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Differentiation of human embryonic stem cells into endothelial and smooth muscle cells as a model for vascular development

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List of abbreviations

bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
Dil-Ac-LDL	Dil- labeled acetyled low density lipoprotein
EB(s)	embryoid bodie(s)
EC(s)	endothelial cell(s)
ECM	extracellular matrix
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ESC(s)	embryonic stem cell(s)
HUVECs	human umbilical vein endothelial cells
ICM	inner cell mass
IGF	insulin-like growth factor
PDGF-BB	platelet- derived growth factor -BB
PECAM 1	platelet endothelial adhesion molecule-1
MEF	mouse embryonic fibroblasts
RT-PCR	reverse transcriptase polymerase chain reaction
SMA	smooth muscle actin
SMC(s)	smooth muscle cell(s)
SM-MHC	smooth muscle myosin heavy chain
TEM	transmission electron microscopy
TGF-β1	transforming growth factor –beta 1
Ve-cad	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VPCs	vascular progenitor cells
Vwf	von Willebrand factor

1. SUMMARY

Aims of the study: We hypothesized that the optimal source of cell for vascular regeneration will be the progenitor cells derived from human embryonic stem cells (ESCs) which can differentiate both into endothelial cells (ECs) as well as vascular smooth muscle cells (SMCs). We propose to test if the population of human ESCs, H9 cell line, can serve this role.

Material and methods: Human ESCs were cocultured with stromal cells S17, M2-10B4 or Wnt1 expressing M2-10B4 cell line to generate a CD34+ cell population. After that, CD34+ cells were sorted and cultured in media containing specific cytokines to generate ECs. To induce SMC differentiation from ECs, culture conditions were changed to media containing platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-beta 1 (TGF-β1). Phenotypic and functional characteristics of these populations were demonstrated by flow cytometry, immunohistochemistry, Q-RT-PCR, tube formation assay, and response to calcium signaling agonists.

Results: CD34+ vascular progenitor cells derived from human ESCs give rise to ECs and SMCs. These two populations express cell specific transcripts and proteins, exhibit intracellular calcium in response to various agonists, and form robust tube-like structures when cocultured in Matrigel. Wnt1 overexpressing stromal cells produced an increased number of progenitor cells.

Conclusions: Here, we demostrate great potential of human ESCs (H9 cell line) to differentiate into both ECs and SMCs in a novel three-phase culture system. The ability to generate large numbers of ECs and SMCs from a single vascular progenitor cell population is promising for therapeutic use. The stepwise differentiation outlined in our work is an efficient, reproducible method with potential for large-scale cultures suitable for clinical applications.

2. INTRODUCTION

There is now growing evidence that human embryonic stem cells (ESCs) provide an important source of cells to define the cellular and molecular mechanism of vascular development, as well as the developmental relationship between endothelial (ECs) and smooth muscle cells (SMCs). Moreover, they are also highlighted as a promising potentially unlimited source of cells for vascular regenerative approaches[1-3].

Human ESCs are in vitro cultured pluripotent cells derived from the inner cell mass of preimplanted blastocysts. They give rise to stable pluripotent cell lines that are capable of unlimited proliferation under specific culture conditions. Human ESCs can be differentiated into representative derivatives of all three embryonic germ layers in vitro and in vivo[4,5]. Since their first derivation by Thomson et al [6], it has been shown that human ESCs can differentiate into various lineages of cells, including hematopoietic cells, neurons, bone, cartilage, muscle, cardiomyocytes, pancreatic cells, hepatocytes and vascular cells.[7] These cells appear to be weakly immunogenic, expressing only moderate amount of major histocompatibility complex (MHC) class I and not any MHC class II proteins.[8] Therefore, human ES cells may play important role in stem cell-based regenerative medicine.

During the last few years, several groups of investigators have explored the endothelial potential of human ESCs. Two main approaches have been used for purifying progenitor ECs from human ESCs: selecting 3-dimensional embryoid bodies (EBs) for specific cell-surface molecules [9,10] and supplementing feeder layers or the medium with various growth factors[11,12]. The first human ESC-derived endothelial progenitors were isolated from 13-day-old EBs by flow cytometry of $PECAM1^{+}$cells cultured in EGM2 media containing specific cytokines[10]. It was shown that human ESC-derived ECs displayed characteristics similar to vascular

endothelium and expressed typical EC markers similar to those expressed in human umbilical vein endothelial cells (HUVECs), such as VE-cad, VWF, PECAM1 and Dil-AC-LDL uptake. Furthermore, these cells displayed the proper organization of endothelial junctions, were capable of forming tubelike structures in vitro and generating capillary structures when embedded in sponges. Their transplantation into immunodeficient mice resulted in the formation of microvessels [3].

The majority of angiogenesis research focus on the regulation of endothelium, but vascular SMCs are also important participants in formation of blood vessels. Vascular SMCs play critical roles in structural and functional support of the vascular network by stabilizing nascent endothelial vessels during vascular development and blood vessel growth[13]. Moreover, differentiation and phenotypic plasticity of vascular SMCs play important role also in many human diseases including atherosclerosis, cancer, and hypertension[14].

SMCs are heterogeneous cells with a wide range of different phenotypes at different developmental stages, and even in adult organisms the SMCs are not terminally differentiated[15]. Phenotypically, SMCs differ from cardiac and skeletal muscle cells not only by their expression of specific contractile proteins including α-SMA, calponin-1, SM22α and smooth muscle myosin heavy chain (SM-MHC), but also by their plasticity or ability to reenter to cell cycle and exhibit a „synthetic“ phenotype, secreting ECM proteins[16,17]. In addition, even for SMCs derived from the same ontogenic cell source, considerable differences in gene expression exist, and little is known about developmental and phenotypic differences between visceral and vascular SMCs[18]. Cultured SMCs could rarely be stably maintained and are limited in the capacity for regulatory mechanism and pathway studies[19], therefore intense researches have been focused on exploring the molecular mechanisms of SMC differentiaton through

inducible in vitro SMC differentiation systems. Major progress has been made in the last decade to differentiate SMCs from mouse ESCs and other types of adult stem cells[20-23]. Although most of these studies have demonstrated the presence of proteins consistent with SMC phenotype, only few have addressed whether the differentiated cells also have functional properties of SMCs. Recently, SMCs were characterized also during human ESC differentiation. For instance, Huang at al [24] established in vitro SMC differentiation system by treating the monolayer-cultivated human ESCs with all-trans retinoic acid.

While we already have some evidence for the existence of a hemangioblast, the common precursor of hematopoietic and ECs, the developmental relationship between ECs and SMCs is still less well characterized. Some works using mouse ESCs suggest the existence of common precursor of ECs and SMCs[25]. Another study with human ESCs described the population of vascular progenitor cells derived from human ESCs which have the ability to differentiate into endothelial-like and smooth muscle-like cells[26]. Despite multiple methods of SMC differentiation, the exact lineage relation between ECs and SMCs has not been elucidated.

Recent advances in stem cell technology suggest that stem cell-derived vasculogenic cells may play important role in vascular regenerative approaches. Since human ES cells can be grown in virtually unlimited numbers, these cells provide an appealing alternative to adult stem cells and may serve as a stable potential source of therapeutic ECs. Human ESCs are advantageous in many aspects when compared with other ECs and SMCs origins, due to their high proliferation capability, pluripotency, and low immunogenity. However, there are still many challanges and obstacles to overcome before the vision of using embryonic vascular progenitor cell in the clinic can be realized.

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3. HYPOTHESES AND AIMS OF THE STUDY

ESCs are emphasized as an important model to better define the cellular and molecular mechanisms of vascular development. They also hold a strong potential to treat or even cure many diseases in the future.

In our study, we hypothesized that the optimal source of cell to regenerate arterioles and arteries in ischemic disease will be a bi-potential cells derived from human ESCs that can differentiate both into endothelial cells as well as vascular smooth muscle cells. We propose to test if the population of human ESCs- H9 cell line can serve this role.

Specific Aim 1: To derive vascular progenitor cells using human ESCs (H9 cell line) that might be ideal for vascular repair strategies.

Specific Aim 2: To define optimal culture conditions for ECs and SMCs differentiation using human ESCs.

Specific Aim 3: To better examine the developmental origin of vascular progenitor cells and reveal the relationship between ECs and SMCs, as well as provide a more complete biological characterization of these cells derived from human ESCs.

4. MATERIALS AND METHODS

4.1. Cell culture:

Undifferentiated hESCs (H9 cell line) were cultured as previously described[6,27]. Human ESCs were maintained as undifferentiated cells by co-culture with mitomycin C inactivated mouse embryonic fibroblasts (MEF) in DMEM/F12 media supplemented with 15% knockout serum replacer, 1% MEM- nonessential amino acids, 2 mM L-glutamine,0.1 mM β-mercaptoethanol, 8 ng/ml basic fibroblast growth factor (bFGF), and 1% Penicilin/Streptomycin. Undifferentiated cells were fed daily with fresh media and passaged onto new MEFs approximately every 5-7 days as needed. hESCs expressing mCherry florescent protein were generated using lentiviral transduction technique and hESCs expressing GFP were generated using sleeping beauty transduction method (Amaxa).

To promote endothelial differentiation, hESC were cultured as previously described[28]. Briefly, the undifferentiated hESCs were passaged onto mitomycin C inactivated mouse bone marrow-derived stromal cell line S17 or M210B4 cells in RPMI 1640 media supplemented with 15% fetal bovine serum (FBS), 1% MEM-nonessential amino acids , 1% L-glutamine and 0.1% β-mercaptoethanol for 13-15 days. After that, differentiated hESCs were dissociated with 1mg/mL collagenase IV , followed by 0.05% trypsin/0.53mM EDTA , a single cell suspension was generated, and the subpopulation of $CD34^+$ cells were isolated using magnetic nanoparticle technology (EasySep Selection Kit, StemCell Technologies).

The $CD34^+$ population was cultured on fibronectin coated tissue culture flasks in EGM2 complete media consisting of basal media (EBM2) supplemented with 5% FBS, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), heparin, and ascorbic

acid. This population, human ESC-derived ECs, were grown to 80% confluence and serially passaged in parallel with HUVEC cells which functioned as a positive control in all experiments.To obtain smooth muscle cells, a portion of the human ESC-derived EC cells established in culture were placed in high glucose Dulbecco modified Eagle media (DMEM) containing 5% FBS, 5 ng/mL platelet-derived growth factor-BB (PDGF-BB)and 2.5 ng/mL transforming growth factor-beta 1 (TGF-β1).

4.2. Flow Cytometry

The dissociated cells were centrifuged, washed and resuspended in FACS buffer (PBS without Ca^{2+} and Mg^{2+} supplemented with 2% FBS and 0.1% sodium azide). The cells were aliquoted and stained with fluorescently (APC, PE or FITC) labeled antibodies against human cell surface antigens: CD34-APC, CD31-PE , Flk1-PE, TIE2-PE, CD144-PE (VE-Cadherin) and the lectins *Ulex europeaus*-FITC, *Helix pomatia*-FITC, and *Griffonia simplicifolia*-FITC. Appropriate isotype-matched controls labeled with the same fluorochromes were used to determine the degree of non-specific staining or using the corresponding competitive sugar when lectin binding was examined.

4.3.Transmission Electron Microscopy (TEM) Assessment of human ESC-derived ECs

Human ESCs-derived ECs were seeded at $4x10^4$ cells/cm^2, onto fibronectin coated 0.4 µm polycarbonate membranes at ~20,000 cells/membrane. Cells were suspended in EGM2 media and incubated at 37°C with 5% CO_2/21% O_2 for 72 to 96-hours post seeding. After incubation, cells were fixed in 3% glutaraldehyde. Specimens were post-fixed with 1% osmium tetroxide, dehydrated with a graded alcohol series, and embedded in PolyBed 812 resin. Semi-thin sections (1 µm) were cut and stained with toluidine blue, and examined

by light microscopy to select regions containing cells. Thin sections (80 nm) were cut from the regions of the membrane containing cells using a diamond knife. Sections were stained with uranyl acetate, counter-stained with Reynold's lead citrate, and examined by TEM using a Philips CM 100.

4.4. Matrigel Tube Formation Assay

A total of $3.5x10^4$cells (hESC-ECs, hESC-SMCs, or 60/40 mixture of ECs/SMCs) in 10ul of PBS were added to each 60 ul aliquot of Matrigel, mixed gently, and added to the pre-warmed plates. Mixtures were added as a single three dimensional drop per well. After Matrigel solidified (approximately 30 minutes), 2.5ml of 50/50 mix of combined EC/SMC media was carefully added to each well and the three dimensional Matrigel drop was maintained. Tube formation was visualized after 3-4 days.

4.5. Immunohistochemical staining

Human ESC-ECs analysis for the acetyled LDL receptor was performed by incubation of the cells in media with 5% FBS containing dil-acetylated low-density lipoprotein (dilAcLDL) for 4 hours. After washing, the cells were observed by fluorescence microscopy. HUVECs were used as a positive control, human ESC- SMCs as a negative control.For detection of von Willebrand factor (vWF) and endothelial nitric oxide sythanse (eNOS) cells were fixed, permeabilized, and incubated at room temperature with primary antibody for one hour followed by incubation with secondary antibody Alexa Fluor 488 for 30 minutes. After a final wash, cells were observed by fluorescence microscopy. CD31 and VE-cadherin were detected using mouse anti-human antibodies. Alexa Fluor 488 goat anti-mouse secondary antibody was used for final detection and visualization. For hESC-SMCs we examined expression SM22, calponin and alpha-smooth muscle actin using primary goat anti-human

SM22and mouse anti-human calponin and detected with the species matched secondary antibodies labeled with Alexa Fluor 488. ProlongGold + Dapi and Hoechst 33258 was utilized for slide preparation and nuclear visualization via fluorescent microscopy.

4.6. Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative RT-PCR (Q-RT-PCR)

Total RNA was extracted from HUVEC, undifferentiated ES cells, human ESC-ECs and human ESC-SMCs using an RNeasy kit with homogenization via Qiashredder according to the manufacturer's instructions. mRNA was reverse transcribed (RT) to cDNA using an Omniscript RT kit following the manufacturer's instructions. Simultaneous RT reactions without reverse transcriptase were performed to control for the transcription of contaminating genomic DNA. cDNA was amplified with a HotStarTaq PCR kit (Qiagen) under the following conditions: initial 95 C for 15 minutes, followed by cycles consisting of 94 C for 1 minute, annealing at variable temperature (as noted in the supplemental table) for 1 minute, 72 C for 1 minute, and 72 C for 10 minutes after the final cycle. 30 cycles were executed for Flk1, 38 cycles for other endothelial primers, and 35 cycles for all SMCs primers. The amplified products were separated on 1.5% agarose gels and visualized via ethidium bromide staining. Additionally, two step Q-RT-PCR was used for quantitative analysis. Relative quantitation of genes expression compared to beta-actin was generated by the comparative threshold cycle (C T) method using Applied Biosystems SDS analysis software (version 1.9.1).

4.7. Measurement of [Ca 2^{+}] in response to calcium signaling agonists

Cells were loaded with fura-2 acetoxymethyl ester (fura-2 AM) in standard culture media at 37 C for 60 minutes in the incubator. After this incubation, the cells were washed with HEPES buffered physiological salt solution (10 mM HEPES, 1.25 mM NaH_2PO_4, 146 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$:$6H_2O$, 10 mM glucose, 2 mM $CaCl_2$, 1 mM Sodium Pyruvate, pH 7.4) and placed on an inverted microscope (Olympus) where they were continuously perfused with HEPES buffered solution for 20 minutes before imaging. The following panel of drugs was tested: Norepinephrine 100 uM, Carbachol 100 uM, Oxytocin 1 uM, Endothelin-1 100 nM, 5-Hydroxytryptamine 10 uM, Vasopressin 100 nM, ATP 10 uM, Bradykinin 1 uM. Each drug was applied for 30 seconds with recovery time between drug applications varied by cellular response. Images were acquired with a 40x oil immersion objective, a CoolSNAP digital camera using an exposure time of 25 ms. Fura-2 was excited alternately at 340 and 380 nm using a computer-controlled filter wheel and shutter (Luld Electronics Corporation, Hawthorne, NY)

4.8. Limiting Dilution Assay

To test the role of Wnt proteins on hESC differentiation, two genetically modified M210 cells lines were used (one over expressed Wnt1 and the other over expressed Wnt5, kindly provided by Dr. Randy Moon, U. of Washington) [25]. On day 14-15, $CD34^{+}$ cells were selected from each stromal cell coculture and plated at limiting dilution in 96 well plates with $4x10^3$, $1.3x10^3$, $4x10^2$, $1.3x10^2$, 40, 13, and 4 cells per well that were pre-coated with fibronectin and cultured in hESC-EC media. Cells received media changes every 4-5 days and after 15 days wells were scored for growth. Progenitor frequencies were calculated and reported graphically as progenitor cells per 1000 cells.

5. RESULTS

5.1.Characterization of endothelial cells derived from human embryonic stem cells

Human ESCs, H9 cell line, were supported to differentiate toward vascular progenitor cells through an initial step utilizing stromal cell co-culture as described in "Materials and Methods". After culture for 13-15 days, $CD34^+$ cells were isolated via immunomagnetic sorting and assessed via flow cytometry for vascular and endothelial surface markers (Figure 5A). For expansion and further endothelial differentiation, these cells were placed on fibronectin coated flasks and cultured in EGM2 media supplemented with a growth enhancing cocktail.Under these culture conditions, after approximately 7-10 days the cells assumed a more uniform "couble stone" morphology similar to ECs isolated from other sources(Figure 7A,B). Human ESC-derived ECs demonstrated typical EC surface antigen expression of CD31, VE-cadherin (CD144), CD146, Flk1, lectins, and intracellular markers vWF and endothelial cell nitrous oxide synthase (eNOS) (Figure 5B). Immunofluorescent staining confirmed EC morphology and expression of endothelial proteins: CD31, VE-Cadherin, vWF, eNOS, as well as uptake of dil-ac-LDL, another characteristic of ECs (Figure 6). Furthermore, RT-PCR was done to demonstrate expression of transcripts for genes commonly expressed by ECs (Figure 8), and the expression level of these transcripts was quantified via Q-RT-PCR (Table 1). Importantly, these human ESC-derived ECs have also functional characteristics of ECs. They formed capillary-like structures when replated on Matrigel (Figure 7 C,D) and they were able to rapidly take up acetyled LDL (Figure 6). Transmission electron micrographs (TEM) of hESC-ECs showed the presence of microparticles being released from the membrane surface of the cell (Figure 9).

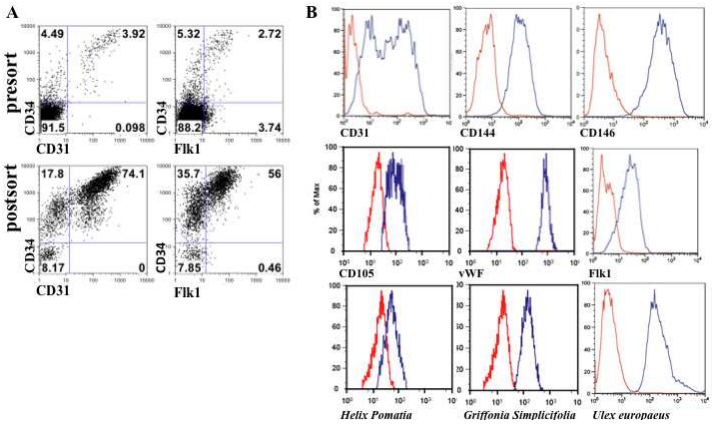

Figure 5. CD34+ Vascular Progenitor Cells Capable of Endothelial Cell Differentiation. (A) human ESC-derived cells after 15 days of differentiation demonstrating expression of CD34, CD31, and Flk1 both before (presort) and after (postsort) immunomagnetic sorting for CD34+ cells.**(B)** After sorting, the CD34+ cells are placed in EC culture medium on fibronectin coated tissue culture flask. After 2-4 passages, expression of EC specific surface markers and lectins can be detected by flow cytometry. Histograms demonstrate red plot as isotype control or corresponding competitive sugar control (for lectins) in each panel, and blue plot is stained for surface antigen or lectin, as indicated.

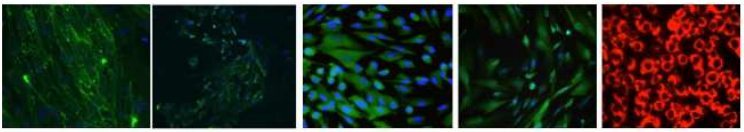

Figure 6. Immunofluorescent staining of human ESC-derived ECs: (left to right) CD31, VE-Cadherin, vWF, eNOS and uptake of dil-ac-LDL.Original magnification 100x for each plot. Blue signal represents DAPI stained nuclei.

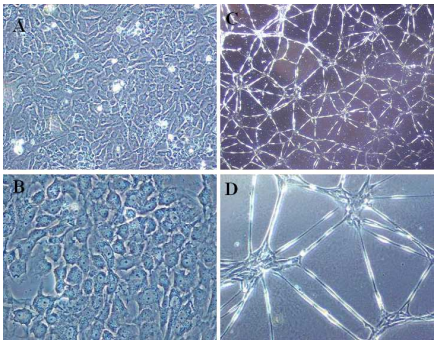

Figure 7. Characteristics of human ESC-derived ECs. (A-B) Morphology of human ESC-derived ECs cultured on fibronectin coated plates in EGM2 media. **(C-D**) These cells form capillary-like structures when replated on Matrigel. Original magnification: x100 (A, D), x 200 (B), x 20 (C).

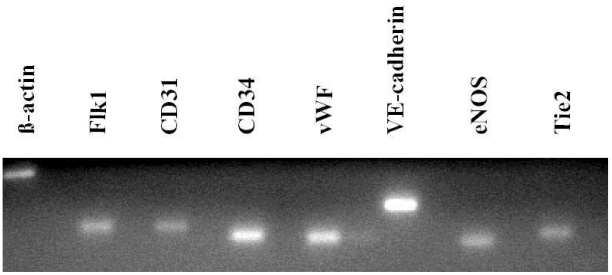

Figure 8. RT-PCR of human ESC-derived ECs. Total cellular RNA was isolated from human ESC-derived ECs and sequence-specific primers for the indicated genes were used for RT-PCR analysis. Here, mRNA expression of 7 transcripts for typical EC genes as indicated above each row. To control for contaminating genomic DNA, reactions were also done under conditions with no reverse transcriptase.

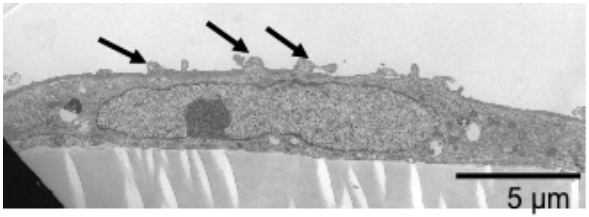

Figure 9. Transmission Electron Micrograph (TEM) images of human ESC derived ECs. Human ESC-derived ECs cultured under endothelial cell culture conditions in EGM2 media show release of microparticles of approximately 100nm in size (as indicated by arrows) from the cell surface.

5.2. Characterization of smooth muscle cells derived from human embryonic stem cells

To generate SMCs, human ESC-derived ECs (passage 3 or 4) are removed from endothelial cell culture media and cultured in media containing TGF-β1 and PDGF-BB (Figure 10). 24 to 36 hours after this change, a complete morphologic change of cells can be observed. The cell population converted rapidly to a flatter morphology and acquired intracellular fibrils as we can see in other SMC cultures (Figure 10). Immunofluorescent staining revealed robust expression of SM22, calponin, and α-smooth muscle actin (Figure 11 A,B,C) Notably, HUVECs cultured under SMC conditions (with TGF-β1 and PDGF-BB) did not convert to SMC morphology. Also, direct culture of the initial human ESC-derived $CD34^+$ population under SMC conditions did not yield stable cultures with SMC morphology or characteristics. Furthermore, RT-PCR demonstrated transcripts of typical SMC genes (Figure12). Next, we used Q-RT-PCR to more

accurately compare expression of transcripts specific for smooth muscle cells with expression of transcripts for endothelial genes in the populations of human ESC-derived ECs and human ESC-derived SMCs (Table 1).When compared to the human ESC-derived EC population, the human ESC-derived SMCs exhibited a remarkable increase in expression of transcripts specific for SMC genes with a concomitant decrease in endothelial gene transcripts. In a corresponding manner, in the human ESC-derived EC population, high levels of endothelial gene transcripts were measured at the same time as a very low expression of SMC gene transcripts. In contrast, HUVECs under SMC culture conditions did not change morphology and they did not show an increased expression of SMC genes (Table 2).

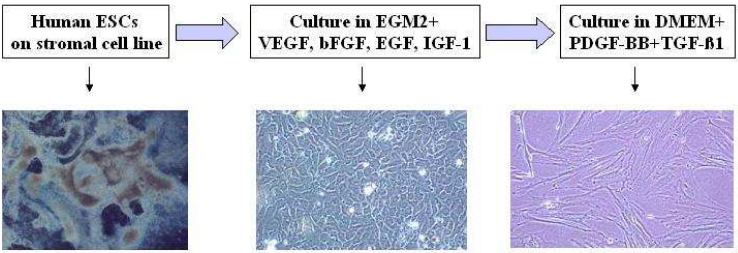

Figure 10. Schema of derivation of ECs and SMCs from human ESCs based on defined culture conditions. First, human ESCs, H9 cell line, were allowed to differentiate by stromal cell co-culture with mouse bone marrow-derived stromal cell line S17 for 10-15 days. Then, endothelial cell development was supported using EGM2 media containing specific cytokines (VEGF, bFGF, EGF and IGF-1). Next, these human ESC-derived ECs were cultured in DMEM media containing TGFβ1 and PDGF-BB to promote SMC differentiation. Photomicrographs show hESC-derived ECs with characteristic EC morphology. (Original magnification 100x). After change to SMC conditions, cells flatten out and show pronounced intracellular fibrils. (Original magnification100x).

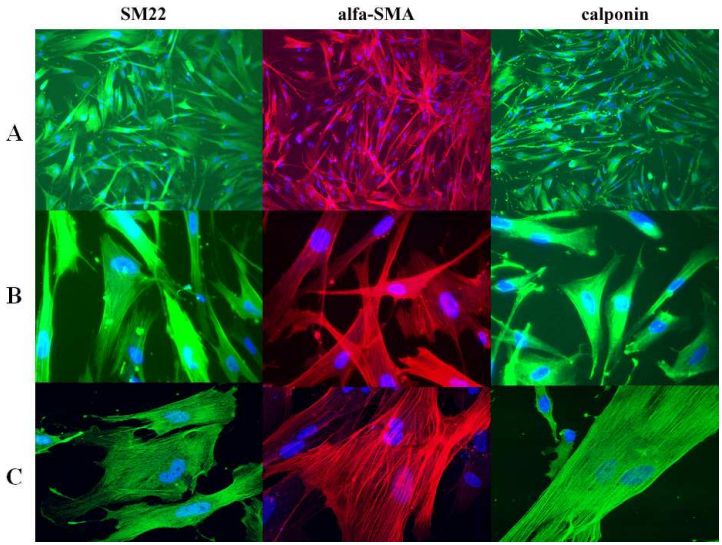

Figure 11. Immunoflouroscent staining of human ESC-derived SMCs (left to right): SM22, α-SMA and calponin. Original magnification 100X(A),200x(B),400x(C) Blue signal represents Hoechst 33258 stained nuclei.

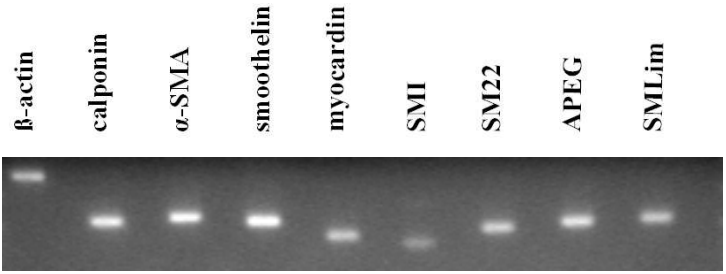

Figure 12. RT-PCR of human ESC-derived SMCs for eight common SMCs genes (and β-actin control), as indicated above each indicated lane. To control for contaminating genomic DNA, reactions were also done under conditions with no reverse transcriptase.

Gene	hESC-ECs	hESC-SMCs
EC Specific Genes		
CD31	46.29±1.02	0.74±0.05
Flk1	7.15±1.56	0.01±0.00
Tie2	9.48±0.14	3.53±2.27
eNOS	7.56±7.54	2.90±4.09
VE-cadherin	4.76±5.23	0.00±0.00
SMC Specific Genes		
calponin	0.67±0.02	3.1±0.40
alpha-SMA	11.24±0.52	47.93±20.38
SM22	0.62±0.06	6.37±1.05
SM1	1.49±0.55	2.30±0.49
myocardin	13.39±2.59	45.95±27.85

Table 1. Q-RT-PCR of human ESC-derived ECs and human ESC-derived SMCs. Human ESC-derived ECs and human ESC-derived SMCs were analyzed for expression of typical EC and SMC genes**.** For ECs and SMCs, all values are means with standard deviations of three RT-PCR analyses of independent experiments**.** Human ESC-derived ECs express EC-specific genes, whereas human ESC-derived SMCs express lower levels of these genes. In contract, human ESC-derived SMCs express SMC-specific genes, and human ESC-derived ECs express lower levels of these genes. mRNA levels were normalized against β-actin.

Gene	HUVECs in EC Culture	HUVECs in SMC Culture
EC Specific Genes		
CD31	12459.86	16612.71
Flk1	4.87	5.91
Tie2	225.97	598.41
eNOS	39.8	37.41
VE-cadherin	6608.01	8902.53
SMC Specific Genes		
calponin	0.01	0.12
alpha-SMA	0.18	0.33
SM22	0.03	0.06
SM1	0.02	0.06
myocardin	0.04	0.22

Table 2. Q-RT-PCR of HUVECs. Q-RT-PCR was performed on HUVECs cultured in EC and SMCconditions. Here, only EC-specific genes are expressed by these cells cultured under either EC or SMC conditions, and SMC-specific genes are not expressed under either condition. mRNA levels were normalized against β-actin.

5.3. Enhanced Endothelial Progenitors from Wnt Expressing Stromal Cells

To better define conditions that support or enhance differentiation of ECs or SMCs from human ESCs, M210 stromal cells over-expressing either Wnt1 and Wnt5 were used to induce differentiation in human ESCs as described[29]. $CD34^+$ cells were isolated and assessed quantitatively for their ability to produce endothelial progenitors. Not only were a greater number of $CD34^+$ cell obtained, the limiting dilution assay also revealed that $CD34^+$ cells isolated from Wnt1 expressing stromal cells yielded a higher number of ECs that could be subsequently induced to form SMCs, as an indication of vascular progenitor cells (Figure13). Wnt5 expressing M210 cells did not have the same effect.

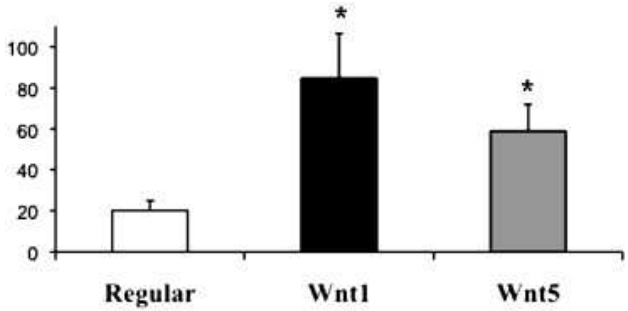

Figure 13.Increased Development of CD34+ Vascular Progenitor Cells From Wnt1 Expressing Stromal Cell Differentiation. Limiting dilution analysis was done to quantify vascular progenitor cells from human ESCs allowed to differentiate on M2-10B4 stromal cells that did not over-express Wnt proteins, or M2-10B4 cells that overexpressed either Wnt1 or Wnt5, as indicated. Numerical values shown as progenitor cells per 10,000 cells. Error bars represent Standard Error of the Mean of n= 4 individual experiments; * Wnt1 ($p=0.0279$) and * Wnt5 ($p=0.0309$).

5.4.Functional characterization of human ESC-derived endothelial and smooth muscle cells

To evaluate the functional characteristics of hESC-ECs and SMCs, nine different pharmacological agonists were used to measure the ability of these cells to respond to stimuli by release with a change in intracellular calcium concentration.(Figure 14). The majority of the SMC population responded to bradykinin, oxytocin and endothelin-1 and fewer cells demonstrated a response to histamine, ATP, serotonin, vasopressin, norephinephrine and carbachol. In contrast, the human ESC-derived ECs responded to endothelin-1, histamine, bradykinin, as well as carbachol, though there was little response to oxytocin or the other agonists (Figure 14C). Not only do these results support the notion that human ESC-derived ECs and human ESC-derived SMCs are distinct populations, but also indicate their ability to function in a physiologically appropriate manner. Undifferentiated human ESCs were also tested and were found only to have a uniform Ca-response to carbachol, ATP, and ET-1. While the three populations each have different response profiles, the lack of response by human ESC-derived ECs and human ESC-derived SMCs to certain agonists indicates these cells have not advanced to a fully mature phenotype.

To evaluate functional interactions between the two cell types, human ESC-derived ECs and human ESC-derived SMCs were cocultured in a Matrigel tube formation assay. Here, distinct difference was evident when the two populations were cultured together as opposed to being cultured as single populations in Matrigel (Figure15).

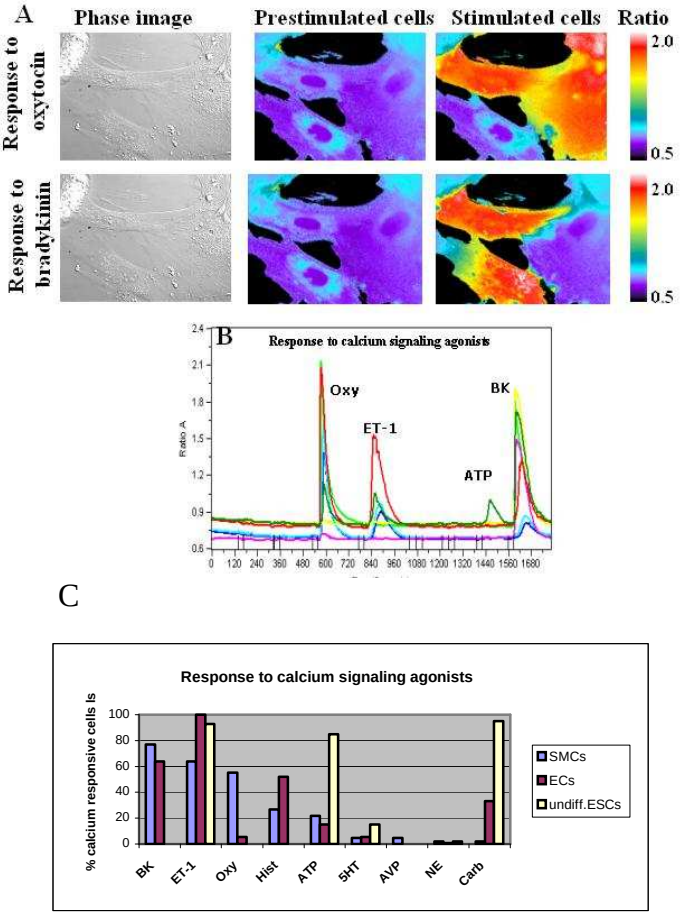

Figure 14. Intracellular calcium response of human ESC-derived ECs and human ESC-derived SMC to pharmacological agonists. Responses of each cell type were measured via a fluorimetric ratio using fura-2**. (A)** From left to right: human ESC-derived SMC phase images, Fura2 loaded human ESC-derived SMCs prior to agonist exposure, fluorometric changes post-agonist exposure (oxytocin and bradykinin) **(B)** Representative of time course graph of seven

individual hESC-SMCs exposed to oxytocin, ET-1, ATP and Bradykinin. **(C)** Graphic summary comparing responses of 100 undifferentiated human ESCs, 100 human ESC-derived ECs , and 105 human ESC-derived SMCs to specific agonsits: bradykinin (BK), endothelin-1 (ET-1), oxytocin (Oxy),histamine (Hist), ATP, serotonin (5-HT), vasopressin (AVP), norepinephrine (NE), and carbachol (Carb).

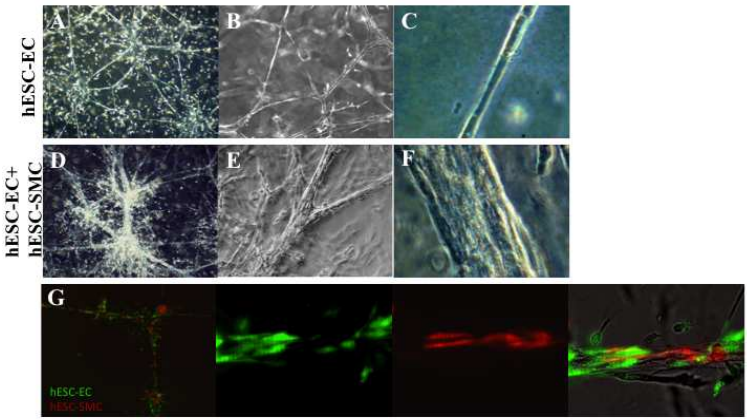

Figure 15. In-vitro Matrigel Tube Formation Assay. Vascular tube structures formed in both hESC-derived ECs and hESC-derived EC/ SMC cultures, with the cocultured cells interacting to form more robust, denser tube structures. **(A-C)** hESC-derived ECs alone **(D-F)** hESC-derived ECs cocultured with hESC-derived SMCs **(G)** Fluorescent image of GFP-expressing hESC-ECs cocultured with mCherry-expressing hESC-SMC,(G) show low power (25X) of both cell populations, as well as higher power (200X) of separate GFP and mCherry expressing cells, as well as colocalization of fluorescent cells with phase image. (Original magnifications: A 40x, B 100x, C 400x, D 40x, E 100x F 400x, G 25x and 200x).

6. DISCUSSION

In our study, we demonstrate an efficient method to differentiate ECs and SMCs from human ESCs in a defined stepwise culture system. Two main approaches have been utilized by our group and others to support differentiation of human ESCs into ECs: stromal cell coculture and EB formation[9,10,30,31]. In contrast to work by Levenberg at al[10] using EB-mediated differentiation, here we used stromal cell co-culture with the mouse bone marrow derived stromal cell lines (M2-10B4 and S17) as an efficient method to derive vascular progenitor cells. Phenotypical and functional characteristics of our human ESC-derived ECs including morphology, expression of cell-surface antigens, uptake of acetylated LDL, and tube formation in Matrigel were consistent with the typical EC characteristics.

Furthermore, based on studies to derive SMCs from other cell populations such as mouse ES cells and adult progenitor cells[20,21,23], we evaluated the SMC potential of this human ESC-derived EC population. In our study, we demonstrated that changing the culture conditions to media containing TGF-β1 and PDGF-BB resulted in a rapid and profound change to SMC morphology confirmed by immunophenotyping and gene expression of SMC markers. Additionally, these human ESC-derived SMCs demonstrate a functional response to calcium signaling agonists similar to responses of SMCs in their physiological environments in vivo. Notably, the response of human ESC-derived ECs to these pharmacological agonists is considerably different than the human ESC-derived SMCs. Despite the fact these two populations are derived from a common $CD34^+$ vascular progenitor cell population, these studies clearly illustrate the difference between the two cell types and highlight their independent contribution in the structure and function of mature vasculature.

While ECs and SMCs have been previously derived and characterized from human ESCs, our results advance these previous results in several important ways. First, we are able to demonstrate the potential of a human ESC-derived $CD34^+$ cells to produce both ECs and SMCs. Second, this is accomplished in a novel, efficient, three phase culture system through the development of human ESC-derived ECs and subsequent differentiation of human ESC-derived SMC cell population. Third, this method of human ESC differentiation is scalable to produce the large EC and SMC populations with little variability. Finally, in addition to demonstrating distinct responses to a variety of pharmacological agents, the two populations of ECs and SMCs were successfully combined in culture to form more robust, enhanced vascular structures.

There are at least two mechanisms that may account for the ability to derive SMCs from a population of human ESC-derived ECs. One possibility is that the initial human ESC-derived ECs, generated from the $CD34^+$ vascular progenitor population contain a very limited number of SMC progenitor cells that remain relatively suppressed under EC culture conditions. Then, upon changing the culture from EC to SMC conditions, the SMC population rapidly expands, and the EC growth is limited, eventually eliminating the human ESC-derived EC population from the culture. These potential SMC progenitor cells could be in either the main $CD34^+$ cell population or the residual $CD34^-$ cells that remain after immunomagnetic sorting. Alternatively, and more likely, the human ESC-derived ECs may be capable of directly converting to an SMC population under the alternative conditions. This second hypothesis is supported by the fact that human ESC-derived ECs cultured under SMC conditions for as little as 24 hours quickly change morphology and no EC-like cells are observed. In attempts to derive human ESC-derived SMC directly from the $CD34^+$ population, cells placed under SMC conditions did not give rise to viable cultures. The

two main factors that contributed to this fate were low plating efficiency and no detectable cell proliferation of plated cells. Moreover, typical EC characteristics such as the expression of EC markers and tube formation capabilities are also quickly diminished. Further studies are required to better define and validate the mechanisms of differentiation operating in these cultures.

Mouse ESCs have been previously used to model both EC and SMC development including characterization of $Flk1^+$ cells capable of producing ECs, SMCs, and hematopoietic cells[25]. While both ECs and SMCs could be derived from this population, they were cultured and expanded as separate populations and the SMCs did not differentiate from the EC population. It is important to note that other more definitive or mature EC populations such as HUVECs are not able to convert to SMCs under the same conditions that induce differentiation from human ESC-derived ECs to human ESC-derived SMCs. This partially clarifies the difference in developmental potential of fetal ECs and human ESC-derived ECs.

A recent study also found separate outgrowth of ECs and SMCs from a $CD34^+$ population selected from EBs[32]. While these populations display phenotypes similar to the cell described in our study, it is important to note the differences in methods and in the secondary characteristics of the populations. The method for generating SMCs featured in our work occurs via a human ESC-derived ECs intermediate in a stepwise differentiation process. By using the stromal cell coculture method outlined in this study, a greater potential to study the $CD34^+$ progenitor population exists.

In addition to defining the phenotypic function of human ESC-derived ECs and SMCs, it is possible to further control the differentiation environment via co-culture through the use of genetically modified stromal cells or additional media supplements. In this study, use of Wnt1- and Wnt5-

overexpressing M210 stromal cells not only increased the quantity of $CD34^+$ progenitors, but also the coexpression of typical EC surface antigens, such as CD31 and Flk1.

The field of cardiovascular regenerative medicine is rapidly progressing. Multiple studies have evaluated the ability of different cell populations to mediate cardiac repair and/or improved function both using model animals and clinical studies.[33-35] Most of these studies use heterogenous or poorly defined cell populations such as myocytes, whole bone marrow or, mesenchymal stem cells, and the mechanisms that lead to improved function are often not clear. While improvement of cardiac function has been demonstrated in rodent models[36,37], these findings do not always translate to similar efficacy in clinical trials[38,39]. Use of human ESC-derived cells can be utilized to better identify cells most effective at cardiovascular repair. Specifically, use of human ESCs with stable expression of fluorescent proteins as described here, and bioluminescent imaging via luciferase-expressing cells, as demonstrated previously[40] , can be used to better define the contribution of defined cell populations in pre-clinical models of ischemia. While human ESC-derived cells are not yet suitable for clinical trials, studies of these cell populations can help to better identify cells from bone marrow or other adult tissue that may be most effective at cardiovascular repair. It is most likely that truly efficient cardiovascular repair will require a combination of all naturally occurring vascular components (ie endothelial, smooth muscle, pericyte, and cardiomyocyte cells). Using a coculture differentiation system such as the one outlined in this study provides a tool for isolating all of these cell types as they arise during the differentiation process. This method is highly effective, reproducible, and fulfills the desire to to derive all cell types from a single source.

6. CONCLUSIONS

In our study, we demostrate great potential of human ESCs (H9 cell line) to differentiate into both ECs and SMCs in a defined stepwise fashion via a novel three-phase culture system. We show that these cells can be expanded in culture and induced to maintain distinct phenotypic and functional characteristics of both ECs and SMCs as demonstrated by detailed analyses. Here, we also demonstrate more complete fuctional characterization of the human ESC-derived ECs and SMCs including calcium imaging to define distinct responses to a panel of nine agonists. We also show an increase in the number and quality of $CD34^+$ vascular progenitor cells derived using Wnt1-expressing M210 stromal cell layers during the initial differentiation period. This population of $CD34^+$ cells produced a higher percentage of endothelial progenitors.

Our human ESC-derived EC and SMC model provides important insight into human vascular development as well as a source of preliminary data for future design of clinical vascular regenerative therapies. Most importantly, this system elucidates more closely the relationship between ECs and SMCs. The stepwise differentiation outlined here is an efficient, reproducible method with great potential for large-scale cultures suitable for clinical applications.

8. LITERATURE

Reference List

(1) Bai H, Wang ZZ. Directing human embryonic stem cells to generate vascular progenitor cells. Gene Ther. 2007.

(2) Hematti P, Obrtlikova P, Kaufman DS. Nonhuman primate embryonic stem cells as a preclinical model for hematopoietic and vascular repair. Exp Hematol. 2005;33:980-986.

(3) Levenberg S, Zoldan J, Basevitch Y, Langer R. Endothelial potential of human embryonic stem cells. Blood. 2007;110:806-814.

(4) Peura TT, Bosman A, Stojanov T. Derivation of human embryonic stem cell lines. Theriogenology. 2007;67:32-42.

(5) Cowan CA, Klimanskaya I, McMahon J et al. Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med. 2004;350:1353-1356.

(6) Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145-1147.

(7) Moon SY, Park YB, Kim DS, Oh SK, Kim DW. Generation, culture, and differentiation of human embryonic stem cells for therapeutic applications. Mol Ther. 2006;13:5-14.

(8) Drukker M, Katchman H, Katz G et al. Human embryonic stem cells and their differentiated

derivatives are less susceptible to immune rejection than adult cells. Stem Cells. 2006;24:221-229.

(9) Wang L, Li L, Shojaei F et al. Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. Immunity. 2004;21:31-41.

(10) Levenberg S, Golub JS, Amit M, Itskovitz-Eldor J, Langer R. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A. 2002;99:4391-4396.

(11) Vodyanik MA, Bork JA, Thomson JA, Slukvin II. Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood. 2005;105:617-626.

(12) Gerecht-Nir S, Ziskind A, Cohen S, Itskovitz-Eldor J. Human embryonic stem cells as an in vitro model for human vascular development and the induction of vascular differentiation. Lab Invest. 2003;83:1811-1820.

(13) Conway EM, Collen D, Carmeliet P. Molecular mechanisms of blood vessel growth. Cardiovasc Res. 2001;49:507-521.

(14) Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev. 2004;84:767-801.

(15) Gittenberger-de Groot AC, DeRuiter MC, Bergwerff M, Poelmann RE. Smooth muscle cell origin and its

relation to heterogeneity in development and disease. Arterioscler Thromb Vasc Biol. 1999;19:1589-1594.

(16) Owens GK. Regulation of differentiation of vascular smooth muscle cells. Physiol Rev. 1995;75:487-517.

(17) Mahoney WM, Schwartz SM. Defining smooth muscle cells and smooth muscle injury. J Clin Invest. 2005;115:221-224.

(18) Sobue K, Hayashi K, Nishida W. Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. Mol Cell Biochem. 1999;190:105-118.

(19) Yoshida T, Owens GK. Molecular determinants of vascular smooth muscle cell diversity. Circ Res. 2005;96:280-291.

(20) Blank RS, Swartz EA, Thompson MM, Olson EN, Owens GK. A retinoic acid-induced clonal cell line derived from multipotential P19 embryonal carcinoma cells expresses smooth muscle characteristics. Circ Res. 1995;76:742-749.

(21) Drab M, Haller H, Bychkov R et al. From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: a retinoic acid and db-cAMP in vitro differentiation model. FASEB J. 1997;11:905-915.

(22) Oishi K, Ogawa Y, Gamoh S, Uchida MK. Contractile responses of smooth muscle cells differentiated from rat neural stem cells. J Physiol. 2002;540:139-152.

(23) Ross JJ, Hong Z, Willenbring B et al. Cytokine-induced differentiation of multipotent adult progenitor cells into functional smooth muscle cells. J Clin Invest. 2006;116:3139-3149.

(24) Huang H, Zhao X, Chen L et al. Differentiation of human embryonic stem cells into smooth muscle cells in adherent monolayer culture. Biochem Biophys Res Commun. 2006;351:321-327.

(25) Yamashita J, Itoh H, Hirashima M et al. Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature. 2000;408:92-96.

(26) Ferreira LS, Gerecht S, Shieh HF et al. Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle like cells and form vascular networks in vivo. Circ Res. 2007;101:286-294.

(27) Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A. 2001;98:10716-10721.

(28) Tian X, Kaufman DS. Hematopoietic development of human embryonic stem cells in culture. Methods Mol Biol. 2008;430:119-133.

(29) Woll PS, Morris JK, Painschab MS et al. Wnt signaling promotes hematoendothelial cell development from human embryonic stem cells. Blood. 2008;111:122-131.

(30) Wang ZZ, Au P, Chen T et al. Endothelial cells derived from human embryonic stem cells form

durable blood vessels in vivo. Nat Biotechnol. 2007;25:317-318.

(31) Zambidis ET, Peault B, Park TS, Bunz F, Civin CI. Hematopoietic differentiation of human embryonic stem cells progresses through sequential hematoendothelial, primitive, and definitive stages resembling human yolk sac development. Blood. 2005;106:860-870.

(32) Ferreira LS, Gerecht S, Shieh HF et al. Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle like cells and form vascular networks in vivo. Circ Res. 2007;101:286-294.

(33) Leor J, Gerecht-Nir S, Cohen S et al. Human embryonic stem cell transplantation to repair the infarcted myocardium. Heart. 2007.

(34) Laflamme MA, Zbinden S, Epstein SE, Murry CE. Cell-based therapy for myocardial ischemia and infarction: pathophysiological mechanisms. Annu Rev Pathol. 2007;2:307-339.

(35) van Laake LW, Passier R, Monshouwer-Kloots J et al. Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. Stem Cell Res. 2007;1:9-24.

(36) Passier R, van Laake LW, Mummery CL. Stem-cell-based therapy and lessons from the heart. Nature. 2008;453:322-329.

(37) Tendera M, Wojakowski W. Cell therapy--success does not come easy. Eur Heart J. 2009;30:640-641.

(38) Laflamme MA, Chen KY, Naumova AV et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol. 2007;25:1015-1024.

(39) He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. Circ Res. 2003;93:32-39.

(40) Wilber A, Linehan JL, Tian X et al. Efficient and stable transgene expression in human embryonic stem cells using transposon-mediated gene transfer. Stem Cells. 2007;25:2919-2927.

9. LIST OF PUBLICATIONS

Seznam publikací vztahujících se k tématu dizertace:

a) s IF

Hill KL*, Obrtlikova P*, Alvarez DF et al. Human embryonic stem cell-derived vascular progenitor cells capable of endothelial and smooth muscle cell function. Experimental Hematology. 2010, 38: 246-257 IF 3.203
**Hill and Obrtlikova contributed equally to this work as co-first authors, the proof in paper*

Hematti P., Obrtlikova P., Kaufman DS. Nonhuman primate embryonic stem cells as a preclinical model for hematopoietic and vascular repair. Experimental Hematology 2005, 33:980-986 , IF 4.681.

Seznam publikací vztahujících se k původnímu tématu dizertace:

a) s IF
Pytlik R., Hofman P., Kideryova L., Cervinkova P., Obrtlikova P., Salkova J., Trneny M., Klener P. Dendritic cells and T lymphocyte interactions in patients with lymphoid malignancies. Physiol Res. 2008, 57: 289-298, IF 1.806.

b) bez IF
Obrtlíková P., Pytlík R., Hofman P., Červinková P. Hodnocení přežití a proliferace T-lymfocytů ve smíšené lymfocytární reakci s dendritickými buňkami metodou diferenciálního gatingu, Epidemiol Mikrobiol Imunol. 2006, 54: 109-115.

Ostatní publikace:

Mocikova H, Obrtlikova P, Vackova B, Trneny M. Positron emission tomography at the end of first- line therapy and during follow-up in patients with Hodgkin lymphoma: a retrospective study. Ann Oncol. 2009 Nov 9. [Epub ahead of print] IF 4,935.

Mociková H, Vacková B, Pytlík R, Obrtlíková P, Trněný M. Analýza absolutního počtu lymfocytů a dalších faktorů ovlivňujících přežívání pacientů s Hodgkinovým lymfomem po autologní transplantaci periferních kmenových buněk. Transfuze a hematologie dnes. 2010, 16: 25-29.

10. POSTERS AND ORAL PRESENTATIONS

Petra Obrtlikova, Jeffrey J. Ross, Aernout Luttun, Joan D. Beckman, Susan A. Keirstead, and Dan S. Kaufman. Transition of Human Embryonic Stem Cell-Derived Endothelial Cells to Smooth Muscle Cells in Culture as a Model for Vasular Development. 47th American Society of Hematology Annual Meeting, 2005, (poster).

Katherine L. Hill, Petra Obrtlikova**,** Diego F Alvarez, Judy A King, Qinglu Li, Jianyi Zhang, and Dan S. Kaufman**.** In Vitro and in Vivo Characterization of Human Embryonic Stem Cell-Derived $CD34^+$ Cells That Function as Pericytes with Endothelial Cell and Smooth Muscle Cell Potential. Blood (ASH Annual Meeting Abstracts, lecture), Nov 2008; 112: 693.

Pytlík R., Purkrábková T., Stehlík D., Hofman P., Červinková P, Obrtlíková P, Zima T., Trněný M.: Growth and

differentiation of mesenchymal stem cells in human sera 31 st Annual meeting of the European Group for Blood and Marrow transplantation 2005 (lecture).

Pytlík R., Purkrábková T., Obrtlíková P., Strnadová H., Karban J., Novotná E., Trněný M., Klener P: Růst a diferenciace mezenchymových kmenových buněk u pacientů s lymfoidními malignitami. Olomoucké hematologické dny 2003 (poster).

Pytlík R., Trč T., Purkrábková T**.,** Obrtlíková P., Strnadová H., Stehlík D., Trněný M., Klener P: Využití mezenchymových buněk v ortopedii. Pražský hematologický den 2003 (přednáška).

P.Obrtlikova, B.Vackova, R.Pytlik, H.Krejcova, P.Klener and M.Trneny , Ist Dept Medicine, Charles University General Hospital, Prague, Czech Republic: Effects of Pretransplantation Treatment with Rituximab on Outcomes of Autologous Stem-Cell Transplantation for Diffuse Large B-Cell Lymphoma.11th Congress of the European Hematology Association, 2006 (poster).

P.Obrtlíková, B.Vacková, R.Pytlík, H.Krejčová, P.Klener and M.Trněný, I. interní klinika VFN a 1. LF UK v Praze: Vysokodávkovaná chemoterapie s autologní transplantací krvetvorných buněk u nemocných s difuzním velkobuněčným lymfomem- analýza výsledků jednoho pracoviště XX. Olomoucké hematologické dny 2006 (přednáška).

P.Obrtlíková, B.Vacková, R.Pytlík, H.Krejčová, P.Klener and M.Trněný, I. interní klinika VFN a 1. LF UK v Praze: Vysokodávkovaná chemoterapie s autologní transplantací krvetvorných buněk v léčbě folikulárního lymfomu –

sledování nemocných léčených v jednom centru. XX. Olomoucké hematologické dny 2006 (přednáška).

P.Obrtlíková, B.Vacková, R.Pytlík, H.Krejčová, P.Klener and M.Trněný, I. interní klinika VFN a 1. LF UK v Praze: Rituximab v předtransplantační léčbě a jeho význam na výsledek léčby po autologní transplantaci u pacientů s difuzním velkobuněčným lymfomem. Brněnské onkologické dny 2006 (přednáška).

David Salek, Ingrid Vasova, Robert Pytlik, David Belada, Tomas Papajik, Katerina Kubackova, Jan Pirnos, Ludmila Boudova, Roman Kodet, Petra Obrtlikova**,** Hana Krejcova, and Marek Trneny **:** Mantle Cell Lymphoma International Prognostic Score Is Valid and Confirmed in Unselected Cohort of Patients Treated in Rituximab Era. Blood (ASH Annual Meeting Abstracts), Nov 2008; 112**:** 3745.

Mociková, Heidi - Obrtlíková, Petra - Skopalová, M. - Vacková, Blanka - Pytlík, Robert - Šálková, Jana - Haber, Jan - Kolešková, Evženie - Žikešová, Eva - Straub, Jan//Cmunt, Eduard//Šišková, Magda//Karban, Josef//Campr, V.//Stříteský, Martin//Trněný, Marek: PET findings after therapy during folow-up in patients with Hodgkin lymphoma- a retrospective study (lecture), 10th International Conference on Malignant Lymphoma 2008 Lugano, Switzerland, June 04-07, 2008.

H.Mociková, P.Obrtliková, B.Vacková, R.Pytlík, J.Šálková,J.Haber, E.Kolešková, J.Straub, E.Žikesová, E.Cmunt, P.Cieslar, M.Šišková, J.Karban a M.Trněný Nálezy v dlouhodobém sledování pacientů s Hodgkinovým lymfomem po první linii léčby, Česko-slovenský hematologický a transfuziologický sjezd 2008, 05.09.2008 - 09.09.2008 Špindlerův Mlýn, Česká Republika.

H.Mociková,P.Obrtliková,Vacková,R.Pytlík,J.Šálková, J.Haber,E.Kolešková, J.Straub, E.Žikesová, E.Cmunt, P.Cieslar, M.Šišková, J.Karban, V. Campr, J Stříteský and M.Trněný: Positron emission tomography at the end of first-line therapy and during follow-up in patients with Hodgkin lymphoma: a retrospective study, poster, 3rd International Symposium on Hodgkin Lymphoma 2009, Prague 17-18 September 2009.

H.Mociková,B.Vacková, R.Pytlík, P.Obrtliková and M. Trněný: Lymphocyte recovery after autologous stem cell transplantation in patients with Hodgkin lymphoma,3rd International Symposium on Hodgkin Lymphoma 2009, poster, Prague 17-18 September 2009.