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DIZERTAČNÍ PRÁCE

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

MUDr. Petra Obrtlíková

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Školitel: Prof. MUDr. Marek Trněný, CSc.

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LIST OF ABBREVIATIONS:

AVP	vasopressin
bFGF	basic fibroblast growth factor
BK	bradykinin
BMP	bone morphogenetic protein
Carb	carbochol
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)
ET-1	endothelin-1
G-CSF	granulocyte colony stimulating factor
Hist	histamin
HUVECs	human umbilical vein endothelial cells
ICM	inner cell mass
IGF	insulin-like growth factor
Ihh	Indian Hedgehoh
Oxy	oxytocin
PDGF-BB	platelet- derived growth factor -BB
PECAM 1	platelet endothelial adhesion molecule-1
PGC	primordial germ cells
Q-RT-PCR	quantitative reverse transcriptase polymerase chain reaction
MEF	mouse embryonic fibroblasts
NE	norephinephrine
RESDECs	rhesus ESC –derived endothelial cells
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor
SCID	severe combined immunodeficient
SMA	smooth muscle actin

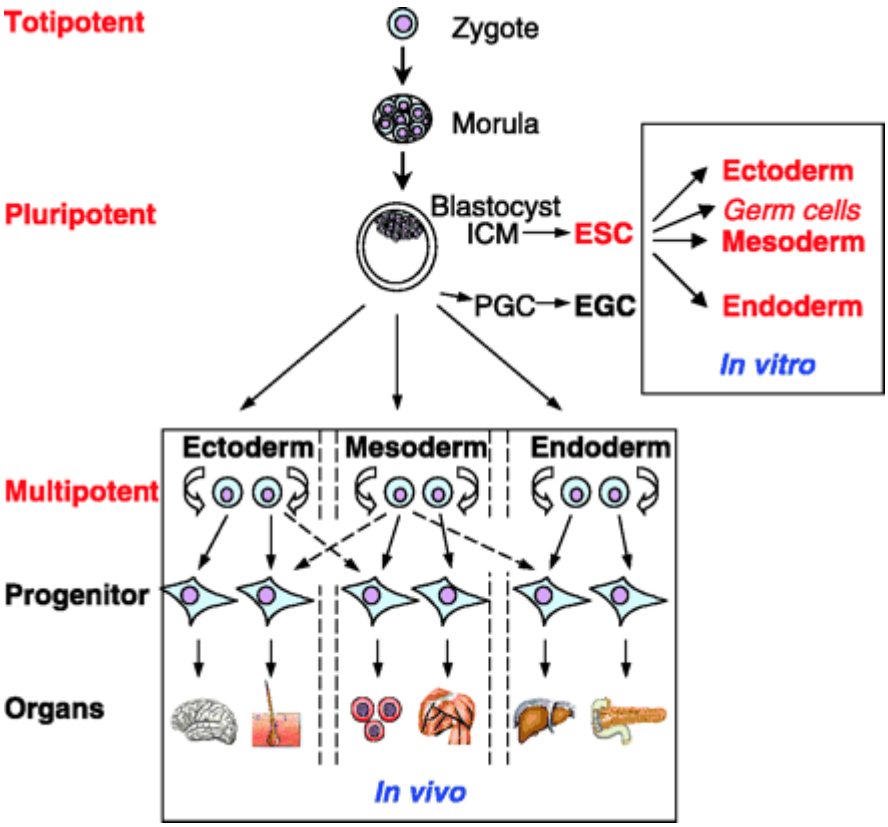
SMC(s)	smooth muscle cell(s)
SM-MHC	smooth muscle myosin heavy chain
SSEA	stage specific embryonic antigen
TEM	transmission electron microscopy
TGF-β1	transforming growth factor –beta 1
Ve-cad	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VPCs	vascular progenitor cells
Vwf	von Willebrand factor

1. INTRODUCTION

There is now growing evidence that human embryonic stem cells (ESCs) provide an important resource to define the cellular and molecular mechanism of vascular development, as well as the developmental relationship between endothelial cells (ECs), hematopoietic and smooth muscle cells (SMCs). Moreover, human ESCs are also highlighted as a promising potentially unlimited source of cells for vascular regenerative approaches including cell transplantation into heart to improve myocardial regeneration, induction of angiogenesis for treatment of regional ischemia, formation of new blood vessels or vascularization of engineered tissue in vitro before transplantation.[1-3]

1.1. Human embryonic stem cells

Human embryonic stem cells 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. These cells can continuously proliferate in an undifferentiated state, while retaining a normal karyotype, maintain a high level of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells.[4-7] Human ESCs can be differentiated into representative derivatives of all three embryonic germ layers (endoderm, ectoderm and mesoderm) in vitro and in vivo. Following their injection into immunodeficient mice, undifferentiated human ESCs can generate teratomas composed of multiple-tissue-type cells, thus demonstrating their pluripotent potential.[4,8,9] Since their first derivation by Thomson et all [4], 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.[10] 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.[11] Therefore, human ES cells may play important role in stem cell-based regenerative medicine.



(Anna M. Wobus et al, Physiol Rev 85:635-678, 2005)

Figure 1. Stem cell hierarchy. Zygote and early cell division stages (blastomeres) to the morula stage are defined as totipotent, because they can generate a complex organism. At the blastocyst stage, only the cells of the inner cell mass (ICM) retain the capacity to build up all three primary germ layers, the endoderm, mesoderm, and ectoderm as well as the primordial germ cells (PGC), the founder cells of male and female gametes. In adult tissues, multipotent stem and progenitor cells exist in tissues and organs to replace lost or injured cells. At present, it is not known to what extent adult stem cells may also develop (transdifferentiate) into cells of other lineages or what factors could enhance their differentiation capability (dashed lines). Embryonic stem (ES) cells, derived from the ICM, have the developmental capacity to differentiate in vitro into cells of all somatic cell lineages as well as into male and female germ cells.

Undifferentiated human ESCs form relatively flat and compact colonies either in a culture on feeder cells or in a feeder-free culture system[10] and express stem cell markers including Oct4, stage specific embryonic antigen (SSEA) -3 and SSEA-4., TRA-1-60, TRA 1-81, and alkaline phosphatase that distinguish them from differentiated cells.[4,9]

One of the most important issues in ESC research is the development of a method for the efficient differentiation of the ESCs into specific cell types. Until now, several differentiation approaches have been developed. First, when the essential factors for the maintenance of undifferentiated states are removed and the cells are cultured in suspension,

the ESCs can be triggered to undergo spontaneos differentiation by forming three-dimensional embryoid bodies (EBs) containing many differentiated cell types. Although the EB is far less organized than an embryo, it can partially mimic the spatial organization of cells in embryo.[1,12] In addition to differentiation via EB formation, ESCs can be differentiated directly via coculturing with specific feeder cells. This method is more efficient for directing the ESCs into specific lineages than are the EB-based protocols.[10,13-15] However, steps for the elimination of feeder cells are necessary, and concerns related to risk of xenocontamination remain. Another option is a differentiation of ESCs in a monolayer on defined substrates such as collagen without feeder cells. This protocol minimizes the influences of unknown factors affecting the differentiation process and present a promising culture option for differentiation of human ESCs. [16,17]

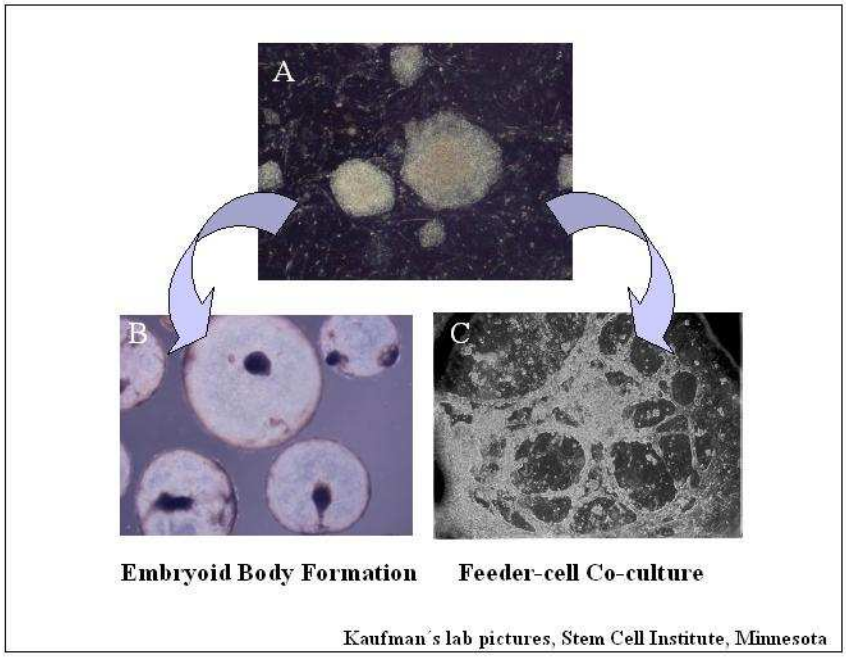


Figure 2. Methods of Embryonic Stem Cell Differentiation
A-undifferentiated human ESCs cultured on mouse embryonic fibroblasts (MEF)
B-differentiation of human ESCs by forming three-dimensional embryoid bodies
C-differentiation of human ESCs in coculture with mouse stromal cell line S17

1.2. ESC-derived endothelial cells

Various differentiation techniques and source of cells have been explored to induce EC diferentiation. Many previous studies have described successful differentiation of ESCs into ECs using mouse or nonhuman primate ESCs.[18-22] Remarkable work by Yamashita et.al. identified $Flk1^+$ population of the cells derived from murine embryonic stem cells which serve as common 'vascular progenitor cells' (VPCs) and can differentiate into two major vascular cell types, endothelial cells and mural cells (pericytes and vascular smooth muscle), and can reproduce the vascular organization process.[18] Subsequent studies by the same group further demonstrated that differentiated vascular cells derived from VPCs can contribute to generation of vascular structures in adult neoangiogenesis.[23]

Another study using nonhuman primate (cynomolgus) ESCs showed that the $Flk1^+VEcadherin^-$ cells give rise to both endothelial and mural cells and can act as VPCs in primates.[19] The authors also demonstrated that $Flk1^+$ cells can form vascular structure in vitro. Successful differentiation of functional ECs derived from nonhuman primate ESCs has also been accomplished using rhesus monkey ESCs.[21] In this study, undifferentiated ESCs exposed to medium containing defined growth factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF) gave rise to a relatively homogenous population of ECs which could be propagated and expanded for approximately 20 population doublings with a consistent phenotype and normal karyotype. These cells express many cell-surface antigens and genes specific for endothelial cells such as CD146, vWF, the integrin $\alpha_V\beta_3$, and Flk1. Additionally, it was shown that they bind the lectin ulex europaeus agglutinin-1, secrete VEGF, take up acetylated low-density lipoprotein and form capillary-like structures in Matrigel. In contrast to experiments with ECs derived from mouse and human ESCs, these rhesus ESC-derived endothelial cells (RESDECs) did not express CD31 and VE-cadherin, two surface antigens commonly, but not uniformly, found on the surface of ECs. Moreover, these cells were shown to produce functional blood vessels in a Matrigel plug implanted subcutaneously in mice.

During the last few years, several groups of investigators have explored the endothelial potential of human ESCs, mainly using the spontaneous differentiation of EBs to

vascular –like structures.[22,24,25] In these studies, it was found that during the differentiation process endothelial specific markers are expressed in sequential steps that reflect in vivo endothelial differentiation during embryonic development. Although the EB system enables investigation of vasculogenesis virtually as it occurs in the embryo, multiple cell lineages in EBs make it difficult to study amd control the behavior of ECs in detail.

Two main approaches have been used for purifying progenitor ECs from human ESCs: selecting 3-dimensional embryoid bodies (EBs) for specific cell-surface molecules[22,24,26] and supplementing feeder layers or the medium with various growth factors.[14,27,28] Multiple markers have been used for characterizing and identifying derived ECs. Expression of vascular endothelial cadherin (VE-cad), platelet endothelial adhesion molecule-1 (PECAM1), CD34, Flk1 (vascular andothelial growth factor receptor 2) and the ability to take up Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL) have been used as markers for identifying endothelial precursors. Mature endothelial cells were identified by selective staining for von Willebrand factor (VWF), endothelial nitric oxide synthase (eNOS), and E-selectin proteins.[29]

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 VEGF, bFGF, IGF and EGF.[24] These cells expressed mature endothelial protein VWF in addition to expressing PECAM1, CD34, Flk1, and VE-cad and being capable of taking up Dil-Ac-LDL. The function of these ECs derived from human ESCs was assessed in severe combined immunodeficient (SCID) mice by subcutaneous implantation of the cells mixed in a synthetic biopolymer scaffold. The section of implants examined after 14 days showed microvessels expressing human CD31 and CD34. This study demonstrates that during EB induction, human ESCs spontaneously differentiated into embryonic ECs, which were isolated and maintained in vitro.

Another study describing ECs from human ESCs identified a subpopulation of primitive cells derived from human ESCs with putative hemangioblast or hemogenic endothelial properties.[22] Here a subset of $PECAM1(CD31)^+$, $FLK1^+$, VE-$cadherin^+$ but $CD45^-$ cells isolated from day 10 human ESC-derived EBs possesed some phenotypic and functional EC properties. However, these cells did not express mature endothelial proteins, such as VWF and eNOS. Therefore, these progenitors were called „primitive endothelial-like cells“. After culture in endothelial cell medium, these progenitor cells gave rise to cells with endothelial morphology and expressed EC markers CD31, VE-cad and VWF. These human ESC-derived ECs were capable of Dil-Ac-LDL uptake, and formed vascular network in

Matrigel. When the progenitor cells were cultured in the presence of hematopoietic growth factors, they expressed CD45 and formed multilineage hematopoietic colonies on methylcellulose. These results suggest that $CD34^+/CD31^+$ population contain hematopoietic and endothelial progenitor cells, although it is not clear that this occurs at clonal level. Whether there is a true hemangioblast population in $CD34^+/CD31^+$ cell fraction remains unclear.

Zambidis et al[25] grew mesodermal-hemato-endothelial colonies from cells isolated from 7- to 12-day-old human EBs seeded onto serum-free methylcellulose medium. These colonies contained adherent and nonadherent cells. Nonadherent cells expressed CD45 and gave rise to hematopoietic colonies. Some adherent cells expressed endothelial marker, CD31, and were capable of Dil-Ac-LDL uptake. When the adherent cells from these colonies were isolated and cultured in endothelial medium (EGM2), they expanded to the cells with endothelial morphology, expressed the endothelial markers, CD31, VE-cad, VWF and were capable of Dil-Ac-LDL uptake. Because EB differentiation in this study was initiated from human ESC clumps, it is unclear whether mesodermal-hemato-endothelial colonies are generated from single cell-bipotential hemangioblast.

In another study, human ESCs were grown on collagen IV-coated dishes in the presence of endothelial differentiation-supporting medium for 6 days and then filtered through a 40-μm mesh strainer resulted in cells expressing specific endothelial progenitor markers, such as PECAM1, CD34, AC133, Tie2, and GATA2.[27] Reculture of the filtered cells on type IV collagen in the presence of VEGF resulted in endothelial-like cells. Reculture in the presence of platelet-derived growth factor-BB, a factor effecting SMC differentiation in mouse ESCs, resulted in a cell population expressing vascular SMC markers. Furthermore, reculture of the filtered cells on matrigel or collagen I in a three-dimensional system in the presence of VEGF resulted in cell sprouting and formation of network structures, providing additional confirmation to the endothelial-like properties of these cells. Although differentiation of human ESCs to vascular cells was demonstrated, the differentiation pathways involving cytokines and extracellular matrix environment remains to be elucidated.

Other researchers have used undifferentiated human ESCs grown on various feeder layers. For instance, Kaufman et al used mouse bone marrow stromal cells (S17 cell line) or mouse yolk-sack ECs (C166 cell line) as feeder layers for promoting hematopoietic differentiation of cultured human ESCs.[13] After 17 days, the cells had differentiated into an early hematopoietic subpopulation of $CD34^+CD3^-CD45^-$ cells. This subpopulation also contained $CD34^+$cells with coexpression of endothelial marker CD31. Similar results were

obtained when human ES cells were grown on bone marrow stromal cells (OP9 cell line) for only 8 to 9 days.[14] However, additional analysis of endothelial behavior or maturation of these cells were not discussed in these studies.

Another study using two-dimensional induction of human ESCs into ECs was published by Wang at al. [15] In this study, human ESCs were places on mouse embryonic feeders for 10 days. By day 10, 5-10% of these cells expressed CD34, a common hematopoietic and endothelial progenitor marker. They enriched the $CD34^+$ cells by magnetic bead sorting to 80-95% purity. When these cells were cultured in endothelial growth medium, the majority of the cells expressed endothelial markers CD31 and VE-cadherin. After transplantation of these cells into SCID mice, these ECs contributed to functional blood vessels that integrated into the host circulatory system.

Recently, study by Kelly at al investigated the molecular regulation of human EC development using human ESCs cultured on OP9 stromal cell line.[30] Their results revealed that exogenous soluble factor Indian Hedgehog (Ihh) promote endothelial differentiation of human ESCs via the bone morphogenic protein (BMP) pathway, and specifically through BMP4. These novel insights into molecular regulation of human EC development may help us in more efficient generation of cells that can be utilized for human clinical therapies in the future. As another culture parametr, it has long been known that shear stress can change endothelial morphology, proliferation and differentiation. [31,32]. Yamamoto and colleagues demonstrated that shear stress, event at low levels, promotes endothelial differentiaton of ESC. [33]

Based on previous studies, 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. PECAM1 is distributed at the intercellular clefts, and the endothelial marker VWF is expressed in the cytoplasm. 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. Importantly, we should noted that endothelial progenitors derived from human ESCs have various phenotypes because of various derivation protocols and the supplementation of growth factors and, more importantly, were isolated at different stages of development.[29]

Mouse, primate and also human ESCs have been used to generate functional ECs that could contribute to formation of stable vasculature and may serve as a feasible novel source of

cells for therapeutic angiogenesis.[15,23] However, many questions still remain to be solved. An important issue concerns the long-term properties of such ESC-derived vessels and if ESCs are a superior source for ECs compared to adult stem cells or progenitors isolated from the patient´s own bone marrow. [34-36]

1.3. Evidence of hemangioblast

Several groups of investigators have been tried to characterize the developmental relationship between ECs and hematopoietic cells. The existence of hemangioblast, the common precursor of hematopoietic and endothelial cells, was first described in the mouse embryo as well as in the mouse ESC differentiation model. [16,37-40] Moreover, further studies provided evidence about hemangioblasts also in human ESC differentiation model as well as in the adult life.[41-45,45,46] Such hemangioblasts were found in the bone marrow, umbilical cord blood or mobilized peripheral blood cells.[44,47] In these studies, the stem cells with hemangioblastic characteristics were often characterized by the expression of VEGF receptor 2 (Flk1) which is also expressed by endothelial progenitor cells. All these stem cells share the ability to differentiate in vitro into hematopoietic and endothelial lineages when exposed to specific differentiating conditions. An interesting demonstration of the existence of hemangioblast in human adult life comes from Gunsilius´study .[48] He showed the presence of Philadelphia chromosome in patients suffering from chronic myeloid leukemie both in bone marrow and in endothelial cells.

Kennedy et al [49] demonstrated for the first time the existence of the human hemangioblast derived from human ESCs using a procedure that consisted of serum-free differentiation in a mixture of cytokines followed by expansion in serum-containing medium. Here, the human hemangioblasts were identified by their capacity to generate blast colonies that display both hematopoietic and vascular potential. These colony-forming cells express the receptor tyrosine kinase (VEGF receptor2 = Flk1) and represent a transient population that develops in BMP-4-stimulated EBs between 72 and 96 hours of differentiation, prior to the onset of the primitive erythroid program. In this study, the clonality of the blast colonies was demonstrated by 2 different strategies and it was shown that the blast colonies with hematopoietic and endothelial potential are derived from single $Flk1^+$ cells.

Recently, Lu et al[50] described a population of bipotential progenitors, known as hemangioblasts, derived from human ESCs using a two-step procedure with different suplements under fully serum-free conditions. The clonal origin of blast colonies was confirmed using limiting-dilution studies. Compared to previous study, these hemangioblasts do not express Flk1 and CD31, suggesting that these human ES-derived blasts have different properties from blasts in the study performed by Kennedy.[49] However, when these cells were induced toward endothelial cell differentiation, Flk1, VE-cad and CD31 expression appeared, suggesting that these human ESC-derived blasts represent a different, earlier and more expandable population of cells. In vivo studies with SCID mice suggest that these cells have the ability to migrate to areas of vascular injury and assimilate with the resident vasculature to restore vascular function.

Despite the different culture procedures used, several research group have shown that timing of the progression toward lineage commitment from human EBs into endothelial and hematopoietic lineages follows a reproducible temporal pattern. First, the undifferentiated human ESCs form EBs, which differentiate into early endothelial/hematopoietic precursor and finally into endothelial/hematopoietic cells. [13,22,25,51-53] (Figure 3)

Although evidence from both mouse and human studies supports the concept of the hemangioblast, only very small numbers of common precursors were found in the examined populations.[53] It is possible that hemangioblasts may require the paracrine interactions for their survival and therefore the clonal analysis has underestimated their frequency as a common precursor. Moreover, because of different research techniques, the human ESC-derived precursors examined to date may be a heterogeneous population containing different stage of endothelial and hematopoietic progenies with different potentials, which may complicate the interpretation of results.

Finally, several in vitro studies suggest that SMCs are also progeny of the hemangioblast at the onset of hematopoietic development.[18,54,55] This issue remains a topic of discussion since our current knowledge about the relationship between SMCs and the other lineages generated by hemangioblast is still limited. Therefore, it will be important to study this question in more details to fully understand how the hemagioblast produces its progeny.

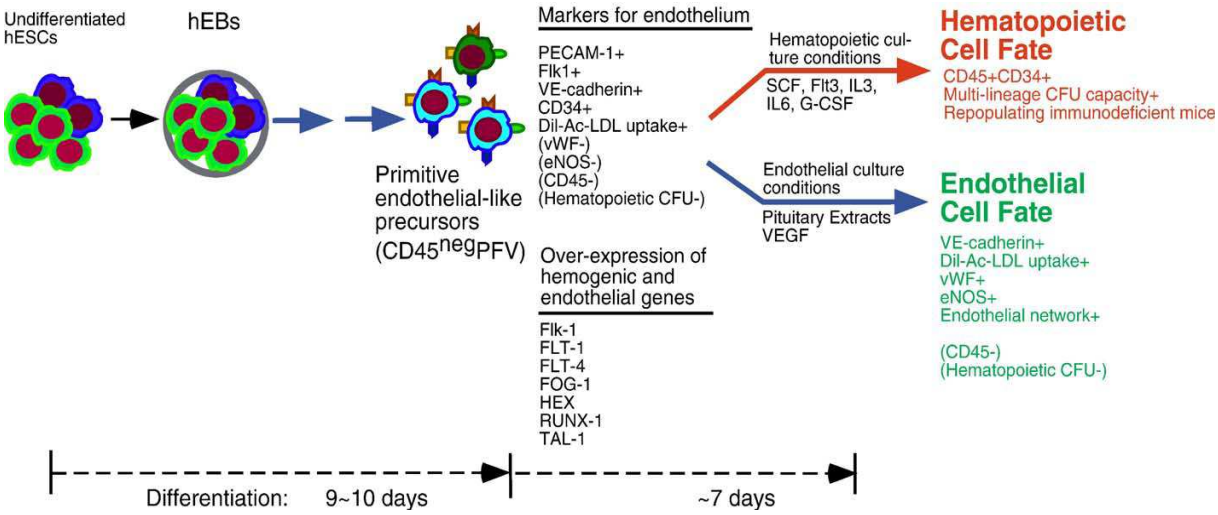


Figure 3. A working model of the current understanding of hematopoietic and endothelial development from human ESCs via EBs. A subset of embryonic endothelia lacking the common leukocyte marker CD45 but expressing surface markers PECAM-1, CD34, Flk-1, and VE-cadherin was identified during approximately 9 to 10 days of hEB development and termed CD45^{neg}PFV. CD45^{neg}PFV cells expressed genes associated with primitive cells with both endothelial and hematopoietic potential. CD45^{neg}PFV cells had either hematopoietic or endothelial cell fates. Clonal analysis demonstrated that a small proportion of CD45^{neg}PFV cells possessed human hemangioblast properties, capable of giving rise to both hematopoietic and endothelial cells.

(Wang, Trends Cardiovasc. Med 2006;16:89–94)

1.4. ESC-derived smooth muscle cells

The majority of the studies of angiogenesis research focus on the regulation of endothelium, but vascular SMCs are also important participants in formation of blood vessels. Endothelial cells alone cannot complete angiogenesis to form mature vasculature. 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.[56] ECs and SMCs interact with each other to regulate not only new vessel growth, but also multiple vascular functions. In adult animals, SMCs regulate growth of blood vessels, vessel tone diameter, vascular permeability and blood flow distribution.[57] Moreover, differentiation and phenotypic plasticity of vascular SMCs play important role also in many human diseases including atherosclerosis, cancer, and hypertension.[58]

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.[59] 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.[60,61] 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[62]. To date, characterization of SMCs often is done by demonstrating the presence of α-SMA, which is also expressed by myofibroblasts, and endothelial cells under certain conditions, and hence not specific for SMCs. SM22α, calponin-1 and SM-MHC are increasingly SMC restrictive, but transient expression in non-SMCs cannot be ruled out.[58] Smoothelin, another cytoskeletal protein found in mature SMCs, is absent in myofibroblast cells, providing a potential marker to distinguish between SMCs and fibroblasts. Cultured SMCs could rarely be stably maintained and are limited in the capacity for regulatory mechanism and pathway studies[63], 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.[64-68] 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.

Retinoic acid has been used to differentiate mouse ESCs into SMCs.[65] In this study, EBs were derived from mouse ESCs under the influence of retinoic acid and dibutyryl-cydic adenosine monophosphate between 7 and 11 days of culture. After that, 67% of the EBs showed formation of spontaneously contracting SMC clusters.

Recently, SMCs were characterized also during human ESC differentiation. In this study, Huang at al [69] established in vitro SMC differentiation system by treating the

monolayer-cultivated human ESCs with all-trans retinoic acid. When human ESCs were cultivated in differentiation medium containing 10 μM retinoic acid, more than 93% of the cells expressed SMC-marker genes along with the steadily accumulation of such SMC-specific proteins as α-SMA and SM-MHC. The fully differentiated SMCs were stable in phenotype and capable of contraction.

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. For instance, recent studies by Yamashita et al.[18] identified $Flk1^+$ population of the cells derived from murine ESCs which serve as common "vascular progenitor cells" and can differentiate into either ECs or mural cells (pericytes and vascular smooth muscle) dependent on culture conditions and can reproduce the vascularization process.

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.[70] In this study, vascular progenitor cells were isolated from EBs grown in suspension for 10 days and were characterized by expression of the endothelial/hematopoietic marker CD34. When these cells were subsequently cultured in EGM-2 media suplemented with vascular endothelial growth factor (VEGF), they gave rise to endothelial-like cells characterized by a cobblestone cell morphology, expression of endithelial markers (PECAM-1, Flk1, VE-cad, VWF), incorporation of Dil-Ac-LDL and formation of capillary-like structures when placed in Matrigel. In contrast, when $CD34^+$cells were cultured in EGM-2 media supplemented with platelet-derived growth factor-BB, they gave rise to smooth muscle-like cells characterized by spindle-shape morphology, expression of SMC markers (α- SM actin, SM22α, calponin-1 and SM-MHC, caldesmon) and the ability to cantract and relax in response to common pharmacological agents such as carbachol and atropin. Implantation studies in nude mice showed that both cell types cotributed to the formation of human microvasculature. Some microvessels contained mouse blood cells, which indicates functional integration with host vasculature. One of the specific limitation of this study is that single cell isolation and parallel divergence of its progeny was not performed, so it is still not clear whether these cells are differentiated from a „common vascular progenitor" which is able to differentiate to either EC or SMCs depending on culture conditions.

Recently, Sone et al tried to clarify the differentiation process from human ESC to mature vascular cells.[71] (Figure 3).After differentiation of human ESCs via coculture with OP9 stromal cells, VEGFR- 2- positive but TRA-1-60 negative population emerged. These cells were also positive for PDGF receptor alfa and beta and could be effectively differentiated into both VE-cadherin $^{+}$ ECs and α SM actin $^{+}$ mural cells. Their transplantation to the hindlim ischemia model of immunodeficient mice contributed to the construction of new blood vessel and improved blood flow.

Despite multiple methods of SMC differentiation, the exact lineage relation between ECs and SMCs has not been elucidated. Human ESC-derived vascular components have been phenotyped and proven to function both in vitro and in vivo in a similar fashion as vascular counterparts isolated from post-natal sources.

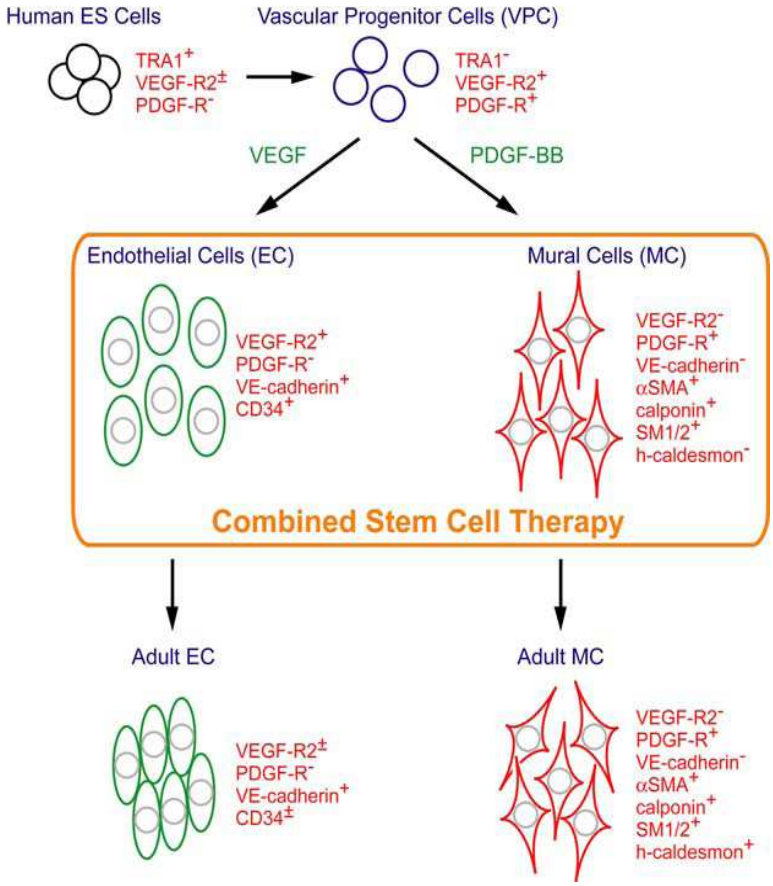


Figure 4. Possible differentiation pathway of vascular cells derived from human ESC via vascular progenitor cells. (Yamahara et al**,** PLoS ONE. 2008;3:e1666.)

1.5.Vasculogenesis and angiogenesis

In the embryo proper, primitive vascular networks are formed de novo by the aggregation of embryonic ECs, a process termed vasculogenesis. The formation of a primitive vascular network from embryonic mesoderm lays the foundations for the pending development of the mature vasculature. In the yolk sac, the precursor for both the hematopoietic and vascular lineages, termed hemangioblast, form aggregates in which the cells in the inner core will differentiate into hematopoietic cells, whereas the cells in the periphery will undergo extensive migration during which they will differentiate into ECs and assemble into a primary capillary plexus. [72,73] Subsequently, the initial vascular network, which consists of ECs that form interconnected vessels, undergoes a succession of morphogenetic events involving sprouting, proliferation and migration of ECs, and remodeling to generate large branched vessels, a process called angiogenesis. [1] It begins with vasodilatation and VEGF-induced increased permeability of the vasculature, which allows extravasation of plasma proteins to the extracellular matrix. This process allows matrix-degrading proteins to enter the extracellular matrix and thus provide the path for the forthcoming migration of the differentiating ECs. [57] This process is followed by loosing of contacts between neighborinh ECs and between ECs and their supporting cells. This enables destabilization of mature vessel, migration of ECs to distant sites, and successive assembly of vessels. [74] Mural cells, including pericytes and vascular SMCs are recruited to the newly formed vessels, causing the vascular networks to be mature and become stable.[57,72]

Vasculogenesis and angiogenesis are largely dependent on the functional changes of proliferation, differentiation and migration of endothelial cells. Unlike vasculogenesis, which is mainly restricted to embryonic development, angiogenesis is common in adult life as well. It is responsible for occurrences of neovascularization in both physiologic processes, such as wound healing and pregnancy, and pathologic conditions, such as cancer and metastases. [73] Disruption of blood vessel formation or function plays a central role in the progression of many disease processes. Thus, controlling vascular cells and angiogenesis, either positively or negatively, could be critical for the treatment of various diseases, including cardiovascular diseases and cancers.

ESCs have become a powerful tool in vascular biology to study the details of vasculogenesis as well as angiogenesis. The study of ESC-derived vascular cells may recapitulate events seen in vascular network formation in vivo and increases the understanding of the molecular mechanisms of vascular development.[1] Stem cells can

cotribute to angiogenesis directly, by participating in new vessel formation[75,76], or indirectly by secreting a broad spectrum of angiogenic and antiapoptotic factors. [77,78] Furthermore, stem cells possess a homing capacity that allows them to migrate toward and engraft into the sites of ischemia or injury. It is important to evaluate this model by comparing features of ECs derived from differentiating stem cells and their responsiveness to external stimuli to those of primary endothelial cells and to in vivo models.

1.6. Therapeutic potential of vascular progenitor cells

Vascular progenitor cells are of great interest because of their potential use in cell therapy for vascular diseases, ischemic tissue and tissue engineering for vascular grafts. However, limited availability and proliferation cabability of cells isolated from patients hampers development of these applications. Recent advances in stem cell technology suggest that stem cell-derived vasculogenic cells may play important role in vascular regenerative approaches.

Several groups have investigated the potential of bone marrow-derived progenitor cells to regenerate infarcted myocardium. [79] In a mouse model, bone marrow cells injected in the contracting wall bordering an infarct could generate de novo myocardium, ameliorating the outcome of coronary artery ligation.[80] Locally transplanted cells led to the formation of new myocytes, endothelial and smooth muscle cells. This therapeutic intervention reduced the infracted area and improved cardiac hemodynamics. Another study of the same group showed that mobilization of bone marrow stem cells by cytokines (G-CSF and SCF) resulted in improved myocardium function and survival of mice.[81] These results have been partially confirmed in several related experiments[82,83] but have not been reproduced in several other reports.[84-86] Indeed, the same cytokine therapy protocol useful for myocardial regeneration in a mouse model, was tested in a rhesus macaque model by the same investigator, but the results did not show clear evidence of myocardial regeneration.[87] Interestingly, other groups working with baboons were also unable to demonstrate myocardial regeneration after cytokine therapy.[88] Based on these results it seems that molecular and cellular mechanisms involved in myocardial regeneration in mice are not induced by this treatment in primates. From mouse to primate models is a major leap in many aspects and we know that in many

cases, the results using mouse model cannot be easily translated to human patients. Initial results from clinical trials with intracoronary infusion of autologous progenitor cells derived from bone marrow in human are encouraging.[89-92] The intracoronary infusion of autologous progenitor cells appears to be safe and effective in limiting postinfarction remodeling processes in humans [89,90] . In antoher clinical study, Chohola at al also published that intra-arterial infusion of autologous bone marrow derived mononuclear cells leads to significant improvement in patients with critical limb ischemia.[93] Larger randomized clinical trials are required to validate these intriguing early results.

Since human ESCs 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. Many attempts to increase vascular repair by stem cells have been successful in small animals models[80-82,94,95], though these results may be difficult to replicate in human clinical trials.

Recently, Yamahara at al[96] reported that human ESC-derived vascular progenitor cells (endothelial and mural cells) transplanted to nude mice with hindlimb ischemia significantly improve the blood flow recovery and capillary density in ischemic tissue. In this study, the combined transplantation of ESC-derived ECs and mural cells was more effective in augmentation of neovascularization in hindlimb ischemia, compared to only ECs or mural cells transplantation, suggesting that interaction between ECs and mural cells is important for vascular regeneration**.** Moreover, this study demonstrated that transplanted vascular cells were effectively incorporated into host circulating vessels as ECs and mural cells to maintain long-term vascular integrity.

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. As we know, it was demonstrated in some works that human ESCs injected subcutaneously, intramusculary or into the testis can form teratocarcinoma-like tumors in adult mice.[4,97] Therefore, any ESC-based therapy has the risk of tumor formation from undifferentiated ES cells. Studies of mouse ESCs indicated that differentiated cells are less likely to generate teratomas after transplantation.[98,99] Therapeutic use of highly differentiated cells is important to minimize the probability of teratomas formation, however, highly differentiated cells often have low proliferation potential. Therefore, one of the major challenges in stem cell research is to identify, characterize and isolate progenitor cells rather than mature cell type that will not

form tumors, and are capable of proliferation and continually producing newly differentiating cells in vivo. Remarkable study using human ESC-derived ECs isolated from CD34+ progenitor cells showed that these cells implanted into SCID mice form stable blood conduits for more than 150 days without teratomas, suggesting that human ESC-derived ECs are safe for potential clinical aplications.[15] Whether or not there is the long term tumorigenicity needs to be further investigated.

Additionaly, another important issues have to be resolved before using human ESCs for clinical applications. In most published studies, the culture of human ESCs in vitro depends mainly on mouse feeder layers or serum from nonhuman sources, therefore the risk of infection transmitted by animal pathogens, as well as immunoreactions caused by animal substances in cell culture can not be excluded. Nowadays, many scientists are undertaking research directed at the development of human ESC derivation and culture conditions that can replace the materials that are currently originate from nonhuman sources with materials of human origin. [100] A large improvement in the basic methods of ESC culture has been the development of the ability to culture human ESCs either in feeder- free conditions or with human-origin feeder cells under serum-free conditions. Xu et al.[101] were the first to report the feeder- free culture system for the growth of human ESCs using conditioned medium from the mouse feeder layer and the serum- free culture medium. However, this culture method does not completely eliminate the possibility that animal pathogens may be transferred to human tissues, owing to the use of mouse embryonic feeder (MEF)-conditioned medium. Since then, several groups have reported the development of culture system completely independent on feeder cells. [102-105]

Another immune problem should also be considered. The immune processes against the differentiated cells after transplantation into immunocompetent patients may occur. To reduce immune rejection when the cells are transplanted into human bodies, human ESCs have to be manipulated by genetic engineering to match the major histocompatibility complex with the patient. [106,107] The genetic manipulation of ESCs, such as somatic cell nuclear transfer, may allow the generation of autologous ESCs, which will be copmatible for clinical therapy.[108,109] It is also very important to examine human ESCs periodically for the occurance of chromosomal abnormalities while culturing. Recently, there have been several reports regarding the appearance of abnormal chromosomes in human ESCs. These abnormalities were dependent on the expansion methods which were used.[110-112]

Methods for efficient differentiation of human ESCs into specific cell types are still preliminary and a better understanding of the signals and genes involved in the differentiation of human ESCs into specific phenotype is strongly needed to improve the development of these induction protocols.

2. HYPOTHESIS AND AIMS OF THE STUDY

ESCs are emphasized as an important model to better define the cellular and molecular mechanisms of vascular development, as well as the developmental relationship between endothelial cells and smooth muscle cells. They also hold a strong potential to treat or even cure many diseases in the future.

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.

3. MATERIALS AND METHODS:

3.1. Cell culture:

Undifferentiated hESCs (H9 cell line obtained from Wicell, Madison, WI) were cultured as previously described.[4,13] Briefly, human ESCs were maintained as undifferentiated cells by co-culture with mitomycin C (Bedford Laboratories, Bedford, Ohio) inactivated mouse embryonic fibroblasts (MEF) in DMEM/F12 media (Invitrogen Corporation, Carlsbad, CA) supplemented with 15% knockout serum replacer (KOSR, Invitrogen), 1% MEM-nonessential amino acids (Invitrogen), 2 mM L-glutamine (Sigma, St Louis, MO), 0.1 mM β-mercaptoethanol (Sigma), 8 ng/ml basic fibroblast growth factor (bFGF, NCI), and 1% (v/v) Penicilin/Streptomycin (Invitrogen). 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, Gaithersburg, MD).

To promote endothelial differentiation, hESC were cultured as previously described.[113] Briefly, the undifferentiated hESCs were passaged onto mitomycin C inactivated mouse bone marrow-derived stromal cell line S17 (kindly provided by Dr. Ken Dorshkind, UCLA) or M210B4 cells (ATCC, Manassas, VA) in RPMI 1640 media (Invitrogen) supplemented with 15% fetal bovine serum (FBS) (Hyclone, Logan, UT), 1% MEM-nonessential amino acids (Invitrogen), 1% L-glutamine, 0.1% β-mercaptoethanol (Sigma) for 13-15 days. After that, differentiated hESCs were dissociated with 1mg/mL collagenase IV (Invitrogen), followed by 0.05% trypsin/0.53mM EDTA (Cellgro, Mediatech), a single cell suspension was generated, and the subpopulation of $CD34^+$ cells were isolated using magnetic nanoparticle technology (EasySep Selection Kit, StemCell Technologies, Vancouver, BC, Canada).

The $CD34^+$ population was cultured on fibronectin (Sigma) coated tissue culture flasks in EGM2 complete media (Lonza, Gaithersburg, Maryland) 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, hESC-ECs, were grown to 80% confluence and serially passaged in parallel with HUVEC (Lonza) cells which functioned as a positive control in all experiments.

To obtain smooth muscle cells, hESC-SMCs, a portion of the hESC-EC cells established in culture were placed in high glucose Dulbecco modified Eagle media (DMEM) (Invitrogen) containing 5% FBS, 5 ng/mL platelet-derived growth factor-BB (PDGF-BB, PeproTech Inc, Rocky Hill, NJ) and 2.5 ng/mL transforming growth factor-beta 1 (TGF-β1, PeproTech Inc). Media was changed every 3-4 days.

3.2. Flow Cytometry

H9 cells differentiated by stromal cell co-culture with mouse bone marrow-derived stromal cell line S17 cell were dissociated with collagenase IV, followed by 0.05% trypsin/0.53mM EDTA, passed through a 85-μm filter to remove cell clumps, centrifuged and washed with basal medium containing 5% FBS and resuspended in FACS buffer (PBS without Ca^{2+} and Mg^{2+} (Cellgro, Herndon, VA) supplemented with 2% FBS and 0.1% sodium azide). The similar procedure was performed with hESCs derived ECs cultured in EGM2. These cells were washed with Ca^{2+} and Mg^{2+}free PBS and detached from the monolayer with 0.05% trypsin/0.53mM EDTA .

The dissociated cells were centrifuged, washed and resuspended in FACS buffer. 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) (BD Pharmingen, San Diego, CA) and the lectins *Ulex europeaus*-FITC, *Helix pomatia*-FITC, and *Griffonia simplicifolia*-FITC (EY Laboratories, San Mateo, CA). 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 (EY Laboratories) when lectin binding was examined. Viable cells were identified by propidium iodide exclusion. Cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) with Cell Quest Pro and FlowJo software.

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

Human ESCs derived ECs were seeded at $4X10^4$ cells/cm^2, onto fibronectin (Becton Dickinson)-coated 0.4 µm polycarbonate membranes (Nalgen Nunc International/Thermo Fisher, Rochester, NY) at ~20,000 cells/membrane. Cells were suspended in EGM2 media supplemented with a cocktail that included hEGF, VEGF, hFGF-B, IGF, hydrocortisone, ascorbic acid and heparin at 200 µL/well. Cells were incubated at 37°C with 5% CO_2/21% O_2 for 72 to 96-hours post seeding. Cells were plated in triplicate for two separate experiments. After incubation, cells were fixed in 3% glutaraldehyde (in cacodylate buffer). 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 (FEI, Hillsboro, OR). All fixative and staining reagents purchased from Polysciences (Warrington, PA). Images are representative from random micrographs obtained from the two separate time points assessed.

3.4. Matrigel Tube Formation Assay

12 well plates were pre-warmed in incubators (30 minutes) set at 37°C, 5% CO_2, before mixtures containing Matrigel (Becton Dickinson) and cells were added. 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 and images were captured using a phase microscope (Olympus, Center Valley, PA) or an inverted fluorescent microscope (Zeiss, Thornwood, NY) equipped with an Olympus camera and AxioVison software.

3.5. Immunohistochemical staining

Human ESC-derived 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 -Molecular Probes, Eugene, OR) for 4 hours. After washing, the cells were observed by fluorescence microscopy. HUVECs were used as a positive control, human ESC- derived 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 (vWF – rabbit anti-human, 1:100, Dako, Carpinteria, CA; eNOS – mouse anti-human, 1:50, Beckton Dickinson) for one hour followed by incubation with secondary antibody Alexa Fluor 488 (vWF – goat anti-rabbit, eNOS – goat anti-mouse, Molecular Probes) for 30 minutes. After a final wash, cells were observed by fluorescence microscopy. CD31 and VE-cadherin were detected using mouse anti-human antibodies (eBioscience, San Diego, CA and BD Pharmingen) diluted 1:100 and 1:50 respectively. Alexa Fluor 488 goat anti-mouse secondary antibody (Invitrogen) diluted 1:600 was used for final detection and visualization.

For human ESC-derived SMCs we examined expression SM22, calponin and alpha-smooth muscle actin using primary goat anti-human SM22 (AbCam, Cambridge, MA) and mouse anti-human calponin (Sigma) and detected with the species matched secondary antibodies labeled with Alexa Fluor 488. Alpha-SMC actin was detected with mouse anti alpha-SMC actin Cy 3 conjugated antibody (Sigma). Mouse and goat IgG isotypes (BD Pharmingen) detected with corresponding secondary Alexa Fluor488 conjugated antibodies were used as negative controls. Cells were fixed, permeabilized, blocked and incubated at room temperature for 1 hour with primary antibodies. For SM22 and calponin, cells were washed and incubated at room temperature for 45 minutes with fluorescently-labeled secondary antibodies. ProlongGold + Dapi (Invitrogen) and Hoechst 33258 (Sigma, Aldrich) was utilized for slide preparation and nuclear visualization via fluorescent microscopy.

3.6. 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-derived ECs and human ESC-derived SMCs using an RNeasy kit (Qiagen, Valencia, CA)

with homogenization via Qiashredder (Qiagen, Valencia, CA) according to the manufacturer's instructions. mRNA was reverse transcribed (RT) to cDNA using an Omniscript RT kit (Qiagen) 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 (CT) method using Applied Biosystems SDS analysis software (version 1.9.1). The reported means reflect three independent Q-RT-PCR experiments conducted for human ESC-derived ECs and human ESC-derived SMCs. Oligonucleotide primer sequences, annealing temperature (Ta) and predicted product size are described in the supplemental table.

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

Cells were grown on fibronectin coated 22x22 mm glass cover slips for 2 days in standard culture media. Cells were then loaded with fura-2 acetoxymethyl ester (fura-2 AM, Invitrogen) 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 (Roper Scientific, Trenton, NJ)

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) Pairs of images were acquired at 2-30 sec. intervals. Background-corrected images were ratioed (340/380) and analyzed using MetaMorph/MetaFluor image acquisition and analysis software (Molecular Devices, Sunnyvale, CA). Changes in ratio in individual cells were measured and plotted versus time using graphing software (Excel, Microsoft, Redmond, WA).

3.8. Limiting Dilution Assay

To test the role of Wnt proteins on human ESC 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.

4. RESULTS

4.1. 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". We found no significant difference in ability to induce endothelial differentiation between M2-10B4 and S17 stromal cell lines.[113] 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). A significant percentage of this population co-expresses typical endothelial markers CD31 and Flk1. For expansion and further endothelial differentiation, these cells were placed on fibronectin coated culture flasks and cultured in EGM2 media supplemented with a growth enhancing cocktail including specific cytokines hEGF, VEGF, bFGF, IGF-1, ascorbic acid, and heparin. 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 6A,B) These differentiated cells with a typical EC morphology could be readily passaged and expanded with a maintenance of a homogeneous appearance for approximately 5-7 passages. After expansion of the cells for 2-5 passages, further assessment and characterization of these cells was performed using flow cytometry, immunohistochemistry, matrigel tubing test and RT-PCR including quantitative RT-PCR. Human ESC-derived ECs demonstrated typical EC surface antigen expression of CD31, VE-cadherin (CD144), CD146, Flk1, lectins *Helix pomatia*, *Griffonia simplicifolia*, and *Ulex europaeus*, 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 (Figure7). Furthermore, RT-PCR was done to demonstrate expression of transcripts for genes commonly expressed by ECs including Flk1, CD31, CD34, vWF, VE-cadherin, eNOS, and Tie2 (Figure 8), and the expression level of these transcripts was quantified via Q-RT-PCR (Table 1). Q-RT-PCR analysis confirmed evident increase in expression of EC transcripts in ES-derived ECs after differentiation in comparison with undifferentiated ES cells. However, the EC-genes expression, specially for CD31, Tie2 and VE-Cadherin ,was lower in comparison with HUVEC. Only for Flk1, we found higher expression of transcripts in ES-derived ECs than in HUVEC (Table 1).

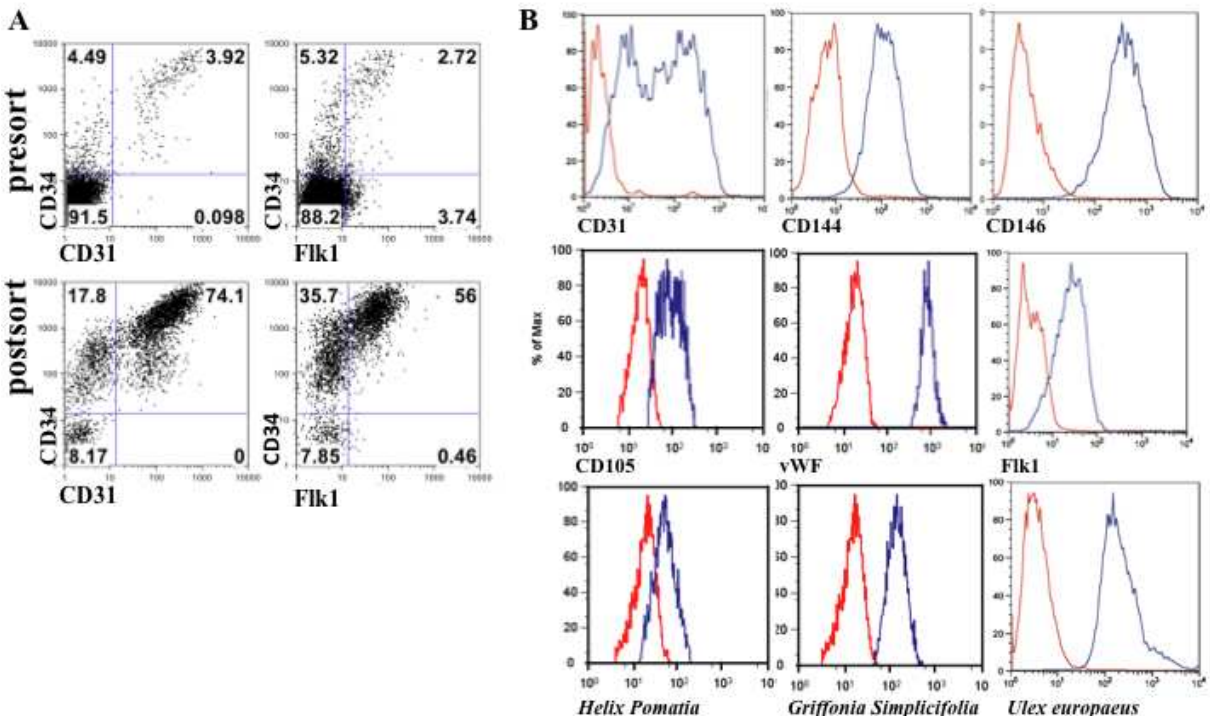


Figure 5. CD34+ Vascular Progenitor Cells Capable of Endothelial Cell Differentiation.
(A) human ESC-derived cells after 15 days of differentiation demonstrating expression of CD34, CD31, and Flk1 both before (presort) and after (postsort) immunomagnetic sorting for CD34+ cells.
(B) After sorting, the 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.

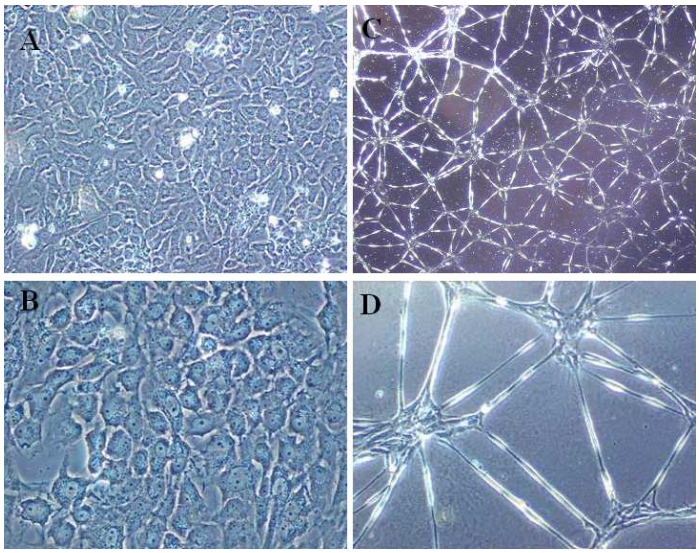


Figure 6. Characteristics of human ESC-derived ECs. (A-B) Morphology of human ESC-derived ECs cultured on fibronectin coated plates in EGM2 media. **(C-D**) These cells form capillary- like structures when replated on Matrigel. Original magnification: x100 (A, D), x 200 (B), x 20 (C).

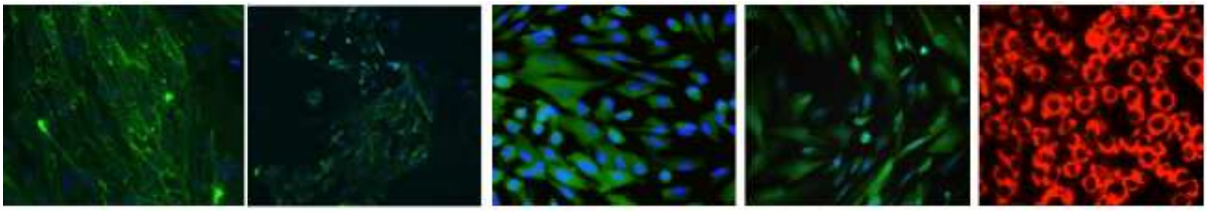


Figure 7. Immunofluorescent staining of human ESC-derived ECs: (left to right) CD31, VE-Cadherin, vWF, eNOS and uptake of dil-ac-LDL.Original magnification 100x for each plot. Blue signal represents DAPI stained nuclei.

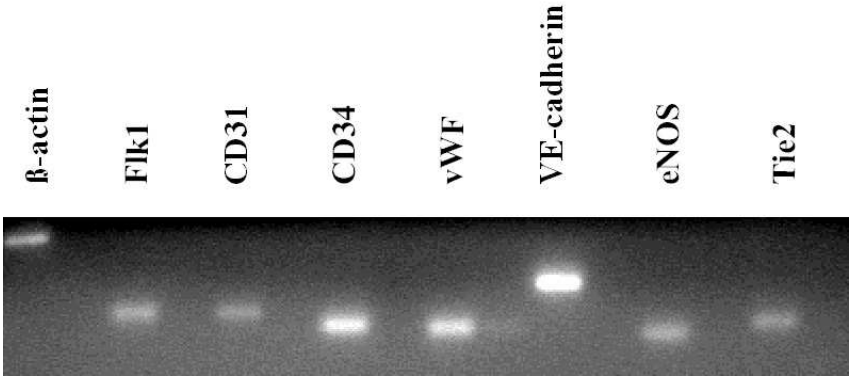


Figure 8.RT-PCR of human ESC-derived ECs. Total cellular RNA was isolated from human ESC-derived ECs and sequence-specific primers for the indicated genes were used for RT-PCR analysis. Here, mRNA expression of 7 transcripts for typical EC genes as indicated above each row. To control for contaminating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

Importantly, these human ESC-derived ECs have also functional characteristics of ECs. They formed capillary-like structures when replated on Matrigel (Figure 6 C,D) similar to those formed by HUVECs or other endothelial cell populations and they were able to rapidly take up acetyled LDL which was confirmed by immunohistochemical staining (Figure 7, picture on the right). Next, both of these functional tests were negative for ESC-derived SMCs which are described in paragraph below.

Transmission electron micrographs (TEM) of hESC-ECs showed the presence of microparticles being released from the membrane surface of the cell (Figure 9). This is another characteristic of endothelial cells, as microparticles play an important role in endothelial cell function.[114,115] The TEM images also revealed that these endothelial cells are in a metabolically active state, displaying an abundance of mitochondria and endoplasmic reticulum as well as nuclear euchromatin.

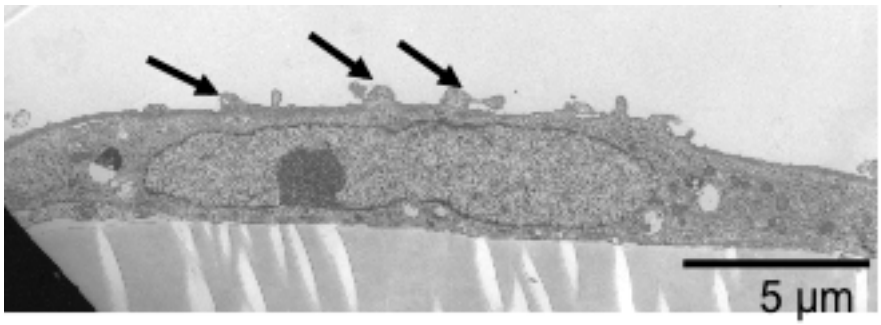


Figure 9. Transmission Electron Micrograph (TEM) images of human ESC derived ECs. Human ESC-derived ECs cultured under endothelial cell culture conditions in EGM2 media show release of microparticles of approximately 100nm in size (as indicated by arrows) from the cell surface.

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

Studies of several human and mouse progenitor cell sources demonstrate that SMC survival and growth is promoted by TGF-β1 and PDGF-BB.[116,117] 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 (Figure10).

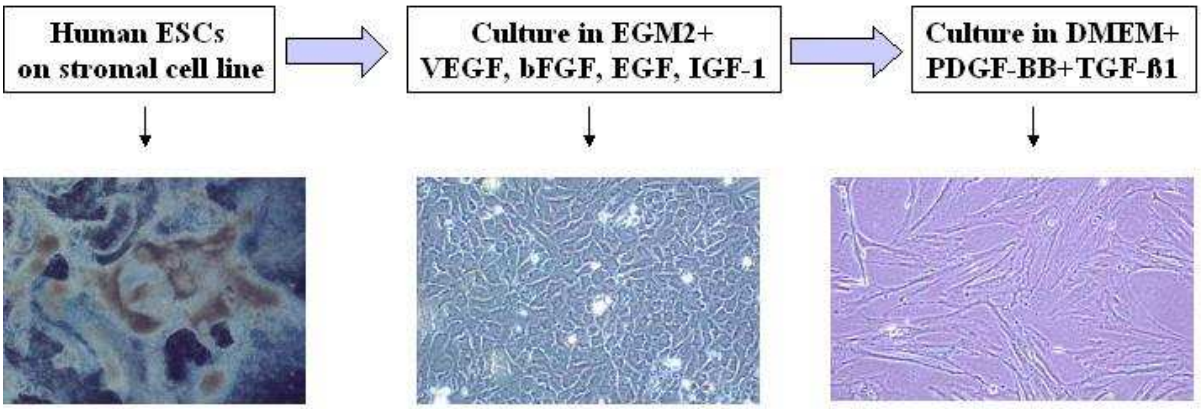
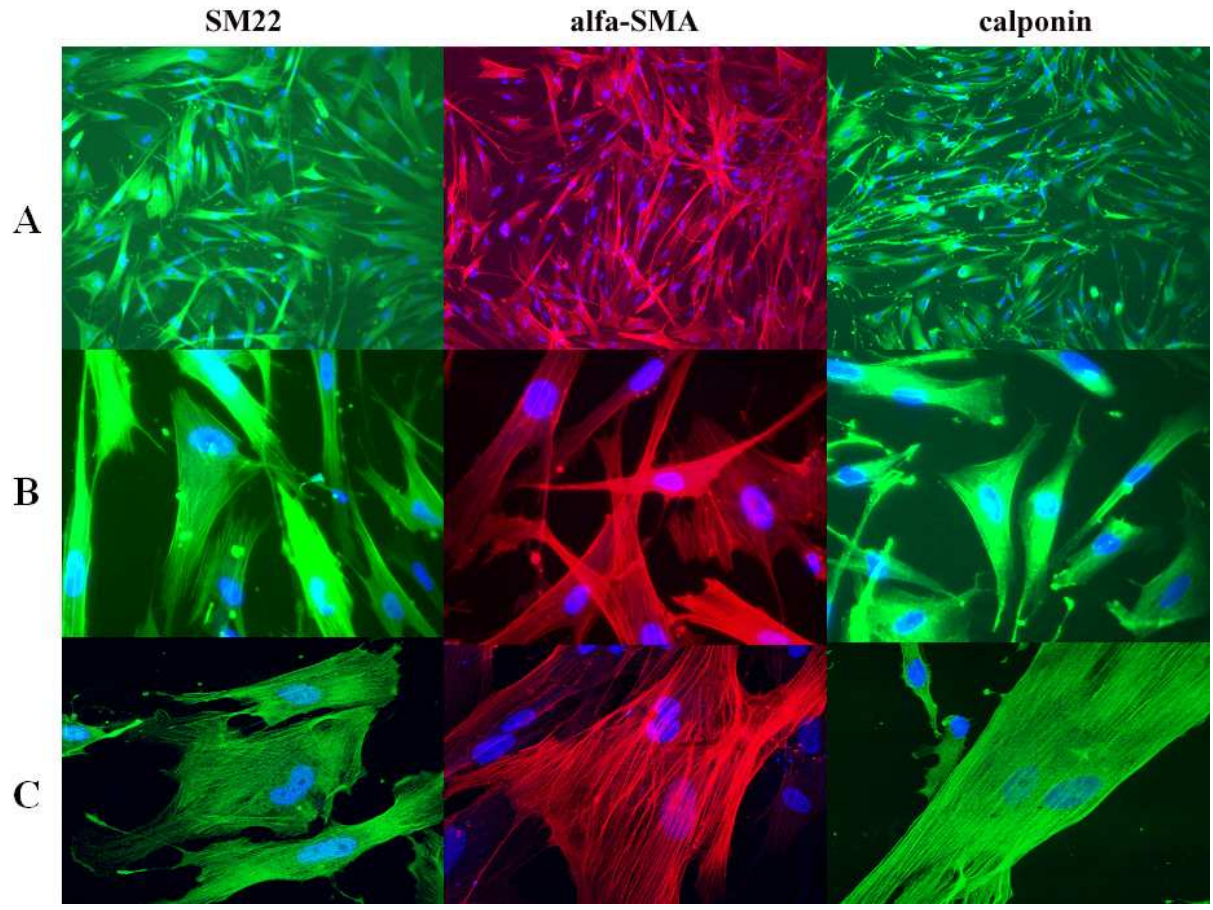


Figure 10. Schema of derivation of ECs and SMCs from human ESCs based on defined culture conditions. First, human ESCs, H9 cell line, were allowed to differentiate by stromal cell co-culture with mouse bone marrow-derived stromal cell line S17 for 10-15 days. Then, endothelial cell development was supported 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).

Immunofluorescent staining revealed robust expression of SM22, calponin, and α-smooth muscle actin (Figure 11A,B,C) and the absence of these markers in human ESC-derived ECs (Figure 11D). 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. Human ESC-derived SMCs continued to proliferate for 2 or 3 passages in the culture.

Human ESC-derived SMCs



Human ESC-derived ECs

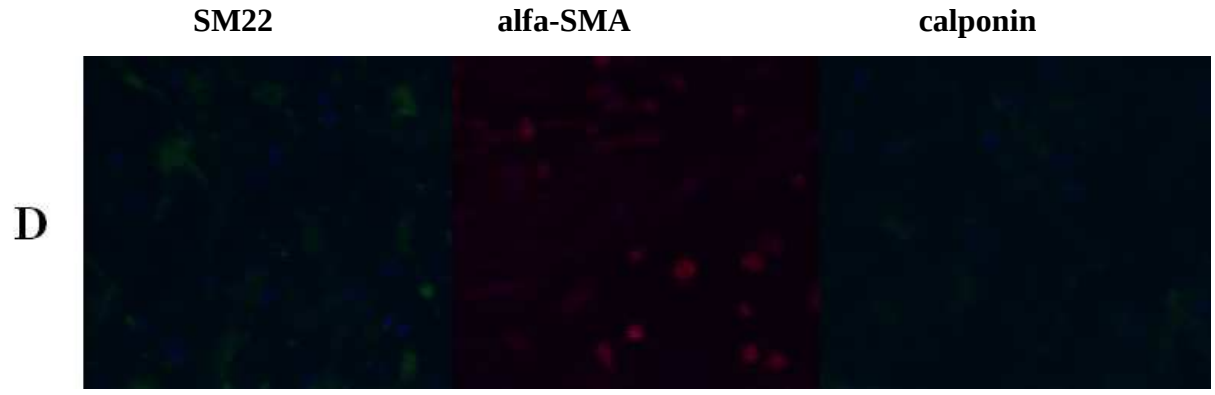


Figure 11. Immunoflouroscent staining of human ESC-derived SMCs (left to right): SM22, α-SMA and calponin. Original magnification 100X(A),200x(B),400x(C)-confocal microskope. **D-Staining of human ESC-derived ECs, which are negative for SMC markers** (left to right): SM22, α-SMA, and calponin. Original magnification 200x.Blue signal represents Hoechst 33258 stained nuclei.

Furthermore, RT-PCR demonstrated transcripts of typical SMC genes including alpha-SMC actin, calponin, SM22, SMI, smoothelin, myocardin (Figure12). We also found concomitant expression of 2 genes APEG-1 and CRP2/SMLIM (Figure 12). These two genes are preferentially expressed in arterial SMCs, indicating these SMCs may be a more specific sub-type of supportive vasculature.

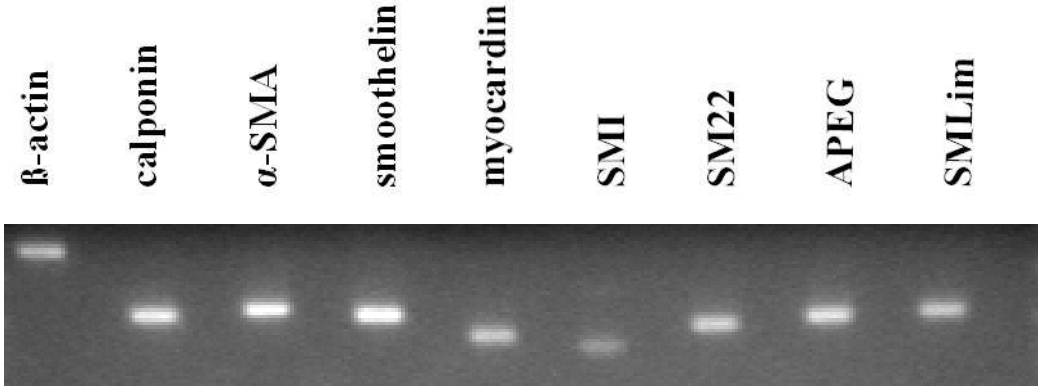


Figure 12. RT-PCR of human ESC-derived SMCs for eight common SMCs genes (and β-actin control), as indicated above each indicated lane. To control for contaminating genomic DNA, reactions were also done under conditions with no reverse transcriptase.

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, respectively (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 (α-smooth muscle actin, calponin, SM22, SMI and myocardin) 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, while HUVECs could survive and proliferate in SMC conditions (media containing TGF-β1 and PDGF-BB), HUVECs under these conditions did not change morphology and they did not show an increased expression of SMC genes (Table 1).

A

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

B

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 1. Q-RT-PCR of human ESC-derived ECs, human ESC-derived SMCs, and HUVECs. 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**. A)** 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. **B)** Q-RT-PCR was also 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.

4.3. Enhanced Endothelial Progenitors from Wnt Expressing Stromal Cells

Studies by our group and others have shown that various stromal cell lines provide lineage specific support for differentiation.[118,119] 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.[120] $CD34^+$ 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. Statistical analysis by unpaired t-test generated the following p values: regular M2-10B4 stromal cells vs Wnt1 expressing M2-10B4 (p=0.0279) and vs.Wnt5 M2-10B4 (p=0.0309).

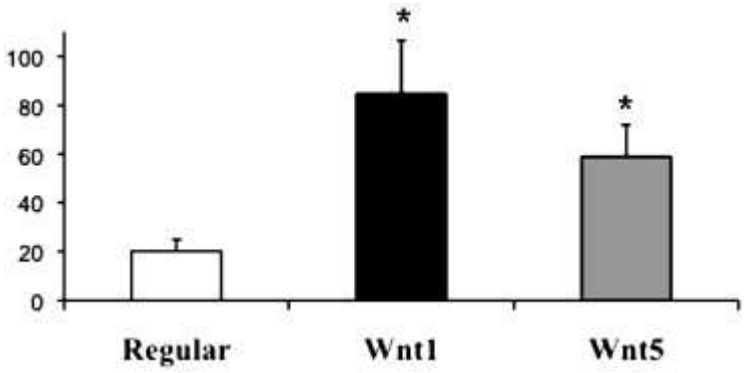


Figure 13. Increased Development of CD34+ Vascular Progenitor Cells From Wnt1 Expressing Stromal Cell Differentiation. Limiting dilution analysis was done to quantify vascular progenitor cells from human ESCs allowed to differentiate on M2-10B4 stromal cells that did not over-express Wnt proteins, or 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**).**

4.4. 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. Fura-2 labeled human ESC-derived ECs and human ESC-derived SMCs were tested and differences in responsiveness to various agonists were evaluated in more than 200 cells. (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. Human ESC-derived ECs cultured alone formed typical relatively thin capilary-like tubes.(Figure 15A-C). Human ESC-SMCs cultured by themselves on Matrigel did not form significant structures (data not shown). However, in the samples consisting of a mixture of human ESC-derived ECs and human ESC-derived SMCs, substantially denser and more robust three-dimensional networks of vasculature like structures were observed. The human ESC-derived ECs and human ESC-derived SMCs could be tracked in culture by their respective fluorochrome expression. The human ESC-derived ECs were derived from a H9 cells with stable GFP expression and the human ESC-derived SMCs were derived from another H9 cell line expressing mCherry. Images show alignment of the cells as well as physical cell-cell interaction of enhanced vascular formation that appears to recapitulate in vivo EC and SMC interactions.

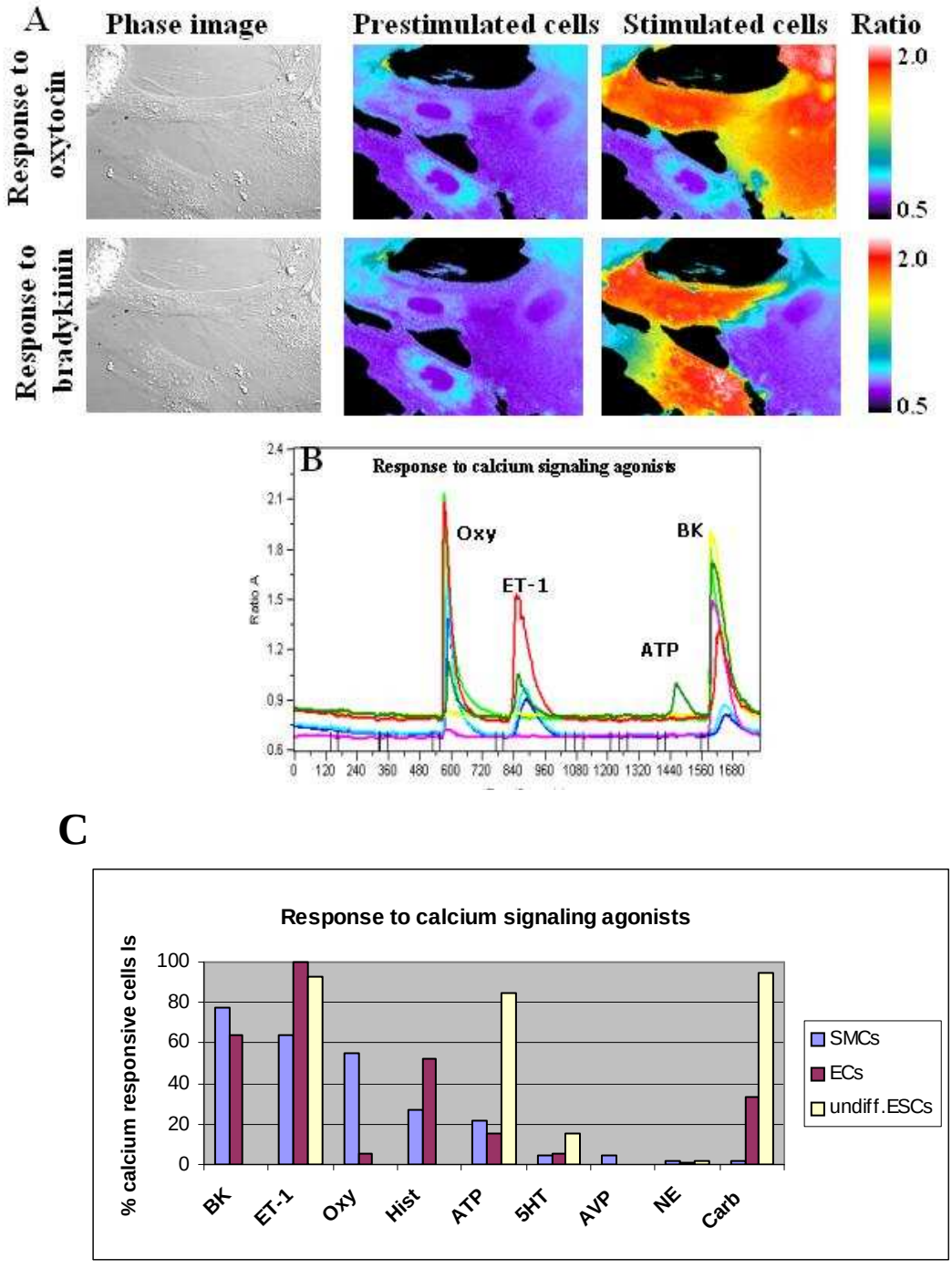


Figure 14. Intracellular calcium response of human ESC-derived ECs and human ESC-derived SMC to pharmacological agonists. Single cell preparations were exposed to pharmacological agonists as indicated. Responses of each cell type were measured via a fluorimetric ratio using fura-2**. (A)** From left 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); pseudocolor scale: low ratios indicated by blue color and high ratios indicated by yellow to red color (original magnification 400x). **(B)** Representative of time course graph of seven individual hESC-SMCs exposed to oxytocin, ET-1, ATP and Bradykinin. Each line represents the ratios obtained

from an individual cell in successive image pairs . **(C)** Graphic summary comparing responses of 100 undifferentiated human ESC, 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). Each population tested was comprised of cells from more than one culture.

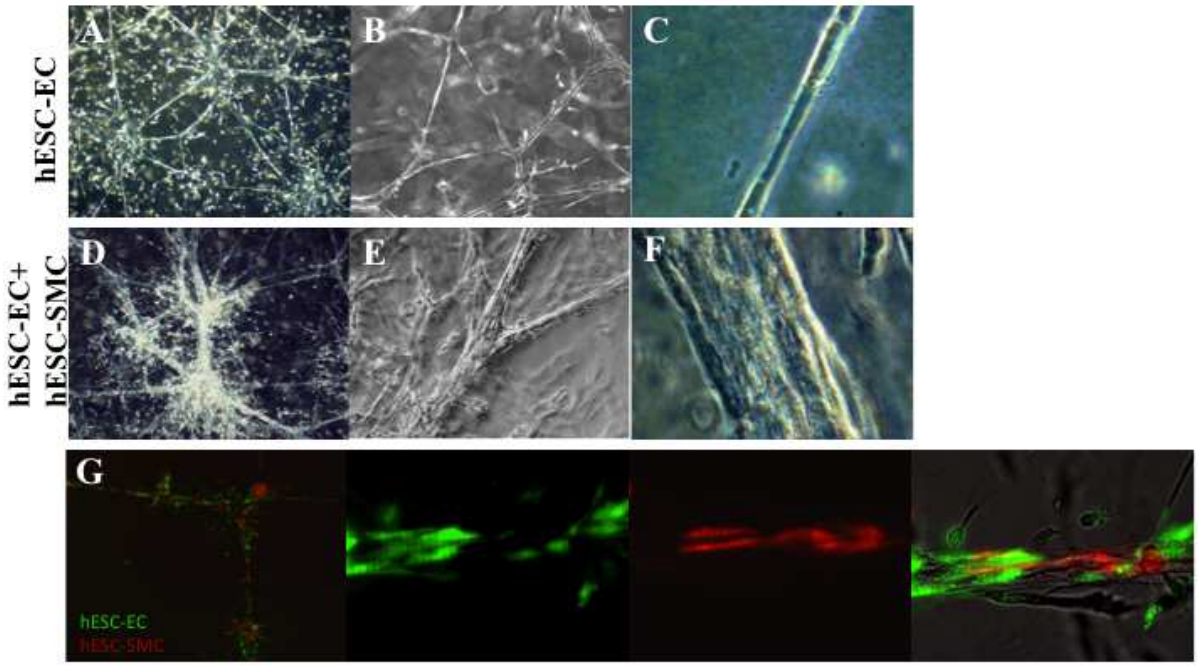


Figure 15. In-vitro Matrigel Tube Formation Assay. To assess functional potential of human ESC-derived ECs and human ESC-derived SMCs in vitro, cells were cultured on Matrigel. Vascular tube structures formed in both hESC-derived ECs and hESC-derived EC/ SMC cultures, with the cocultured cells interacting 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 demonstrate close interaction between the two cell populations in these in vitro Matrigel cultures images in (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).

5. 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. [15,22,24,25,27] In contrast to work by Levenberg at al[24] 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. We found no significant difference in ability to induce endothelial differentiation between M2-10B4 and S17 two stromal cell lines. 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. Transmission electron micrograph images of these ECs confirmed the release of microparticles from the surface of the ECs, which is also associated with functional endothelium.

Furthermore, based upon studies to derive SMCs from other cell populations such as mouse ES cells and adult progenitor cells[64-66,68], 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. Other populations, such as mesenchymal stem cells and pericytes, have been cited as SMC precursors.[121-123] The hallmark characteristics of these populations are controversial and, in some cases, overlap with SMCs. Further studies to allow definitive characterization will be necessary to determine the relationship, if any, between these cell types.

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.[18,124] 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.[124] 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.

Other recent studies using mouse ESCs demonstrate that Flk1 positive and/or Isl1 positive cells are able to give rise to not only ECs and SMCs, but also cardiomyocyte progenitor cells.[125,126] Another study demonstrated that Nkx 2.5-positive cells derived from mouse ESCs could form both cardiac tissue (vascular and conductive) and SMCs, though not ECs.[127] Also, while many studies have now demonstrated hematopoietic development from human ESCs, one recent study demonstrates a putative bi-potential hemato-endothelial (hemangioblast) development from a $Flk1^+$ cell population.[128] ECs and SMCs are known to have differences in phenotype and gene expression depending on anatomic location or developmental source.[129-131] Therefore, it will now be of interest to evaluate the potential for the human ESC-derived populations described in our studies not only for cardiomyocyte potential but also for their potential contributions in other tissues and organs. Furthermore, these cells can now be better evaluated for functional capacity using in vivo models of cardiac or peripheral vascular ischemia.[71,132]

A recent study also found separate outgrowth of ECs and SMCs from a $CD34^+$ population selected from EBs.[70] 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. The $CD34^+$ population emerges between days 7 and 10 and continues to develop through days 22-28. While the $CD34^+$ population is present during all stages of differentiation, the co-expression of other surface antigens better define the progenitor cell population(s).

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 effort to more accurately recreate native stem cell niches, several groups have reported the use of stromal cell lines that over express various components thought to promote or inhibit lineage-specific differentiation.[120] 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 (also termed KDR or VEGFR2). It is possible to further enhance the method of differentiation presented here by a similar addition of cytokines

to media components either throughout the differentiation time course or at various points as other groups have found useful for more specific and direct differentiation.[133] It is advantageous to apply defined cytokines to stromal cell coculture differentiation models due to the fact that the cells undergoing differentiation in a two dimensional environment have more total surface area and hence more exposure to the medium. Moreover, it is possible that additional phenotypic and functional cardiac and vascular progenitors could be produced at different stages of differentiation by utilizing these methods.

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.[134-139] 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[135,139,140], these findings do not always translate to similar efficacy in clinical trials.[141-144] In most cases where there is functional improvement, it is uncertain whether this is due to the exogenous cells generating functional tissue, or if these injected cells stimulate endogenous repair. 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 (GFP, dsRed, or others) as described here, and bioluminescent imaging via luciferase-expressing cells, as demonstrated previously[145], 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 functinal 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.

7. SUPPLEMENTARY DATA

Supplementary Table 1.

Gene	Primers (S=forward, AS=reverse)	Product size (bp)	Ann.Temp.©
B-ACTIN	S:5´- TACCTCATGAAGATCCTCA-3´	267	51
	AS:5´-TTCGTGGATGCCACAGGAC-3´		
	AS:5´-TCCCGTCCATGAAGCCTTTGG-3´		
CALPONIN	S:5´-TTTTGAGGCCAACGACCTGT-3´	91	63
	AS:5´-TCCTTTCGTCTTCGCCATG-3´		
MYOCARDIN	S:5´-AACCAGGCCCACTCCCAC-3´	81	62
	AS.5´-CAGGCAAGCCCCGAATT-3´		
SM22	S:5´-GGCAGCTTGGCAGTGACC-3´	101	63
	AS:5´-TGGCTCTCTGTGAATTCCCTCT-3´		
aSMC-ACTIN	S:5´-CTACAATGAGCTTCGTGTTGC-3´	130	59
	AS:5´-ATGGCTGGGACATTGAAAG-3´		
SMOOTHELIN	S:5´-ACTGGTGTCGAGCCAAGACT-3´	119	60
	AS:5´-GCCTCAGGGAAGAAGTTGTG-3´		
SM-LIM	S:5´-GTGCAAAGTGTGGGAAGAGT-3´	119	58
	AS:5´-TCCTTGGCCATAGCCAAATC-3´		
APEG-1	S:5´-GAGACCTGGCCGCGAAC-3´	111	62
	AS:5´-CTGGTCCATAAGTGAGACCTTGAA-3´		
CD31	S:5´-ACTGCACAGCCTTCAACAGA-3´	92	60
	AS:5´-TTTCTTCCATGGGGCAAG-3´		
CD34	S:5´-TCCAGAAACGGCCATTCAG-3´	69	62
	AS:5´-CCCCACCTAGCCGAGTCA-3´		
VE-CADHERIN	S:5´-CTCTGCATCCTCACCATCAC-3´	179	59
	AS: 5´-GAGTTGAGCACCGACACATC-3´		
VWF	S:5´-GTCGAGCTGCACAGTGACAT-3´	64	60
	AS:5´-CCACGTAAGGAACAGAGACCA-3´		
FLK1	S:5´-CGGCTCTTTCGCTTACTGTT-3´	98	60
	AS:5´-TCCTGTATGGAGGAGGAGGA-3´		
eNOS	S:5´-GGCTGCTCAGCACCTTGGCA-3´	56	68
	AS:5´-GAGGGCCTCCAGCTCCTGCT-3´		
TIE 2	S:5´-TGCCCAGATATTGGTGTCCT-3´	73	60
	AS:5´-CTCATAAAGCGTGGTATTCACGTA-3´		

Supplementary Table 1.
Q-RT-PCR primers, product sizes, and annealing temperatures

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

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

(6) 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.

(7) Kim HS, Oh SK, Park YB et al. Methods for derivation of human embryonic stem cells. Stem Cells. 2005;23:1228-1233.

(8) Heins N, Englund MC, Sjoblom C et al. Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells. 2004;22:367-376.

(9) Lu J, Hou R, Booth CJ, Yang SH, Snyder M. Defined culture conditions of human embryonic stem cells. Proc Natl Acad Sci U S A. 2006;103:5688-5693.

(10) 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.

(11) 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.

(12) Leahy A, Xiong JW, Kuhnert F, Stuhlmann H. Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation. J Exp Zool. 1999;284:67-81.

(13) 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.

(14) 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.

(15) 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.

(16) Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. Development. 1998;125:1747-1757.

(17) Ying QL, Stavridis M, Griffiths D, Li M, Smith A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol. 2003;21:183-186.

(18) 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.

(19) Sone M, Itoh H, Yamashita J et al. Different differentiation kinetics of vascular progenitor cells in primate and mouse embryonic stem cells. Circulation. 2003;107:2085-2088.

(20) Hirashima M, Kataoka H, Nishikawa S, Matsuyoshi N, Nishikawa S. Maturation of embryonic stem cells into endothelial cells in an in vitro model of vasculogenesis. Blood. 1999;93:1253-1263.

(21) Kaufman DS, Lewis RL, Hanson ET et al. Functional endothelial cells derived from rhesus monkey embryonic stem cells. Blood. 2004;103:1325-1332.

(22) 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.

(23) Yurugi-Kobayashi T, Itoh H, Yamashita J et al. Effective contribution of transplanted vascular progenitor cells derived from embryonic stem cells to adult neovascularization in proper differentiation stage. Blood. 2003;101:2675-2678.

(24) 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.

(25) 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.

(26) Nourse MB, Halpin DE, Scatena M et al. VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering. Arterioscler Thromb Vasc Biol. 2010;30:80-89.

(27) 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.

(28) Nakahara M, Nakamura N, Matsuyama S et al. High-efficiency production of subculturable vascular endothelial cells from feeder-free human embryonic stem cells without cell-sorting technique. Cloning Stem Cells. 2009;11:509-522.

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

(30) Kelly MA, Hirschi KK. Signaling hierarchy regulating human endothelial cell development. Arterioscler Thromb Vasc Biol. 2009;29:718-724.

(31) Riha GM, Lin PH, Lumsden AB, Yao Q, Chen C. Review: application of stem cells for vascular tissue engineering. Tissue Eng. 2005;11:1535-1552.

(32) Nerem RM, Girard PR. Hemodynamic influences on vascular endothelial biology. Toxicol Pathol. 1990;18:572-582.

(33) Yamamoto K, Sokabe T, Watabe T et al. Fluid shear stress induces differentiation of Flk-1-positive embryonic stem cells into vascular endothelial cells in vitro. Am J Physiol Heart Circ Physiol. 2005;288:H1915-H1924.

(34) Yamahara K, Itoh H. Potential use of endothelial progenitor cells for regeneration of the vasculature. Ther Adv Cardiovasc Dis. 2009;3:17-27.

(35) Li Z, Han Z, Wu JC. Transplantation of human embryonic stem cell-derived endothelial cells for vascular diseases. J Cell Biochem. 2009;106:194-199.

(36) Li X, Claesson-Welsh L. Embryonic stem cell models in vascular biology. J Thromb Haemost. 2009;7 Suppl 1:53-56.

(37) Shalaby F, Rossant J, Yamaguchi TP et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995;376:62-66.

(38) Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. Development. 1998;125:725-732.

(39) Vittet D, Buchou T, Schweitzer A, Dejana E, Huber P. Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. Proc Natl Acad Sci U S A. 1997;94:6273-6278.

(40) Fehling HJ, Lacaud G, Kubo A et al. Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. Development. 2003;130:4217-4227.

(41) Grant MB, May WS, Caballero S et al. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. Nat Med. 2002;8:607-612.

(42) Bailey AS, Jiang S, Afentoulis M et al. Transplanted adult hematopoietic stems cells differentiate into functional endothelial cells. Blood. 2004;103:13-19.

(43) Cogle CR, Wainman DA, Jorgensen ML et al. Adult human hematopoietic cells provide functional hemangioblast activity. Blood. 2004;103:133-135.

(44) Loges S, Fehse B, Brockmann MA et al. Identification of the adult human hemangioblast. Stem Cells Dev. 2004;13:229-242.

(45) Pelosi E, Valtieri M, Coppola S et al. Identification of the hemangioblast in postnatal life. Blood. 2002;100:3203-3208.

(46) Schatteman GC. Adult bone marrow-derived hemangioblasts, endothelial cell progenitors, and EPCs. Curr Top Dev Biol. 2004;64:141-180.

(47) Gunsilius E, Duba HC, Petzer AL et al. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. Lancet. 2000;355:1688-1691.

(48) Gunsilius E, Duba HC, Petzer AL et al. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. Lancet. 2000;355:1688-1691.

(49) Kennedy M, D'Souza SL, Lynch-Kattman M, Schwantz S, Keller G. Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. Blood. 2007;109:2679-2687.

(50) Lu SJ, Feng Q, Caballero S et al. Generation of functional hemangioblasts from human embryonic stem cells. Nat Methods. 2007;4:501-509.

(51) Chadwick K, Wang L, Li L et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood. 2003;102:906-915.

(52) 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.

(53) Wang L. Endothelial and hematopoietic cell fate of human embryonic stem cells. Trends Cardiovasc Med. 2006;16:89-94.

(54) Lancrin C, Sroczynska P, Serrano AG et al. Blood cell generation from the hemangioblast. J Mol Med. 2010;88:167-172.

(55) Pearson S, Sroczynska P, Lacaud G, Kouskoff V. The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. Development. 2008;135:1525-1535.

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

(57) Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med. 2000;6:389-395.

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

(59) 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.

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

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

(62) 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.

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

(64) 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.

(65) 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.

(66) Jain MK, Layne MD, Watanabe M et al. In vitro system for differentiating pluripotent neural crest cells into smooth muscle cells. J Biol Chem. 1998;273:5993-5996.

(67) 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.

(68) 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.

(69) 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.

(70) 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.

(71) Sone M, Itoh H, Yamahara K et al. Pathway for differentiation of human embryonic stem cells to vascular cell components and their potential for vascular regeneration. Arterioscler Thromb Vasc Biol. 2007;27:2127-2134.

(72) Risau W. Mechanisms of angiogenesis. Nature. 1997;386:671-674.

(73) Goldberg C, I, Beck G, Ziskind A, Itskovitz-Eldor J. Human embryonic stem cells for vascular development and repair. Isr Med Assoc J. 2006;8:573-578.

(74) Coussens LM, Raymond WW, Bergers G et al. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Genes Dev. 1999;13:1382-1397.

(75) Cho SW, Moon SH, Lee SH et al. Improvement of postnatal neovascularization by human embryonic stem cell derived endothelial-like cell transplantation in a mouse model of hindlimb ischemia. Circulation. 2007;116:2409-2419.

(76) Kocher AA, Schuster MD, Szabolcs MJ et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med. 2001;7:430-436.

(77) Kinnaird T, Stabile E, Burnett MS et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res. 2004;94:678-685.

(78) Crisostomo PR, Abarbanell AM, Wang M et al. Embryonic stem cells attenuate myocardial dysfunction and inflammation after surgical global ischemia via paracrine actions. Am J Physiol Heart Circ Physiol. 2008;295:H1726-H1735.

(79) Orlic D. The strength of plasticity: stem cells for cardiac repair. Int J Cardiol. 2004;95 Suppl 1:S16-S19.

(80) Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infarcted myocardium. Nature. 2001;410:701-705.

(81) Orlic D, Kajstura J, Chimenti S et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci U S A. 2001;98:10344-10349.

(82) Tang YL, Zhao Q, Zhang YC et al. Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. Regul Pept. 2004;117:3-10.

(83) Jackson KA, Majka SM, Wang H et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest. 2001;107:1395-1402.

(84) Murry CE, Soonpaa MH, Reinecke H et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature. 2004;428:664-668.

(85) Balsam LB, Wagers AJ, Christensen JL et al. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature. 2004;428:668-673.

(86) Nygren JM, Jovinge S, Breitbach M et al. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med. 2004;10:494-501.

(87) Gillio AP, Gasparetto C, Laver J et al. Effects of interleukin-3 on hematopoietic recovery after 5-fluorouracil or cyclophosphamide treatment of cynomolgus primates. J Clin Invest. 1990;85:1560-1565.

(88) Kawai T, Cosimi AB, Wee SL et al. Effect of mixed hematopoietic chimerism on cardiac allograft survival in cynomolgus monkeys. Transplantation. 2002;73:1757-1764.

(89) Assmus B, Schachinger V, Teupe C et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation. 2002;106:3009-3017.

(90) Strauer BE, Brehm M, Zeus T et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation. 2002;106:1913-1918.

(91) Tse HF, Kwong YL, Chan JK et al. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. Lancet. 2003;361:47-49.

(92) Perin EC, Dohmann HF, Borojevic R et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation. 2003;107:2294-2302.

(93) Chochola M, Pytlik R, Kobylka P et al. Autologous intra-arterial infusion of bone marrow mononuclear cells in patients with critical leg ischemia. Int Angiol. 2008;27:281-290.

(94) Yoshioka T, Ageyama N, Shibata H et al. Repair of infarcted myocardium mediated by transplanted bone marrow-derived CD34+ stem cells in a nonhuman primate model. Stem Cells. 2005;23:355-364.

(95) Shen G, Tsung HC, Wu CF et al. Tissue engineering of blood vessels with endothelial cells differentiated from mouse embryonic stem cells. Cell Res. 2003;13:335-341.

(96) Yamahara K, Sone M, Itoh H et al. Augmentation of neovascularizaiton in hindlimb ischemia by combined transplantation of human embryonic stem cells-derived endothelial and mural cells. PLoS ONE. 2008;3:e1666.

(97) Odorico JS, Kaufman DS, Thomson JA. Multilineage differentiation from human embryonic stem cell lines. Stem Cells. 2001;19:193-204.

(98) Plachta N, Bibel M, Tucker KL, Barde YA. Developmental potential of defined neural progenitors derived from mouse embryonic stem cells. Development. 2004;131:5449-5456.

(99) Brustle O, Spiro AC, Karram K et al. In vitro-generated neural precursors participate in mammalian brain development. Proc Natl Acad Sci U S A. 1997;94:14809-14814.

(100) Skottman H, Dilber MS, Hovatta O. The derivation of clinical-grade human embryonic stem cell lines. FEBS Lett. 2006;580:2875-2878.

(101) Xu C, Inokuma MS, Denham J et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol. 2001;19:971-974.

(102) Klimanskaya I, Chung Y, Meisner L et al. Human embryonic stem cells derived without feeder cells. Lancet. 2005;365:1636-1641.

(103) Rosler ES, Fisk GJ, Ares X et al. Long-term culture of human embryonic stem cells in feeder-free conditions. Dev Dyn. 2004;229:259-274.

(104) Rajala K, Lindroos B, Hussein SM et al. A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. PLoS One. 2010;5:e10246.

(105) Unger C, Skottman H, Blomberg P, Dilber MS, Hovatta O. Good manufacturing practice and clinical-grade human embryonic stem cell lines. Hum Mol Genet. 2008;17:R48-R53.

(106) Hwang WS, Ryu YJ, Park JH et al. Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. Science. 2004;303:1669-1674.

(107) Hwang WS, Roh SI, Lee BC et al. Patient-specific embryonic stem cells derived from human SCNT blastocysts. Science. 2005;308:1777-1783.

(108) Drukker M, Benvenisty N. The immunogenicity of human embryonic stem-derived cells. Trends Biotechnol. 2004;22:136-141.

(109) Cowan CA, Atienza J, Melton DA, Eggan K. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science. 2005;309:1369-1373.

(110) Inzunza J, Sahlen S, Holmberg K et al. Comparative genomic hybridization and karyotyping of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation. Mol Hum Reprod. 2004;10:461-466.

(111) Mitalipova MM, Rao RR, Hoyer DM et al. Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol. 2005;23:19-20.

(112) Buzzard JJ, Gough NM, Crook JM, Colman A. Karyotype of human ES cells during extended culture. Nat Biotechnol. 2004;22:381-382.

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

(114) Jimenez JJ, Jy W, Mauro LM et al. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. Thromb Res. 2003;109:175-180.

(115) Freyssinet JM. Cellular microparticles: what are they bad or good for? J Thromb Haemost. 2003;1:1655-1662.

(116) Hirst SJ, Barnes PJ, Twort CH. PDGF isoform-induced proliferation and receptor expression in human cultured airway smooth muscle cells. Am J Physiol. 1996;270:L415-L428.

(117) Kirschenlohr HL, Metcalfe JC, Weissberg PL, Grainger DJ. Proliferation of human aortic vascular smooth muscle cells in culture is modulated by active TGF beta. Cardiovasc Res. 1995;29:848-855.

(118) Vodyanik MA, Slukvin II. Directed differentiation of human embryonic stem cells to dendritic cells. Methods Mol Biol. 2007;407:275-293.

(119) Mummery CL, Ward D, Passier R. Differentiation of human embryonic stem cells to cardiomyocytes by coculture with endoderm in serum-free medium. Curr Protoc Stem Cell Biol. 2007;Chapter 1:Unit.

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

(121) Cai X, Lin Y, Friedrich CC et al. Bone marrow derived pluripotent cells are pericytes which contribute to vascularization. Stem Cell Rev. 2009;5:437-445.

(122) Lozito TP, Taboas JM, Kuo CK, Tuan RS. Mesenchymal stem cell modification of endothelial matrix regulates their vascular differentiation. J Cell Biochem. 2009;107:706-713.

(123) Covas DT, Panepucci RA, Fontes AM et al. Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with CD146+ perivascular cells and fibroblasts. Exp Hematol. 2008;36:642-654.

(124) Ema M, Faloon P, Zhang WJ et al. Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. Genes Dev. 2003;17:380-393.

(125) Moretti A, Caron L, Nakano A et al. Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell. 2006;127:1151-1165.

(126) Christoforou N, Miller RA, Hill CM et al. Mouse ES cell-derived cardiac precursor cells are multipotent and facilitate identification of novel cardiac genes. J Clin Invest. 2008;118:894-903.

(127) Wu SM, Fujiwara Y, Cibulsky SM et al. Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. Cell. 2006;127:1137-1150.

(128) Chung YS, Zhang WJ, Arentson E et al. Lineage analysis of the hemangioblast as defined by FLK1 and SCL expression. Development. 2002;129:5511-5520.

(129) Kawai Y, Hosaka K, Kaidoh M et al. Heterogeneity in immunohistochemical, genomic, and biological properties of human lymphatic endothelial cells between initial and collecting lymph vessels. Lymphat Res Biol. 2008;6:15-27.

(130) Mellgren AM, Smith CL, Olsen GS et al. Platelet-derived growth factor receptor beta signaling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations. Circ Res. 2008;103:1393-1401.

(131) Garlanda C, Dejana E. Heterogeneity of endothelial cells. Specific markers. Arterioscler Thromb Vasc Biol. 1997;17:1193-1202.

(132) Stevens KR, Pabon L, Muskheli V, Murry CE. Scaffold-free human cardiac tissue patch created from embryonic stem cells. Tissue Eng Part A. 2009;15:1211-1222.

(133) Yang L, Soonpaa MH, Adler ED et al. Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. Nature. 2008;453:524-528.

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

(135) 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.

(136) 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.

(137) Dai W, Field LJ, Rubart M et al. Survival and maturation of human embryonic stem cell-derived cardiomyocytes in rat hearts. J Mol Cell Cardiol. 2007;43:504-516.

(138) Caspi O, Huber I, Kehat I et al. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J Am Coll Cardiol. 2007;50:1884-1893.

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

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

(141) Kehat I, Kenyagin-Karsenti D, Snir M et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest. 2001;108:407-414.

(142) 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.

(143) Mummery C, Ward-van Oostwaard D, Doevendans P et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation. 2003;107:2733-2740.

(144) 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.

(145) 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. APPENDICES I

9.1.1. 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.

b) bez IF

9.1.2. 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.

9.2. 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.

9.3. Stručný souhrn výsledků k původnímu tématu postgraduálního studia 2002-2004

Původní téma: Využití dendritických buněk v protinádorové terapii

Během prvních 3 let mého postgraduálního studia jsem se v Laboratoři buněčné terapie na I. interní klinice VFN zabývala metodami přípravy dendritických buněk v co největší míře prezentujících idiotypový antigen cytotoxickým T- lymfocytům s cílem jejich potencionálního využití k protinádorové terapii v preklinických studiích.

Dendritické buňky byly získávány z periferní krve kultivací z monocytů. Nezralé dendritické buňky byly pěstovány 5-8 dní v kultivačním mediu RPMI 1640 s 10% fetálním telecím sérem, GM-CSF a IL 4, poté byly pulsovány tetanovým toxoidem a vyzrávány následnou kultivací s TNF-alfa po dalších 48 hodin, následně byla prováděna kontrola dendritického fenotypu pomocí imunofenotypizace.

V rámci našeho výzkumu bylo hlavním cílem projektu zjišťování schopnosti dendritických buněk stimulovat T lymfocyty u pacientů s chronickou lymfocytární leukemií před a po konvenční léčbě s cílem zjistit, zda po intenzivní chemoterapeutické léčbě mají pacienti s chronickou lymfocytární leukemií, s ohledem na těžkou imunosupresi, alespoň teoretickou schopnost navodit protinádorovou odpověď proti svým nádorovým buňkám. Tato otázka byla i předmětem grantu Fondu rozvoje vysokých škol (FRVŠ), jehož jsem byla řešitelem a který byl v roce 2005 úspěšně dokončen.

V rámci řešení tohoto projektu byla zavedena metoda generace dendritických buněk z CD 14-monocytů a jejich imunofenotypická charakterizace z hlediska prezentace antigenu. Byla spolehlivě zvládnuta metoda kokultivace dendritických buněk s CD 4+ a CD 8+ lymfocyty v různých experimentálních uspořádáních (autologní a allogení smíšené lymfocytární reakce) a zavedena metoda diferenciálního gatingu jako způsobu používání průtokové cytometrie, která umožňuje bližší charakterizaci zkoumaných buněčných populací pouze na základě side a forvard scatterů. Tuto práci jsme publikovali v roce 2005 (Epidemiol. Mikrobiol. Imunol.- viz seznam publikovaných prací).

V další fázi výzkumu jsme prokázali funkčnost dendritických buněk prostřednictvím smíšené lymfocytární reakce (MLR) u pacientů s chronickou lymfocytární leukemií před i po léčbě ve srovnání se zdravými dobrovolníky. U všech neléčených pacientů s chronickou lymfocytární leukemií autologní dendritické buňky podporovaly přežití a proliferaci jak CD4, tak CD8 lymfocytů, zatímco pouze 70-80% léčených pacientů jsme pozorovali stejný efekt na CD4 a CD 8 lymfocyty. U 3 z 5 neléčených pacientů, kde dendritické buňky byly

pulsovány tetanovým toxoidem, došlo k posílení odpovědi CD 4 lymfocytů, což nebylo možno pozorovat u pacientů s léčenou chronickou lymfocytární leukemií. Stejný vliv dendritických buněk pulsovaných tetanovým toxoidem na CD 8 lymfocyty se nepodařilo prokázat v žádné skupině vyšetřovaných pacientů. Výsledky jsou podrobně popsány v rámci článku publikovaném v roce 2008 (Physiol. Res. – viz seznam publikovaných prací).

V dalším průběhu mého postgraduálního studia nebylo dále v tomto výzkumném projektu v Laboratoři buněčné terapie na I. interní klinice pokračováno a díky možnosti výzkumu na poli lidských embryonálních kmenových buněk jsem zažádala o změnu tématu mého postgraduálního studia na současný projekt s názvem: Diferenciace lidských embryonálních kmenových buněk v buňky endotelové a hladké svalové jako model cévního vývoje (viz disertační práce).

9.4. 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.