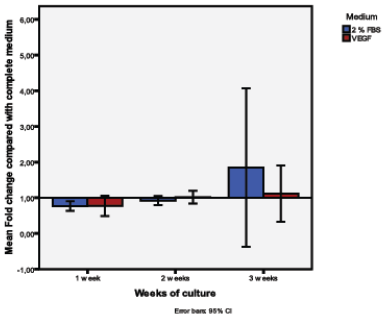
vWF



CD31



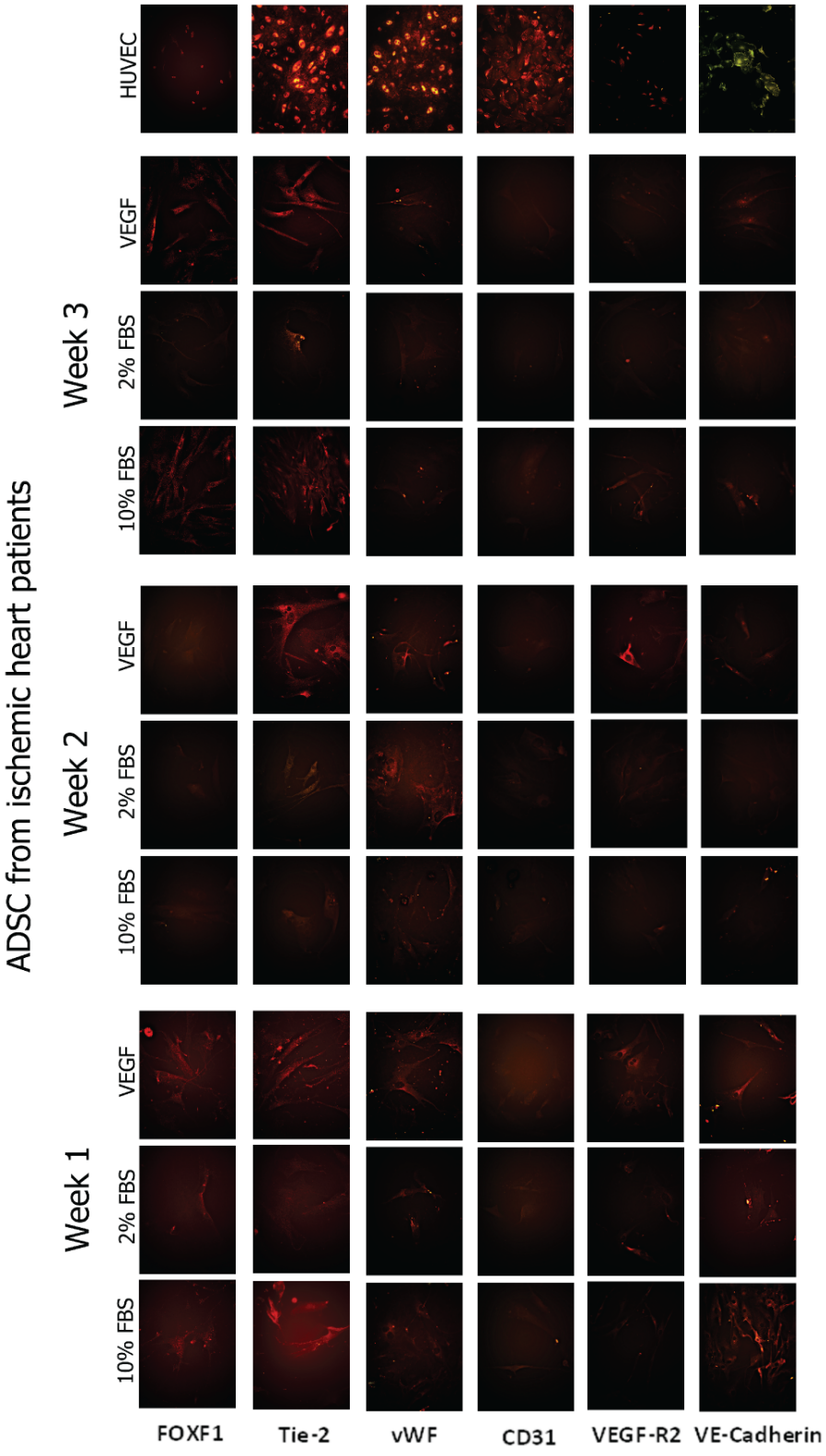
CD105

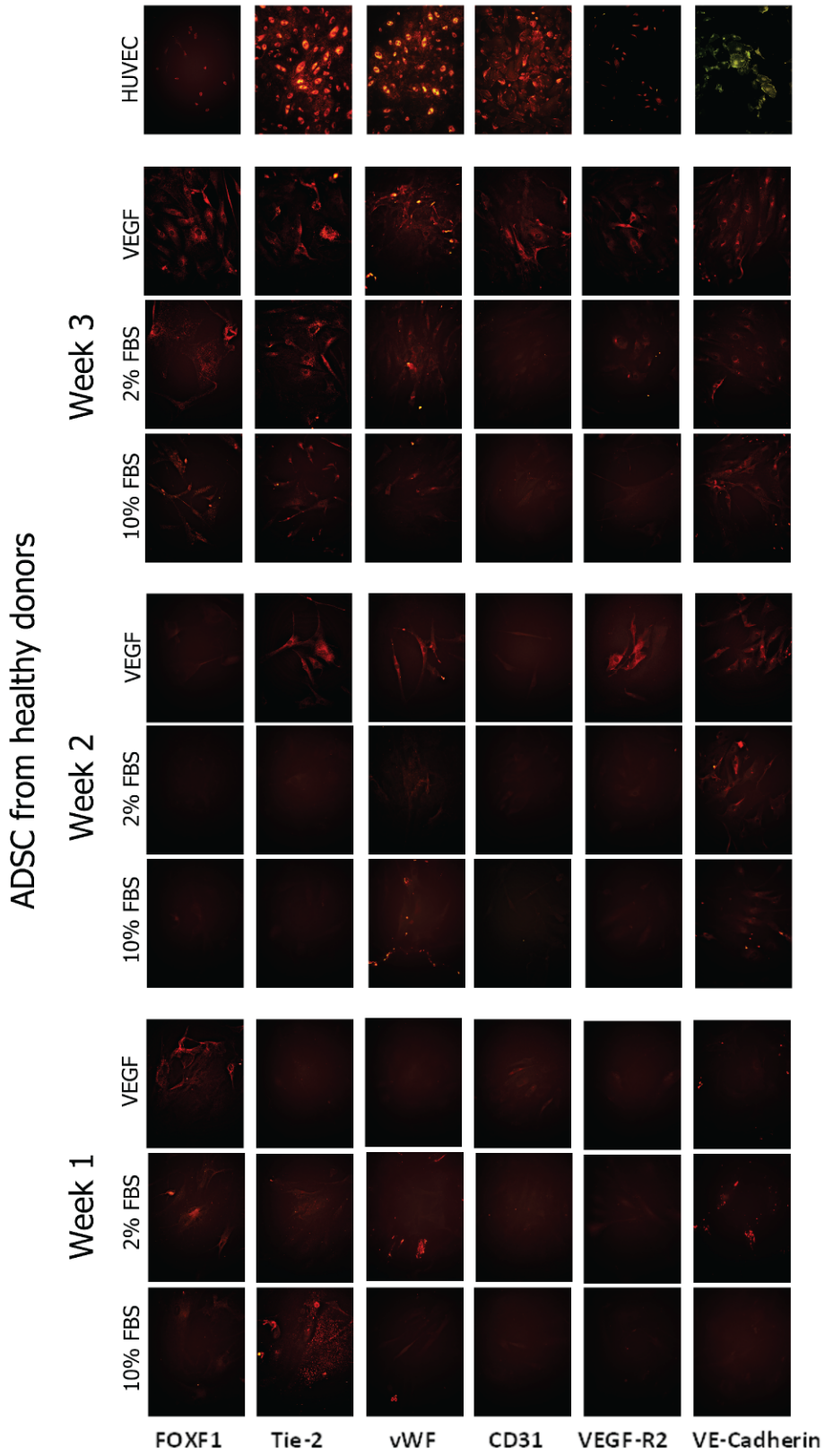


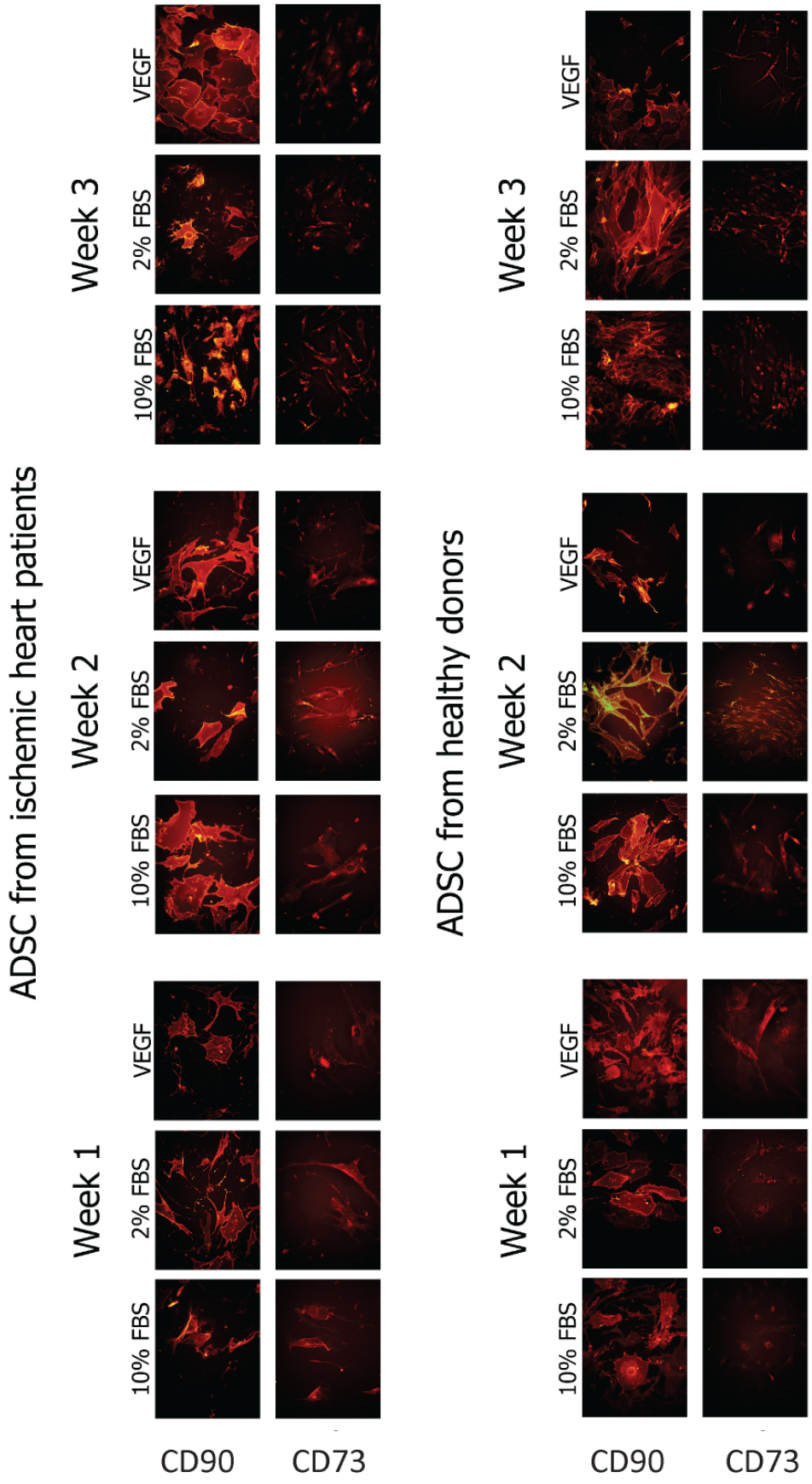
Appendix O: Table of all FC results

		IHD patients			Healthy donors		
		1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks
CD105	10%	95,00 ± 2,18	93,29 ± 7,59	83,12 ± 6,20	93,65 ± 5,99	98,66 ± 0,42	93,95 ± 3,52
	2%	94,35 ± 2,71	96,74 ± 1,03	81,03 ± 15,96	97,46 ± 0,39	98,81 ± 0,82	96,52 ± 4,88
	VEGF	97,31 ± 1,68	89,85 ± 4,52	62,10 ± 37,50	88,91 ± 5,47	98,67 ± 0,81	94,02 ± 5,13
CD90	10%	77,43 ± 2,84	77,74 ± 10,90	48,44 ± 19,79	76,61 ± 25,06	94,13 ± 3,23	78,16 ± 16,91
	2%	69,98 ± 7,56	75,13 ± 12,61	61,67 ± 18,21	72,55 ± 39,10	94,08 ± 3,14	90,38 ± 7,51
	VEGF	88,05 ± 6,94	71,22 ± 13,27	66,24 ± 11,40	76,10 ± 24,68	93,86 ± 1,27	91,12 ± 5,99
CD73	10%	87,92 ± 9,12	88,53 ± 8,65	63,99 ± 17,00	91,88 ± 6,22	98,47 ± 1,15	80,61 ± 25,32
	2%	90,94 ± 5,61	73,96 ± 32,21	71,70 ± 24,37	89,33 ± 12,23	97,04 ± 2,05	89,58 ± 14,28
	VEGF	95,99 ± 9,12	89,22 ± 8,65	73,97 ± 17,00	91,47 ± 6,22	96,57 ± 1,15	91,40 ± 25,32
CD13	10%	89,04 ± 7,06	93,08 ± 3,44	70,60 ± 17,66	94,36 ± 0,71	97,45 ± 1,69	83,31 ± 19,07
	2%	91,72 ± 3,87	90,96 ± 4,04	68,79 ± 25,30	63,56 ± 50,98	96,18 ± 2,58	85,20 ± 17,94
	VEGF	95,60 ± 3,60	91,09 ± 1,66	73,20 ± 17,34	94,05 ± 1,28	94,71 ± 4,50	85,73 ± 14,92
CD45	10%	7,26 ± 8,02	6,33 ± 10,21	17,02 ± 29,07	5,53 ± 5,61	13,52 ± 8,48	13,66 ± 9,14
	2%	0,75 ± 0,27	1,68 ± 2,29	8,26 ± 11,51	2,33 ± 1,62	7,06 ± 3,48	8,67 ± 5,66
	VEGF	6,88 ± 8,79	2,81 ± 2,80	38,50 ± 23,34	3,13 ± 2,45	8,04 ± 4,50	13,80 ± 11,41
CD34	10%	1,55 ± 1,24	8,10 ± 6,70	6,98 ± 5,22	20,07 ± 31,76	18,85 ± 28,04	23,26 ± 35,00
	2%	4,59 ± 3,26	6,41 ± 4,90	10,25 ± 10,41	14,78 ± 19,04	14,74 ± 14,45	22,52 ± 31,29
	VEGF	4,70 ± 3,44	7,76 ± 5,49	5,37 ± 5,83	22,02 ± 32,40	22,28 ± 29,63	28,45 ± 39,09
HLA-DR	10%	0,06 ± 0,08	0,02 ± 0,02	0,06 ± 0,06	0,00 ± 0,01	0,00 ± 0,00	0,16 ± 0,22
	2%	0,02 ± 0,03	0,01 ± 0,01	0,05 ± 0,09	0,01 ± 0,01	0,05 ± 0,08	0,06 ± 0,06
	VEGF	0,00 ± 0,01	0,02 ± 0,02	0,08 ± 0,10	0,04 ± 0,06	0,03 ± 0,04	0,18 ± 0,22
CD19	10%	0,22 ± 0,26	0,62 ± 1,04	0,13 ± 0,10	0,26 ± 0,22	1,48 ± 1,63	0,41 ± 0,55
	2%	0,04 ± 0,01	0,05 ± 0,06	0,12 ± 0,04	0,03 ± 0,01	0,38 ± 0,25	1,62 ± 2,15
	VEGF	0,18 ± 0,20	0,17 ± 0,14	0,33 ± 0,18	0,47 ± 0,72	0,20 ± 0,19	0,45 ± 0,51
CD14	10%	0,56 ± 0,29	1,45 ± 1,65	0,80 ± 0,51	1,23 ± 1,16	2,99 ± 1,40	4,56 ± 4,49
	2%	0,49 ± 0,20	1,13 ± 1,56	1,03 ± 1,05	0,56 ± 0,30	2,69 ± 1,70	5,80 ± 8,84
	VEGF	1,55 ± 1,08	1,29 ± 1,07	1,89 ± 0,91	0,76 ± 0,71	2,58 ± 1,28	2,54 ± 3,69
VEGFR2	10%	0,25 ± 0,36	0,20 ± 0,17	0,07 ± 0,01	0,63 ± 1,05	0,36 ± 0,26	0,11 ± 0,03
	2%	0,33 ± 0,35	0,09 ± 0,06	0,09 ± 0,03	0,49 ± 0,72	0,72 ± 0,51	0,16 ± 0,15
	VEGF	0,44 ± 0,56	0,15 ± 0,06	0,12 ± 0,05	0,47 ± 0,60	0,24 ± 0,19	0,17 ± 0,14
VE-Cadh	10%	0,27 ± 0,29	0,31 ± 0,19	0,08 ± 0,05	0,11 ± 0,05	0,62 ± 0,33	0,64 ± 0,79
	2%	0,39 ± 0,53	0,10 ± 0,02	0,10 ± 0,07	0,52 ± 0,74	0,61 ± 0,33	0,17 ± 0,17
	VEGF	0,28 ± 0,22	0,20 ± 0,16	0,17 ± 0,04	0,52 ± 0,71	0,34 ± 0,27	0,27 ± 0,14
Tie-2	10%	0,04 ± 0,29	0,63 ± 0,19	0,03 ± 0,05	0,92 ± 0,05	8,88 ± 0,33	0,31 ± 0,79
	2%	0,03 ± 0,03	0,03 ± 0,01	0,13 ± 0,16	0,22 ± 0,13	0,84 ± 0,86	8,22 ± 8,07
	VEGF	0,24 ± 0,22	0,02 ± 0,16	0,09 ± 0,04	0,27 ± 0,71	0,64 ± 0,27	0,30 ± 0,14
CD31	10%	0,19 ± 0,22	0,09 ± 0,13	0,08 ± 0,08	0,28 ± 0,47	0,40 ± 0,36	0,17 ± 0,19
	2%	0,08 ± 0,11	0,06 ± 0,06	0,11 ± 0,09	0,25 ± 0,16	0,12 ± 0,04	1,15 ± 1,76
	VEGF	0,06 ± 0,07	0,07 ± 0,07	0,07 ± 0,05	0,20 ± 0,26	0,13 ± 0,11	0,08 ± 0,07

Appendix P: ICC results

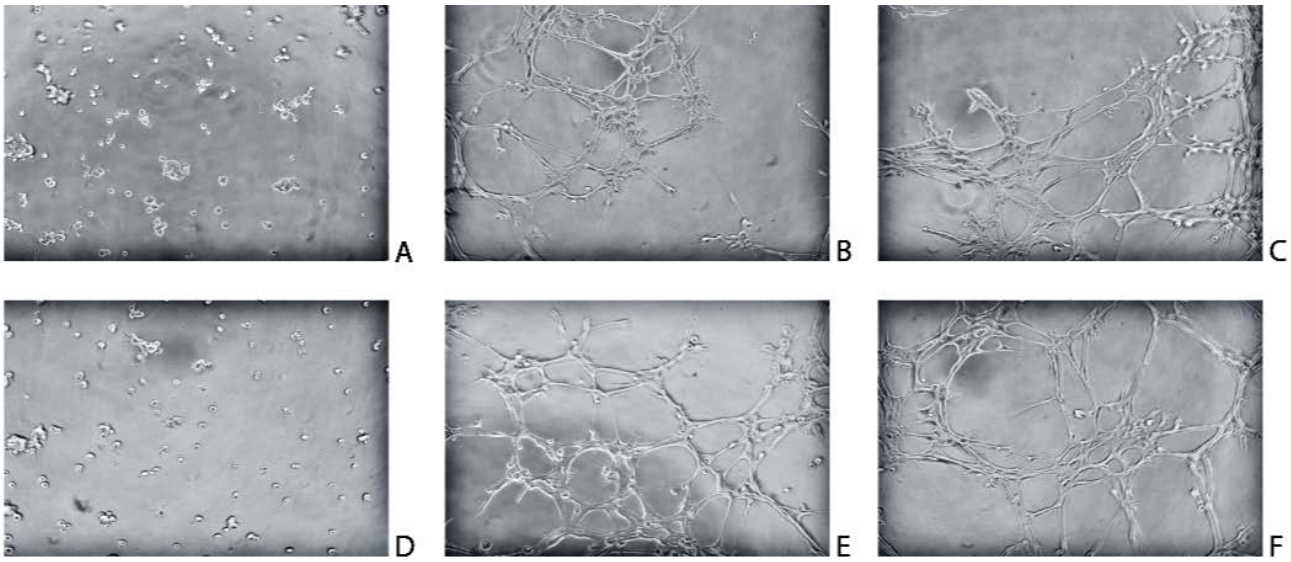



ADSC from healthy donors
Week 1
Week 2
Week 3
VEGF
2% FBS
10% FBS
HUVEC
FOXF1
Tie-2
vWF
CD31
VEGF-R2
VE-Cadherin


ADSC from ischemic heart patients
ADSC from healthy donors
Week 1
Week 2
Week 3
10% FBS
2% FBS
VEGF
CD90
CD73

Appendix Q: Polygons observed on ECMatrix

IHD patients



Healthy donors