

Identification of Arterial Phenotype in Endothelial Cells Derived From Human Pluripotent Stem
Cells

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Table of Contents

I.	List of Tables	iii
II.	List of Figures	iv
III.	Introduction	1
IV.	Methods	9
V.	Results	16
	a. Stromal cell co-culture differentiation	16
	i. Figure 1. Stromal cell co-culture differentiation of endothelial cells.	17
	b. Spin EB differentiation	18
	i. Figure 2. Spin EB differentiation of endothelial cells from hESCs.	20
	ii. Figure 3. Endothelial cells expand outward from EBs but do not differentiate well on fibronectin.	22
	iii. Table 1. . Comparison of endothelial and hematopoietic marker expression between BPEL + VBF and VBS-VBF media	23
	c. Characterization of differentiated endothelial cells	24
	i. Figure 4. hESC-derived ECs express endothelial markers and are functionally similar.	25
	d. Smooth muscle cell differentiation from sorted endothelial cells.	27
	i. Figure 5. Smooth muscle cells from hESCs express SMC-specific markers and can form tubes with ECs <i>in vitro</i> .	28
	e. Apelin enhances endothelial differentiation	27
	i. Figure 6. Apelin and DAPT promote higher expression of hemato-endothelial markers.	31
	f. DAPT enhances expression of endothelial markers	30
	i. Figure 6. Apelin and DAPT promote higher expression of hemato-endothelial markers.	31
	g. Characterization of arterial, venous, and lymphatic endothelial cell phenotypes	30
	i. Figure 7. Comparison of expression of lineage-specific genes between H9-ECs, HUAECs, HUVECs, and HLECs.	35
	h. PI3K inhibition along with cAMP increases CXCR4 expression	34
	i. Figure 8. PI3K inhibitors and cAMP increase CXCR4 expression after 24 hours.	38
	ii. Figure 9. Arterial gene expression varies following treatment with LY294002 and/or cAMP.	42
	i. Cyclic AMP can counteract alterations to gene expression caused by NOTCH inhibition	41
	i. Figure 10. Notch inhibition limits CXCR4 expression but can increase <i>Notch1</i> transcription.	43
VI.	Discussion	46
VII.	References	49

List of Tables

Table 1. Comparison of endothelial and hematopoietic marker expression between BPEL + VBF and VBS-VBF media	23
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List of Figures

Figure 1. Stromal cell co-culture differentiation of endothelial cells.	17
Figure 2. Spin EB differentiation of endothelial cells from hESCs.	20
Figure 3. Endothelial cells expand outward from EBs but do not differentiate well on fibronectin.	22
Figure 4. hESC-derived ECs express endothelial markers and are functionally similar.	25
Figure 5. Smooth muscle cells from hESCs express SMC-specific markers and can form tubes with ECs <i>in vitro</i> .	28
Figure 6. Apelin promotes higher expression of hemato-endothelial markers.	31
Figure 7. Comparison of expression of lineage-specific genes between H9-ECs, HUAECs, HUVECs, and HLECs.	35
Figure 8. PI3K inhibitors and cAMP increase CXCR4 expression after 24 hours.	38
Figure 9. Arterial gene expression varies following treatment with LY294002 and/or cAMP.	42
Figure 10. Notch inhibition limits CXCR4 expression but can increase <i>Notch1</i> transcription.	43

Introduction

The isolation and successful culturing of human pluripotent stem cells over a decade ago has proven to be a truly revolutionary moment in modern scientific research. We are now able to study and manipulate cells that are capable of differentiating into nearly every cell type present in the adult body. Initial studies using embryonic stem cells (ESC) have led to development of methods to reprogram terminally differentiated cells into induced pluripotent stem cells (iPS). One such area of intrigue using these technologies is vascular development and how applying knowledge of the cells that make up this vital network can be used to improve the lives of those that suffer from maladies that afflict it. The primary component of any vasculature system is the endothelial cell (EC) which constitutes the framework for every vessel that serves to deliver nutrients, important signaling molecules, and immune response capability to every cell in the body. Interactions with other vasculature-associated cells (also known as mural cells) such as smooth muscle cells (SMC) and pericytes provide stable vessels that allow for consistent blood flow as well as invasion into the local tissue by immune cells. While this process, known as vasculogenesis, is established during embryonic development, it is also undertaken when needed in adult tissues where it is referred to as angiogenesis. Whether it is in response to injury or a result of tissue growth, the vascular system is dynamic in nature in order to meet the needs of the organism.

Characterization of mature endothelial cells can be accomplished by analysis of the expression of extracellular proteins or markers which, when taken together, can identify the endothelial phenotype. While these markers can aid in identifying endothelial cells, they cannot do so individually as the majority are not expressed solely on ECs. Most are required for vessel integrity and prevention of leakiness in the absence of inflammation while others provide essential enzymatic functions. One of the most important and reliable markers used is platelet endothelial cell adhesion molecule 1 (PECAM1, also known as CD31). This surface protein is involved in cell to cell interactions known as adherens junctions and is required for maintenance of the integrity of vessels. It is, however, not specific to endothelial cells as it is also expressed on leukocytes and developing hematopoietic stem cells (HSC)¹. Another essential intercellular adhesion molecule involved in vessel stability for ECs is vascular endothelial cadherin (VE-cadherin, also known as CD144). Like CD31, CD144 is also expressed on a subset of hematopoietic cells during development which makes using it to identify distinctions between

early ECs and HSCs difficult². A third glycoprotein involved in cell to cell adhesion that is co-expressed on ECs and HSCs is CD34. Little is known about its exact function but like CD31 and CD144 it is expressed on early hematopoietic/vascular progenitors. Intercellular adhesion molecule 2 (ICAM2, also known as CD102) is yet another important adhesion protein that is highly expressed on endothelial cells but can also be found on leukocytes and hematopoietic progenitors³. The enzyme 5'-nucleotidase (5'-NT, also known as CD73) is expressed on the plasma membrane of ECs and is involved in the metabolism of extracellular adenosine monophosphate (AMP) to adenosine as well as leukocyte trafficking in vessels⁴. This enzyme is also reliably expressed on mesenchymal stem cells (MSC) which is not surprising given the close relationship between this type of cell and ECs⁵. Melanoma cell adhesion molecule (MCAM, also known as CD146) is also expressed on both MSCs and ECs and may be involved in leukocyte extravasation⁶. While identification of these markers on a cell surface are useful in determination of an endothelial phenotype, they must be used in conjunction with functional assays and gene expression profiles in order to conclusively characterize differentiated ECs.

During embryonic development, endothelial cells and the vascular cells that they interact with are differentiated from mesoderm precursors that are capable of committing to the hematopoietic lineage. Immediate precursors to endothelial cells are known as angioblasts and first arise in humans within the blood islands of day 7-13.5 post-conception embryos and are also involved in later hematopoietic development⁷. These cells are also capable of differentiation into smooth muscle cells as the vascular system matures in the developing embryo⁸. Early cells that express endothelial cell surface markers that give rise to initial hematopoietic precursors are known as the hemogenic endothelium (or hemangioblasts) and are essential for proper hematopoietic system generation⁹. Given the shared precursor of both endothelial and hematopoietic cells, *in vitro* differentiation of both can be done under similar conditions. Specific protocols for endothelial generation have been well established^{10,11} but the differences between mature cells and endothelial progenitors make isolation of pure populations difficult although not impossible. We sought to establish our own protocol that would provide more directed endothelial differentiation and increase the overall yield of differentiated cells. Our initial method of differentiation involved co-culture of embryonic and induced pluripotent stem cells with mouse stromal cells that are able to support hematopoietic commitment. This method also utilized a media that contained fetal bovine serum (FBS) which did not specifically induce differentiation of any one specific cell type. This lack of specificity resulted in low overall yields of cells that expressed EC-associated surface proteins which were used to identify and isolate the

desired populations. The majority of these cultures became stromal in nature and were accompanied by extensive extracellular matrix proteins which made dissociation into a single cell solution difficult. In order to improve upon this, we turned to a previously established system for hematopoietic development through the use of a defined media in the absence of stromal feeder cells¹². Instead of co-culturing undifferentiated pluripotent cells with murine fibroblasts, this system utilizes formation of embryoid bodies (EB) in the absence of feeder cells along with a defined culture media. Although this system requires more media components and is subject to its own issues of variability, it has proven to be beneficial by reducing the time needed for differentiation as well as honing the lineage commitment towards hematopoietic precursors. In addition to refining the differentiation process, this system eliminated any xenobiological effects from growing human cells on mouse cells which is necessary for future development towards viable cell therapies in human patients. Because this protocol was designed for definitive hematopoietic development, we needed to adjust it to suit our goals of endothelial differentiation. To this end, we modified the cytokine components and examined the use of small polypeptides and small molecule inhibitors in order to further enhance mature endothelial development.

The course of differentiation towards specific cell lineages is, for the most part, determined by temporal exposure of pluripotent cells to growth factors which induce signaling processes that influence the expression of lineage-specific genes. The first of these lineage commitments that occur in the generation of endothelial cells is towards the mesoderm germ layer as opposed to ectoderm or endoderm. *In vitro* studies have shown that the presence of bone morphogenic protein 4 (BMP4), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) are able to support development of a progenitor population from mesodermal cells which are capable of becoming hematopoietic, endothelial, mesenchymal, cardiomyocyte, and smooth muscle cells¹³. Each of these cytokines plays an important temporal role as BMP4 is essential for efficient mesoderm formation, bFGF for development of hemangioblasts, and VEGF (along with thrombopoietin) for hematopoietic commitment¹⁴. While these cytokines are certainly present in the FBS used in stromal cell co-culture differentiation, the availability of other cytokines that are involved in commitment to various other cell lineages reduced the efficiency of that process. By limiting the differentiation media to only those components that are involved in efficient hemangioblast differentiation, we hypothesized that the efficiency of endothelial cell generation would be increased.

We have also examined the effects of inhibiting notch receptor signaling in order to further enhance endothelial commitment of differentiating cells. Notch is an evolutionarily

conserved receptor that is involved in generating signals based on cell to cell interactions which help to define various differentiation processes¹⁵. The ligands for notch, Delta-like (DLL) and Jagged (JAG), are expressed on the surface of neighboring cells which drives the cell to cell signaling complex. It has been established that cells can express both ligand and receptor and the two are capable of acting in a *cis* manner to initiate the signaling process¹⁶. Activation of notch results in cleavage of the intracellular domain (NICD) which subsequently interacts with other transcription factors in the nucleus in order to enhance the expression of notch target genes¹⁷. While this signaling pathway plays a role in multiple lineage commitment phases during development, our research has focused on the ability of notch to direct hemangioblast fate. Along with the hedgehog transcription factor, notch signaling has been shown to be an important element of commitment to hematopoietic progenitors from the hemangioblast precursor¹⁸. Furthermore, inhibition of NICD cleavage by the γ -secretase inhibitor DAPT (N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester) has been shown to reduce hematopoietic development in hemangioblasts and enhance endothelial development in *in vitro* studies¹⁹.

Depending on the type of vessel they are a part of, endothelial cells in the mature vascular system can be arterial, venous, or lymphatic. These designations are not arbitrary as each EC type has unique phenotypic and genotypic components which determine the functional ability of each in order to maintain the integrity of the vasculature. *In vitro* studies have relied on the use of human umbilical arterial endothelial cells (HUAEC), venous endothelial cells (HUVEC), and lymphatic endothelial cells (HLEC) as standards for identifying the differences in phenotypes. Arteries and veins are essential for normal blood flow throughout the body while lymphatic vessels play an important role in trafficking of immune cells and lymph fluid. The determination of which type an endothelial cell will become occurs early in development before the vasculature is fully formed and blood flow is initiated. Early EC progenitors accumulate in a region called the intermediate cell mass (ICM) which is ventral to the notochord and between the somites²⁰. It is here (or possibly during migration) that these progenitors undergo arterial-venous specification as they form the dorsal aorta and posterior cardinal vein²¹. One of the most important determinants of this specification is the expression of ephrin family proteins, specifically ephrin B2 (EPHB2) and its receptor ephrin B4 (EPHB4). The presence of this receptor-ligand pair is not equally distributed as *EPHB2* is more highly expressed on arterial cells whereas *EPHB4* is more venous in nature. A study using a mutant *Ephb2-LacZ* reporter mouse model has indeed shown that *EPHB2* is restricted to developing arteries whereas *EPHB4* is only

found in venous endothelial cells²². This same model showed that while early vessel development was possible, progression to a mature vasculature was defeated by impaired interactions between arterial and venous vessels which resulted in embryonic lethality. It is theorized that developing ECs have already committed to either arterial or venous identity prior to interactions between the two vessel types which would explain the delay in lethality in this mouse model.

Another defining component of arterial-venous specification is the aforementioned notch signaling pathway. It has been established that Notch 1, Notch 4, and their ligands are restricted to arterial ECs and are absent from venous ECs²³. It has also been shown that mice heterozygous for the Notch ligand DLL4 have increased EPHB4 and reduced EPHB2 expression which results in a lack of arterial commitment by endothelial progenitors²⁴. Analysis of Notch expression during zebrafish development further elucidates its important role in arterial specification as loss of Notch decreases arterial marker expression as well as increases expression of venous markers on the dorsal aorta. This condition also leads to vessel development defects primarily due to incomplete arterial-venous differentiation. Conversely, increased Notch expression diminishes venous specification in the developing embryo²⁵. While mature vessel development is severely impacted by loss of Notch, this occurs after the formation of primary vessels which implies that it is not necessary for aggregation of hemangioblast precursors during initial vasculogenesis. Also of note is that zebrafish lacking Notch show expression of some arterial-specific markers which provides evidence for upstream elements of Notch influencing the commitment to an arterial phenotype. Downstream products of Notch signaling have been shown to be influential in expression of arterial specific genes. Hairy/enhancer-of-split related with YRPW motif protein (HEY) and hairy/enhancer-of-split (HES) family proteins are transcription factors whose expression is dependent on Notch-mediated transcription. Mice that are *Hey1* and *Hey2* deficient are embryonic lethal due to cardiovascular defects caused by improper arterial specification²⁶. Similar experiments using *Hes1* knockout mice showed reduced vascular remodeling and lack of arterial ECs in embryos²⁷. Inhibition of Notch in cultured cells can be performed without needing to generate mutant knockdowns or knockouts as small molecule drugs have been developed to disrupt the signaling pathway. DAPT is a widely used inhibitor of γ -secretase activity which is required for cleavage of the intracellular portion of the Notch receptor²⁸. It has been used to examine the role of this enzyme in the formation of amyloid β -peptide plaques which are implicated in Alzheimer's disease as well as cancer development but has also shown usefulness for identifying the mechanisms of Notch function in cardiovascular development. The

importance of the Notch signaling pathway is preserved in the mature vasculature as it is required for recruitment of smooth muscle cells and pericytes to interact with endothelial cells and form stable arterial vessels²⁹.

In contrast to notch-dependent arterial specification, venous development requires transcriptional activation of specific genes by a unique transcription factor. Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) was identified as a mediator of venous identity and is expressed only in venous and not arterial ECs³⁰. Experiments with COUP-TFII knockout mice resulted in embryonic death due to severe hemorrhage and enlarged blood vessels as well as underdeveloped atria and malformed cardinal veins³¹. *In vitro* experiments with targeted disruption of COUP-TFII in mouse vein ECs caused an upregulation of arterial specific genes *EphB2*, *Jag1*, *Notch1*, and neuropilin-1 (*Nrp1*) while ectopic expression *in vivo* results in fusion of arteries and veins which is similar to the findings of notch^{-/-} mice³². It is theorized that COUP-TFII regulates venous identity by decreasing notch signaling which releases EphB4 and VEGFR3 (described later) from being repressed by it³⁰. A second, less understood mediator of venous identity is apelin (APLN), a polypeptide that activates the apelin receptor (APJ). APJ has been shown to be essential for maturation and proper development of *in vivo* vascular networks³³ as well as hematopoiesis³⁴. Stimulation of APJ, a G-protein coupled receptor, activates a signaling pathway that enhances activation of myocyte enhancer factor 2 (MEF2) which has been shown to be essential for endothelial and cardiovascular development³⁵. Analysis of the developing retinal vasculature in mouse embryos has shown early expression of APJ on venous ECs which is not present on arterial ECs³⁶. APJ expression is maintained through maturation and is implicated in the induction of a hypotensive state in blood vessels due to increased nitric oxide (NO) production following activation³⁷. *Apj*^{-/-} mice also showed significant cardiac deficiencies due to the lack of Krüppel-like factor-2 (KLF2) expression³⁸, an APJ-dependent regulator of cardiac development.

Arguably the most important growth factor for endothelial differentiation, growth, and survival is vascular endothelial growth factor (VEGF). A secreted cytokine, VEGF has multiple isoforms (VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E) which bind to different extracellular receptors which include fetal liver kinase-1 (Flk1, also known as VEGFR2), fms-like tyrosine 1 (Flt1, also known as VEGFR1), fms-like tyrosine 4 (Flt4, also known as VEGFR3), and neuropilins 1 and 2 (NP1, NP2)³⁹. VEGFR1 acts as a negative regulator of vasculogenesis during embryonic development and has a high binding affinity for the VEGF-A isoform compared to VEGFR2 and VEGFR3⁴⁰. VEGF-B binds only to VEGFR1 while VEGF-C

and VEGF-D can bind to VEGFR2 and VEGFR3⁴¹. The C and D isoforms have a strong affinity for VEGFR3 and are involved in the regulation of lymphangiogenesis, the development of lymphatic vessels. Expression of VEGFR1 and VEGFR2 is not restricted to endothelial cells as the former is also found on osteoclasts, dendritic cells, pericytes and trophoblasts^{42,43} while the latter has been found to be expressed on neuronal cells, osteoblasts, pancreatic duct cells, megakaryocytes, and retinal progenitor cells⁴⁴. VEGFR2 has also been found on certain populations of circulating cells that co-express hematopoietic markers and are believed to be endothelial progenitor cells that are involved in neo-angiogenesis⁴⁵. Mice that are null for VEGFR1 are embryonic lethal and show disorganized blood vessels and EC overgrowth. The ligand-binding domain, not the kinase domain, may be responsible for this effect by reducing the amount of free VEGF-A available to bind to VEGFR2 and deliver cell growth and proliferation signals⁴⁶. VEGFR2 is expressed highly on hemangioblasts so mouse models in which gene expression is inactivated are embryonic lethal due to undeveloped vasculature as well as diminished hematopoietic development⁴⁷. It is also upregulated during neo-angiogenesis in mature vessels on tip cells which are the lead of new sprouting vessels. While VEGFR3 is limited to lymphatic endothelial cells in the mature vasculature, it has been shown to be essential during development as gene inactivation leads to embryonic lethality in mice due to improper vessel remodeling⁴⁸. The variability of VEGF receptor signaling shows that it is an intricate system of regulated and temporal expression involved in the development of a functional vasculature.

The signaling receptors described are only the initial elements of the signaling pathways that ultimately determine the arterial, venous, or lymphatic fate of endothelial precursors. While Notch activation has been implicated as a primary driver of arterial specification, its signaling can be augmented by other kinases present. VEGFR activation has been shown to activate two downstream effectors, extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3 kinase (PI3K) which have opposite and competing effects on cell fate. Chemical screens using a zebrafish *hey2* ortholog mutant model, named gridlock (*grl*), have been able to identify small molecule inhibitors that target these kinases and identify their role in arterial-venous specification⁴⁹. Inhibition of ERK or upstream signaling molecules results in decreased arterial cell differentiation and malformation of the dorsal aorta whereas constitutive PI3K/AKT signaling induces more commitment to the venous fate by hemangioblasts. It is thought that PI3K/AKT activity is capable of disrupting ERK signaling in some as yet undefined manner. Interestingly, some studies done *in vitro* suggest that PI3K activates notch and Dll4 which is in

contrast to the *in vivo* findings^{50,51}. Application of GS4012, a flavone small molecule, to the *grl* mutant zebrafish model rescued the normal vascular development by activation of the VEGF signaling pathway⁵². A structurally dissimilar molecule identified in chemical screens, GS4898, achieved the same phenotype rescue by inhibiting a downstream effector of the PI3K signaling pathway, AKT⁵³. To this end, various other inhibitors of PI3K signaling have been identified including wortmannin, LY294002⁵⁴, and PI-103 which is also capable of inhibiting the mammalian target of rapamycin (mTOR), a kinase involved in the PI3K pathway⁵⁵. Investigation of these pathways have revealed influences on a variety of protein expression patterns in different cell types and their functional consequences. Many studies have examined the use of PI3K inhibitors for treatment of various cancers, some by affecting the ability of solid tumors to vascularize their tissue⁵⁶. From this research, a relationship between the chemokine receptor CXCR4 and PI3K signaling has been established albeit with limited focus on how this interaction functions in endothelial cells. CXCR4 is expressed on a variety of cell types and along with its ligand, stromal-derived-factor-1 (SDF-1, also known as CXCL12), is involved in chemotaxis and homing in hematopoietic cells⁵⁷. Mice deficient for CXCR4 or SDF-1 show an inability to form arterial networks in the developing small intestine while the venous capillaries remained intact, potentially identifying a role for the receptor and its ligand in angiogenesis⁵⁸. It has also been established that ECs expressing CXCR4 at high levels are more effective during repair of ischemic limbs in mice⁵⁹. An effective positive regulator of CXCR4 expression in various cell types is cyclic adenosine monophosphate (cAMP)^{60,61}. Activation of signaling pathways involving this small molecule have also been implicated in upregulation of notch and subsequent arterial specification via VEGF signaling enhancement^{62,63}.

According to the CDC, roughly 600,000 people die of cardiovascular disease each year which represents about one in every four deaths⁶⁴. In addition to the human cost of this type of disease, it is estimated that it costs the United States \$108.9 billion in healthcare costs and lost productivity⁶⁵. It has become abundantly clear that traditional treatments will need to be augmented with newer technologies to address the severity of this problem. Ongoing research into the efficacy of cellular therapies has benefitted greatly from the ability to generate vascular cells from embryonic or induced pluripotent stem cells. For this reason we investigated the effect of transplantation of our endothelial and smooth muscle cells into mouse and pig ischemic models to analyze their ability to improve the repair of the damaged tissue and provide therapeutic benefit. This was done in collaboration with Dr. Jay Zhang's laboratory at the University of Minnesota who performed the actual surgeries and post-surgical analysis. While this type of

therapy has shown promising results in other investigations⁶⁶, we sought to determine the potential benefits of using a fibrin-based patch in conjunction with delivery of the cells to the ischemic site. Given the dynamic nature of the surface of the heart, the patch was expected to provide stability for the transplanted cells at the ischemic site and improve interaction with the endogenous tissue. This collaboration has resulted in multiple publications that have demonstrated the therapeutic benefit of this system as well as providing an *in vivo* model with which to determine the functional efficacy of our ES/iPS-derived ECs and SMCs⁶⁷⁻⁶⁹.

The primary goals of our research have been twofold: first, we sought to optimize our established differentiation protocol in order to enhance the development of mature endothelial cells that are capable of being cultured for multiple passages while maintaining endothelial functionality and phenotype. Generation of ECs from pluripotent stem cells is not a novel process, but discussions with others in this area of research have revealed the difficulty in growing cells that are able to mimic primary endothelial cells in extended culture conditions. We hypothesized that, based on established research, the treatment of undifferentiated cells with cytokines known to induce hemangioblast differentiation at temporally-sensitive intervals would not only increase the yield of mature ECs but also allow for longer culturing before replicative senescence or transformation to a more fibroblast-like phenotype. Our second goal was to determine if the ECs generated were genotypically and phenotypically similar to primary arterial, venous, or lymphatic endothelial cells. In addition to this, we sought to identify methods that could potentially influence this fate determination in developed ECs towards a more arterial phenotype which we theorize would be more effective in *in vivo* models of ischemic repair.

Methods

Maintenance of undifferentiated pluripotent stem cells

Undifferentiated ES and iPS cells for use in stromal co-culture differentiation were grown in 6-well plates that were coated with a monolayer of irradiated mouse embryonic fibroblasts (MEF) at a density of 180,000 per well (high density). Each plate was stored in incubators at 37°C and 5.0% CO₂, hereby referred to as standard conditions. ES and iPS cells were passed on to fresh MEF plates every 6-7 days depending on confluency and amount of colonies that showed signs of differentiation (non-linear borders, 3-dimensional growth, discoloration). If it was estimated that more than 10% of the colonies were undergoing differentiation, glass picking sticks would be used to remove the unwanted colonies. Wells were fed daily with 2.5mL of ES media containing 15% knockout serum replacement, 1% non-essential amino acids (NEAA), 1%

L-glutamine, 0.5% pen/strep, 1x β -mercaptoethanol, and 8ng/mL bFGF. Removal of undifferentiated cells was done by incubating each well in 1mL of 1mg/mL collagenase IV in DMEM-F12 media for 5 minutes. Cells were mechanically scraped off with a glass pipette re-suspended in ES media, and centrifuged for 5 minutes at 1500 RPM. For undifferentiated cells, the supernatant was aspirated and the cells were re-suspended in ES media at concentrations of 1:3 to 1:10 (based on well to number of milliliters of ES media used to re-suspend) and then added to 6 well plates containing new MEFs.

Stromal cell co-culture differentiation

M210 cells were grown to confluency on 150cm² flasks and fed approximately every other day with M210 media containing 10% fetal bovine serum (FBS) and 1% pen/strep in RPMI. Expansion and passage of M210s was performed by removing the media from the flasks, washing with DPBS, and incubating in 0.05% trypsin-EDTA for 2-4 minutes. The trypsin was quenched with FBS-containing media as cells were collected and centrifuged at 1500 RPM for 5 minutes. The supernatant was aspirated and the cells were resuspended at the desired concentration in fresh M210 media. Upon reaching confluency, the M210 media was collected and temporarily stored in a 50mL conical tube. Mitomycin C was added to this media for a final concentration of 10ng/mL and returned to the M210 flask. The M210 cells were incubated for 3 hours at standard conditions in the mitomycin C-treated media, after which the media was collected and stored according to proper waste disposal guidelines. The cells were washed three times with DPBS which was also stored with the mitomycin C media when collected. M210 cells were then incubated with 0.05% trypsin-EDTA until they were non-adherent and collected in FBS-containing media to quench the trypsin. Each well of a 6 well plate was seeded with 160,000 M210 cells and allowed to adhere overnight.

ES/iPS cells were harvested according to the methods previously described for passaging cells and were added to the confluent M210 6 well plates at a ratio of 1:6-1:10. The cells for differentiation were fed with R15 media containing 16% characterized FBS, 1% NEAA, 1% L-glutamine, 1% pen/strep, and 1x β -mercaptoethanol in RPMI. The media was changed every other day for 12-14 days at which point the cells were collected and sorted by MACS. CD34+ and CD31PE+ kits (Stemcell Technologies, Vancouver) and CD31+ kits (Miltenyi Biotech, Auburn CA) were used according to the manufacturer's protocols.

Primary endothelial cell culture

Human umbilical arterial endothelial cells (HUAEC) and Human lymphatic endothelial cells (HLEC) were obtained from ScienCell, Human umbilical venous endothelial cells (HUVEC) were obtained from LifeLine. Cells were thawed and plated on to 25cm² flasks that were pre-coated with 10ng/mL fibronectin. Each flask was fed with EGM-2MV media (Lonza) every other day and cells were passed when roughly 80% confluency was achieved. When cells reached passage 10 or greater they were not used for any experiments and were discarded.

Spin EB differentiation

ES and iPS cell lines were adapted through routine passage with TrypLE (Life Technologies, Carlsbad) on low density (90,000/10cm² well) MEFs. At roughly 12-15 passages of adaptation, cells were harvested and re-suspended in standard spin EB media containing 10% de-ionized bovine serum albumin, 5% polyvinyl alcohol, 1x synthechol solution, 1x linoleic acid, 1x linoleic acid, 5mg/mL ascorbic acid 2-phosphate, α -Monothioglycerol (13 μ L in 200mL of media), 1mM glutamax I, 1x pen/strep, 5% (v/v) protein-free hybridoma mix, and 1x insulin-transferrin-selenium. 96-well plates were prepared by adding sterile water to the outer wells prior to adding the cells in spin EB media to the rest of the wells at a concentration of 3,000 cells/100 μ L of media in each well. The plates were then centrifuged at 1400 RPM to collect the cells at the bottom of the wells and form embryonic bodies. Following one day of incubation, 70 μ L of spin EB media was removed from each well and replaced with an equal volume of differentiation media #1 containing 2% serum replacement media 3 (Sigma-Aldrich, St. Louis), 1% L-glutamine, 1% NEAA, 0.004% α -Monothioglycerol, and 50ng/mL each of VEGF (R&D Systems, Minneapolis), bFGF (Peprotech, Rocky Hill NJ), and BMP-4 (Peprotech) in IMDM basal media. At day 6 of differentiation, 70 μ L of differentiation media #1 were removed and replaced with 70 μ L of differentiation media #2 which is the same as #1 except it contains no BMP-4. On Day 7, the Spin EBs were harvested and magnetically sorted.

Magnetic Sorting (Miltenyi Kit)

Prior to sorting, cells were dissociated with trypsin and were filtered with 70 μ M filters to reduce clumping. In the case of spin EBs, the EBs were collected with a multichannel pipette and were incubated with trypsin in a 15mL conical. Following neutralization of the trypsin with FBS-containing media, cells are resuspended in Easy Sep media containing 2% FBS and 0.2% EDTA (1mM) in dPBS. After removing a small amount of cells to be used in pre-sort flow cytometry

analysis, the whole cell population is resuspended at a maximum concentration of 1×10^7 cells per 60 μ L of Easy Sep media. 20 μ L of FcR blocking reagent is added per 1×10^7 cells and mixed briefly before adding 20 μ L of CD31 microbead mixture which consisted of anti-CD31 antibodies conjugated to magnetic beads. This mixture is incubated at 4°C for 15 minutes. Cells are then washed with at least 1mL of Easy Sep media to remove unconjugated antibodies. MACS separation LS columns (Miltenyi) were prepared by inserting them into the mini MACS magnet and by allowing 1mL of Easy Sep media to completely run through the column. Following this, the cells (resuspended in 500 μ L of Easy Sep media) are added to the column and allowed to run through. When the media has all flowed through, 500 μ L of media is added and allowed to flow through three times. After the third time, the column is removed from the magnet and placed in a fresh 5mL round bottom tube. 1mL of media is added and the plunger provided is used to rapidly flush the cells from the column into the round bottom tube. Cells are counted and some are allocated for post-sort flow cytometry analysis while the majority are resuspended in EGM-2MV (supplemented with 50ng/mL of VEGF) and plated on dishes coated with fibronectin.

Magnetic Sorting (EasySep kit)

Cells are collected the same way as described in the Miltenyi method above. Total cell populations totaling less than 5×10^7 were re-suspended in 250 μ L of Easy Sep media, populations totaling between $5 \times 10^7 - 2 \times 10^8$ were re-suspended at a concentration of 2×10^8 cells/mL. The EasySep Positive Selection Cocktail was added at a concentration of 100 μ L/mL of cells and incubated at room temperature for 15 minutes. Following this, EasySep Magnetic Nanoparticles were added at a concentration of 50 μ L/mL and incubated at room temperature for 10 minutes. 2.5mL of Easy Sep media was added after this time and the mixture was gently pipetted up and down to dissociate any clumps. If large aggregates remained, the cells would be passed through 100 μ m mesh filters. The tube containing cells for selection was then placed inside of the EasySep magnet for 5 minutes. After this time the tube was inverted while still in the magnet in order to pour off the supernatant fraction. The tube was then removed from the magnet and a fresh 2.5mL of EasySep media was added and the tube was replaced in the magnet. This process was repeated for a total of three times altogether. Following the final inversion, the tube was removed from the magnet and the cells remaining were resuspended in endothelial growth media and plated on fibronectin-coated flasks or set aside for flow cytometry analysis.

Immunocytochemistry

Cells to be analyzed were harvested and re-plated on 8-chambered slides (Nunc, Rochester NY) that had been pre-coated with fibronectin (10ng/mL). After about a day of growth, each chamber was washed with dPBS and 10% formalin was used to fix the cells for 15 minutes at room temperature. The chambers were washed with dPBS again and the cells were permeabilized with 0.2% (v/v) Triton-X in dPBS for no more than 5 minutes. A third wash with dPBS was performed and the cells were then incubated with blocking serum (10% (v/v) FBS and 2% (w/v) BSA in dPBS) for one hour at room temperature. Primary antibodies were diluted to roughly 1-2ng/mL in blocking serum and added to each chamber to incubate overnight at 4°C. Following another wash with dPBS the next day, secondary antibodies diluted to the same concentration in dPBS containing 10% of the serum of the host animal the antibody was obtained from were added to the chambers. These antibodies incubated on the cells for one hour at room temperature and were washed with dPBS following incubation. Chamber dividers were removed and ProLong Gold with DAPI (Life Technologies) was added prior to visualization. Antibodies used are mouse anti-human CD31 (eBioscience, San Diego), mouse anti-human CD34 (BD Biosciences, San Jose), mouse anti-human Von Willebrand Factor (Dako, Carpinteria), mouse anti-human eNOS/NOS type III (BD Biosciences), mouse anti-human CD144 (eBiosciences), mouse IgG1 iso control (eBiosciences), rabbit anti-human α -smooth muscle actin (abcam, Cambridge, UK), mouse anti-human SM22 (abcam), mouse anti-human calponin (Sigma Aldrich, St. Louis), donkey anti-mouse Cy3-conjugated IgG (Jackson ImmunoResearch Laboratories, West Grove), donkey anti-rabbit FITC-conjugated IgG (Jackson ImmunoResearch Laboratories), donkey anti-rabbit Cy3-conjugated IgG (Jackson ImmunoResearch Laboratories), goat anti-mouse alexafluor 488 IgG (Life Sciences), donkey anti-rabbit alexafluor 555 (Life Sciences).

Flow Cytometry

Cells prepared for flow Cytometry were filtered and resuspended in FACS buffer (2% (v/v) FBS and 1% (v/v) of 10% sodium azide in dPBS) at roughly 10,000 to 50,000 cells per sample. Prior to analysis, cells were incubated with fluorophore-conjugated primary antibodies specific for cell surface antigens at 4°C for 20 minutes. Following incubation, cells were washed with FACS buffer and the supernatant was aspirated following centrifugation at 1500 RPM for 3-5 minutes to remove any excess unbound antibody. Immediately prior to analysis, 7-aminoactinomycin (7AAD, Sigma Aldrich) was added to a final concentration of 2ng/mL. Cells were analyzed with either a FACScalibur or LSR II (BD Biosciences) flow cytometer system. Collected data was

analyzed using Flowjo software (TreeStar, Ashland). All antibodies used for flow cytometry applications are mouse anti-human from BD Pharmingen (San Jose) unless otherwise noted. APC-conjugated antibodies used are CD31 mouse anti-human (Thermo Scientific, Waltham MA) and CD73 mouse anti-human (eBioscience). PE-conjugated antibodies used are CD102 mouse anti-human (Biolegend, San Diego), CD144 mouse anti-human (eBiosciences), APLNR rabbit anti-human (Bioss, Boston), VEGFR3 mouse anti-human (Biolegend), Neuropilin-2 rabbit anti-human (Bioss), Neuropilin-1 mouse anti-human (R&D Systems), Streptavidin (BD Biosciences). Biotin-conjugated antibody used was CXCR4 mouse anti-human (eBioscience).

PI3K-inhibitor Assays

Cells were plated at a concentration of 1.0×10^5 /well on Nunc 6-well plates and allowed to recover for 24 hours or until confluency was reached. 24 hours prior to analysis, LY294002 (Cayman Chemical, Ann Arbor), PI-103 (gifted from Dr. David Largespada's lab) and/or 8-Br-cAMP (Enzo Life Sciences, Farmingdale NY) were added to EGM-2MV media at pre-determined volumes which resulted in the desired experimental concentrations. Cells were incubated with or without PI3K inhibitor/cAMP for 24 hours. After this time, cells were collected with trypsin if used for qrt-PCR analysis or non-enzymatic dissociation solution (Sigma Aldrich) if used for flow cytometry as trypsin can cleave the CXCR4 protein. Cells used for flow cytometry were prepared as described earlier and first incubated with a biotin-conjugated CXCR4 mouse anti-human antibody (eBioscience) diluted 1:50 in Easy Sep buffer. Following incubation on ice for 20 minutes, the cells were washed twice in Easy Sep buffer. PE-conjugated streptavidin beads (BD Biosciences) were then added to the cells at a concentration of 1:100 in Easy Sep buffer and incubated on ice for 15 minutes. These cells were then washed twice in Easy Sep buffer and prepared and analyzed under standard flow cytometry protocol.

Quantitative Real-Time Polymerase Chain Reaction (qrt-PCR)

Cells to be analyzed were collected and resuspended in buffer RLT and added to Qiashredder tubes (Qiagen, Germantown MD). Use of these tubes followed the manufacturer's instructions. Following this, the flow through was added to RNeasy Mini Kit (Qiagen) tubes and manufacturer's instructions were followed. RNA was analyzed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific) for concentration and purity. RNA not immediately being converted to cDNA was stored at -80°C . The Superscript III First Strand system (Life Technologies) was used to generate cDNA from the RNA collected during which the manufacturer's instructions were followed. Analysis of gene expression was done with SYBR

Green (Life Technologies) in 96-well Thermo-Fast Detection Plates (Thermo Scientific) using the Mastercycler Realplex² thermocycler (Eppendorf) and the associated software. Primers used for this analysis were:

GAPDH forward	CCACTCCTCCACCTTTGAC	FOXC1 forward	GGCGAGCAGAGCTACTAC
GAPDH reverse	ACCCTGTTGCTGTAGCCA	FOXC1 reverse	TGCGAGTACACGCTCATGG
HES1 forward	CAGGACACCGGATAAACCAAA	RBPJ forward	AACAAATGGAACGCGATGGTT
HES1 reverse	CTTTCATTTATTCTTGCTCTTCGTCTT	RBPJ reverse	GGCTGTGCAATAGTCTTTCCTT
CXCR4 forward	ACCACAGTCATCCTCATCCTG	NOTCH1 forward	GAGGCGTGGCAGACTATGC
CXCR4 reverse	CACACCCTTGCTTGATGATTT	NOTCH1 reverse	CTTGACTCCGTCAGCGTGA
COUPTFII forward	ATAGTCCTGTTACCTCAGATGC	NOTCH4 forward	TGTGAACGTGATGTCAACGAG
COUPTFII reverse	CCTACCAAACGGACGAAAAA	NOTCH4 reverse	ACAGTCTGGGCCTATGAAACC
PROX1 forward	GGGAAGTGCAATGCAGGA	JAG1 forward	GTCCATGCAGAACGTGAACG
PROX1 reverse	GTA CTGGTGACCCCATCGTT	JAG1 reverse	GCGGGACTGATACTCCTTGA
EPHB2 forward	AGAAACGCTAATGGACTCCACT	JAG2 forward	TGGGACTGGGACAACGATAC
EPHB2 reverse	GTGCGGATCGTGTTCATGTT	JAG2 reverse	AGTGGCGCTGTAGTAGTTCCTC
EPHB4 forward	CGCACCTACGAAGTGTGTGA	DLL4 forward	GCCCTTCAATTTACACCTGGC
EPHB4 reverse	GTCCGCATCGCTCTCATAGTA	DLL4 reverse	CAATAACCAGTTCTGACCCACAG

Matrigel Tube-Forming Assay

Prior to cells being harvested, 24-well Nunc plates were coated with recently thawed growth factor-reduced matrigel (BD Biosciences) supplemented with VEGF (R&D Systems) to a concentration of 50ng/mL. The matrigel was spread with a P1000 pipette tip while avoiding creation of bubbles. After evenly spreading the matrigel along the bottom of each well (~100µL/well), the plate was incubated for at least 30 minutes at 37°C. After this time, harvested cells resuspended in EGM-2MV were added to each well at a concentration of 50,000-100,000 per well. Analysis of tube formation was done 18-24 hours later except where indicated.

Results

Stromal Cell Co-Culture Differentiation

In order to generate endothelial and smooth muscle cells, H9 embryonic stem cells as well as induced pluripotent stem cells generated from neonatal human dermal fibroblasts (miPS16 line) and umbilical cord blood cells (UCBiPS7 line) were cultured according to established pluripotent stem cell protocols. Both iPS lines were generated in the Kaufman lab and all lines were previously characterized for expression of stem cell markers as well as ability to form teratomas *in vivo*. All lines were used for experiments that showed their ability to differentiate into various cells of the hematopoietic lineage such as natural killer cells and mesenchymal stem cells. The initial differentiation strategy involved co-culture of undifferentiated cells with inactivated M210 mouse bone marrow stromal cells in media containing 20% FBS. While the media did not necessarily direct differentiation specifically towards an endothelial lineage, M210s have been shown to increase the development of hematopoietic cells through cell to cell interactions that replicate the hematopoietic niche to a degree. Because of this approach, pluripotent colonies expanded rapidly and differentiated quickly with cells at the center of colonies forming 3-dimensional structures while cells at the edges tended to remain as a monolayer. For this reason it was important to limit the number of cells that were initially plated as too dense of a starting population would result in poor differentiation of endothelial cells.

On day 12-14 of differentiation, cells were harvested and sorted magnetically initially for positive selection of CD34⁺ cells and later for CD34⁺/CD31⁺ cells. The pre-sort populations typically consisted of about 2-8% double positive cells for these markers (**Fig. 1A**) so in order to get a usable post-sort population large quantities of cells were needed. Given the prolific nature of the differentiating cells this was not an issue as anywhere from 3.0×10^7 to 1.0×10^8 total cells could be collected for sorting. However, limitations of the Easy Sep sorting system made use of anything less than 7.0×10^7 ideal due to clogging of the sorting column and inefficient magnetic binding. Analysis of the pre-sort population showed that the majority of cells (~90%) were negative for any endothelial markers with the exception of small populations that were CD73⁺/CD34⁻ and CD146⁺/CD34⁻. CD73 and CD146 are not unique to endothelial cells, as they are expressed on a variety of hematopoietic cells with the former present on regulatory T cells⁷⁰ and mesenchymal stem cells⁷¹ while CD146 is found on a subset of activated lymphocytes⁷² as well as MSCs and fibroblasts⁷³. Despite this shared expression among unique

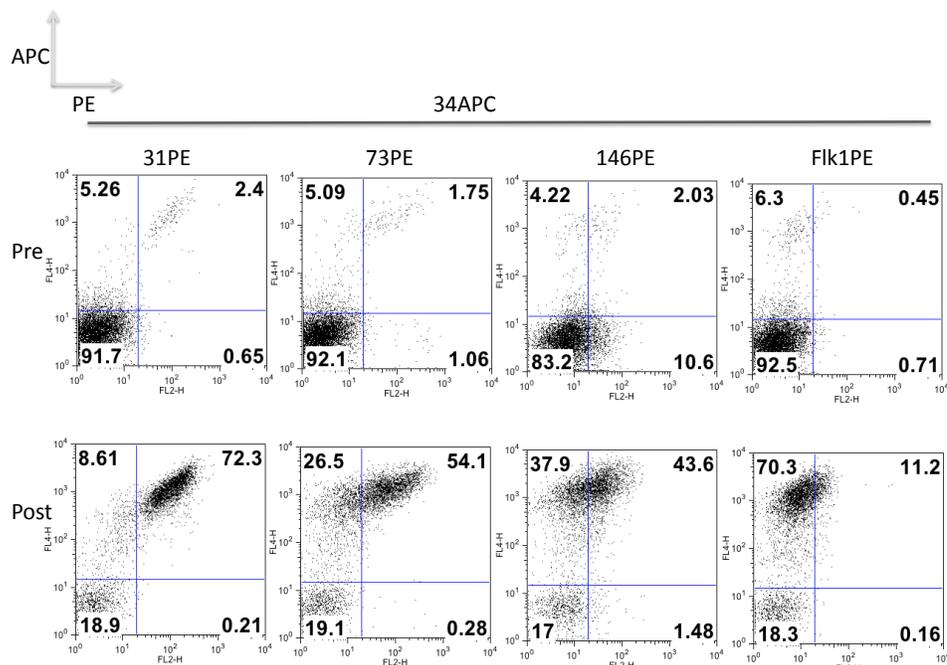
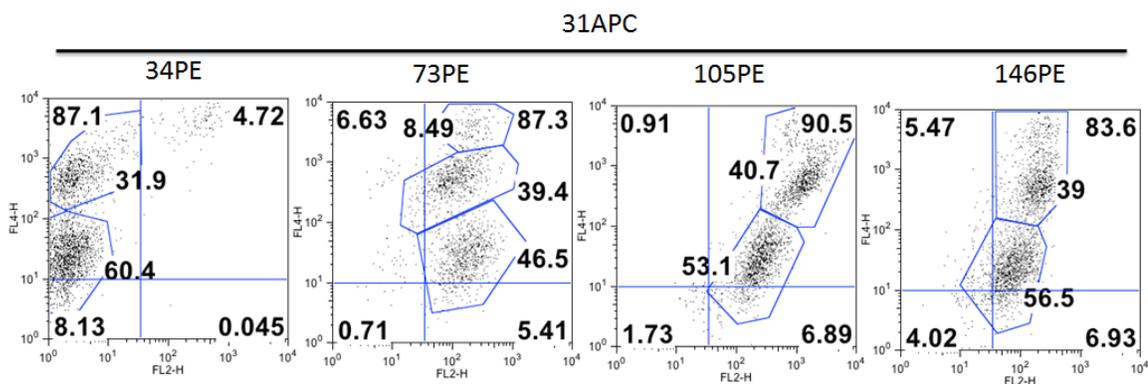
Figure 1**A****B**

Figure 1. Stromal cell co-culture differentiation of endothelial cells. (A) Flow cytometry data from a CD34⁺ MACS sort following 12 days of differentiation in stromal cell co-culture. **(B)** Flow cytometry analysis following two passages post-sort

cell types, mature endothelial cells possess a CD31+/CD73+/CD146+ phenotype so it was determined that the small populations seen with this phenotype were committed to that lineage. Flk1 is the primary receptor for vascular endothelial growth factor on endothelial cells, making it a useful marker for EC identification. While only a small percentage of the cells showed CD31+/Flk1+ expression (**Fig. 1A**) this may be attributed to a weak antibody as later experiments showed more robust expression during analysis but does not entirely rule out lower levels of Flk1 present using this differentiation method. As noted earlier, efficiency of the Easy Sep magnetic sorting system decreased if the starting population was too numerous. Roughly 9.0×10^7 cells were used in the sort shown in **figure 1** but only about 0.6% were recovered after the sort. This inefficiency can also be seen in that roughly 20% of the post-sort cells are negative for the endothelial markers being analyzed. This problem became more evident as the sorted population was allowed to expand in standard endothelial conditions resulting in complete loss of endothelial phenotype after only a few passages (**Fig. 1B**).

Spin EB Differentiation

The inefficiency of the differentiation using the stromal co-culture method, compounded with the overgrowth of sorted cultures by fibroblast-like cells, forced us to reconsider our methods and try to develop a new protocol for endothelial differentiation. Our lab had shown success in generating NK cells using a feeder free method that was adapted from a previously published protocol utilizing a defined differentiation media to support spin embryoid bodies (spin EB)^{12,74}. This system not only increased the efficiency of hematopoietic differentiation, but it was also beneficial in reducing the time needed for differentiation as well as eliminating the need for co-culture with murine fibroblasts. Another essential benefit to this method is the use of only recombinant proteins in the media which eliminates the need for animal products to be used in the culture. Also, by using a defined number of cells in the culture, the problems that arose from differentiation cultures being too dense and thus affecting the capacity to induce endothelial differentiation were eliminated. For these reasons, this system was utilized in an effort to standardize and improve endothelial differentiation efficiency as well as purity of sorted populations.

While this spin EB system was designed with hematopoietic differentiation in mind, it's efficacy of generating viable endothelial populations was not initially realized using the standard protocol. The typical hematopoietic differentiation in the BPEL (bovine serum albumin polyvinyl alcohol essential lipids) media utilized the cytokines VEGF, SCF, and BMP-4 and was

allowed to proceed for twelve days . Analysis of day 5 differentiation showed much more robust expression of CD31+/CD73+/CD34+ populations than were ever seen in stromal cell co-culture differentiations (**FIG2 A**). By day 7 we could see roughly 56% CD31+ expression in the whole cell population along with about 39.4% CD31+/CD34+, 10.9% CD31+/CD73+, and 14.5% CD31+/CD146+. By using the spinEB system we had greatly improved our ability to generate cells expressing endothelial markers in half the time it took to differentiate cells using the stromal co-culture method. At this point we also tried a new magnetic sorting method using Miltenyi columns and CD31 magnetic antibodies instead of CD34 antibodies used in co-culture sorts. Considering that nearly all CD34+ cells collected from those previous sorts were also 31+, this alternate method increased post-sort yields by reducing stress on the cells from being out of normal conditions as well as seemingly performing better at retaining cells bound by the magnetic antibodies during the process (**Fig. 2B**).

Inspired by these results, we sought ways to further refine the process and increase yields of endothelial cells that would remain viable for multiple passages post-sort. We noticed that ECs collected from SpinEBs using the standard BPEL differentiation media would only expand for a few passages before a reduction in endothelial markers was observed followed by senescence. Since this media is meant for definitive hematopoietic differentiation, we needed to adjust it to drive differentiation towards the hematoendothelial lineage. At this time we were also running experiments using a defined media containing VEGF, BMP-4, and basic fibroblast growth factor (bFGF), hereafter referred to as VBF, as opposed to the VEGF, BMP-4, SCF (VBS) cytokine composition of the standard media. This VBF media did not contain the same non-cytokine components essential for formation of EBs as VBS. As a result, using it in place of the latter resulted in negligible embryoid bodies which were unsustainable for differentiation. To address this, we tried using VBF cytokines in the standard BPEL media. By replacing SCF with bFGF, we observed roughly similar levels of CD31, CD34, and CD73 expression but a noticeable increase in CD146 expression (**Fig. 2C**). We also began analyzing cells at this point for expression of CD102, also known as intercellular adhesion molecule 2 (ICAM2), which was found to be reliably expressed at high levels on mature endothelial cells. Modifying the cytokines used also showed little effect on increasing long term viability of the sorted population as the early senescence seen using the VBS media was also evident in the VBF media differentiation. In order to obtain the benefits to differentiation of the spin EB system and increase the viability of the sorted ECs, we next tried setting up the cells for differentiation initially in the standard VBS media followed by switching to the VBF media after one day.

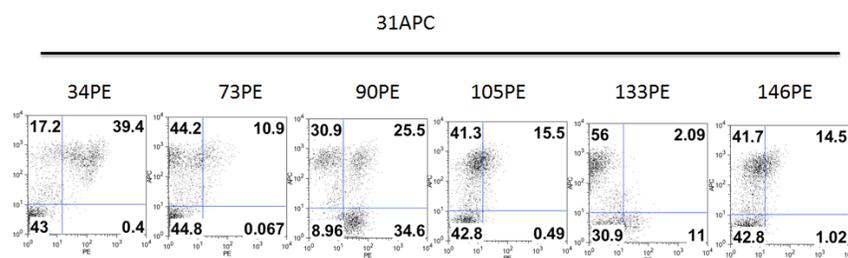
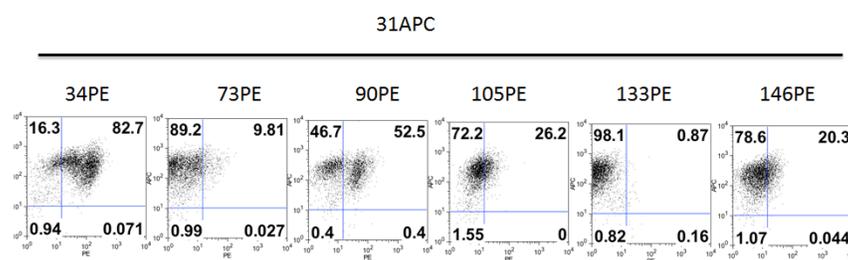
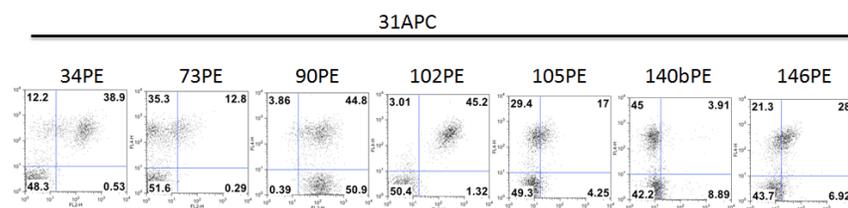
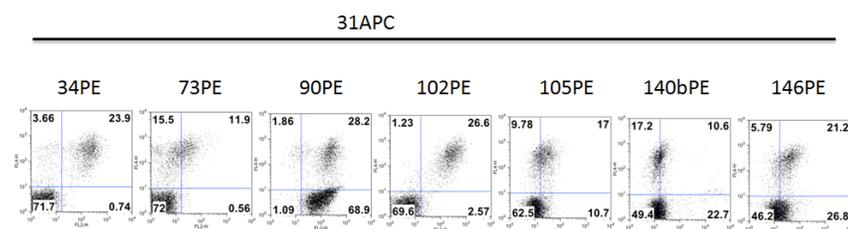
Figure 2**A****B****C****D**

Figure 2. Spin EB differentiation of endothelial cells from hESCs (A) Phenotype of cells at D5 of differentiation using BPEL media. **(B)** Expression of extracellular markers analyzed by flow cytometry following MACS sorting for CD31⁺ cells. **(C)** Pre-sort data for cells in VBF differentiation media instead of BPEL media. **(D)** Pre-sort data for cells differentiated for one day in VBS media and switched to VBF at D1.

It was theorized that allowing the embryoid bodies to develop in the VBS media, which contains the necessary components for EB formation, we could then switch to the VBF media in order to drive differentiation towards a more committed EC lineage. Initially, we found that switching the media at D1 did not increase marker expression to the levels we had anticipated (**Fig. 2D**). Comparing the expression profile of cells differentiated with either BPEL + VBS or BPEL + VBS switched to VBF (VBS-VBF) at day 1, we see that there tends to be higher expression of these double positive cells in BPEL + VBS versus the latter method (**Table 1**). While the increase of EC markers makes it seem that BPEL + VBS is the better of the two, it should be noted that the early hematopoietic marker CD43 is vastly increased in comparison to VBS-VBF as well as rising CD45, a more definitive hematopoietic marker, at later dates. This increase of CD31+/CD43+ and CD31+/CD45+ cells may contribute to the deficiencies of long term viability of cells sorted for CD31. We did find that cells differentiated in VBS-VBF were able to maintain an endothelial phenotype for more passages as well as avoid senescence so we adopted this method for our future differentiation experiments. Following removal of the EBs for sorting, it was noticed that cells with an endothelial morphology that had been expanding outward from the EB (**Fig. 3A**) were left in the well. These cells were fed with standard endothelial growth media (EGM-2MV) and allowed to expand. After a week, these cells showed strong expression of endothelial markers albeit with a small portion of CD31-/CD73+/CD90+/CD102-/CD105+ cells which is indicative of a mesenchymal-like population(**Fig. 3B**). Considering that our mature endothelial cells were grown on fibronectin, we investigated whether differentiating EBs on fibronectin-coated wells would increase the growth of these ECs that emanated from the embryoid body. We found that this actually greatly diminished the capacity for endothelial differentiation (**Fig. 3C**) and interestingly reduced the expression of $\alpha V\beta 3$ integrin, a receptor known to bind fibronectin and promote binding and cellular expansion⁷⁵. Since addition of fibronectin showed a negative effect on EC differentiation, we examined whether we could further increase EC differentiation as well as increase long term viability in our spin EB system by modifying the temporal exposure to specific cytokines. While the three cytokines of VBF differentiation media are all required for mesoderm commitment as well as subsequent hematoendothelial commitment, BMP-4 has been shown to be unnecessary beyond this second stage as it does not contribute to further development along these lineages⁷⁶. Considering the success of VBF media in generating cells that were phenotypically hemangioblasts, a precursor capable of both endothelial and hematopoietic lineage commitment, we examined whether removal of BMP-4 prior to sorting at day 7 would increase endothelial commitment at the expense of hematopoietic commitment. By switching the VBF media to media without BMP-4

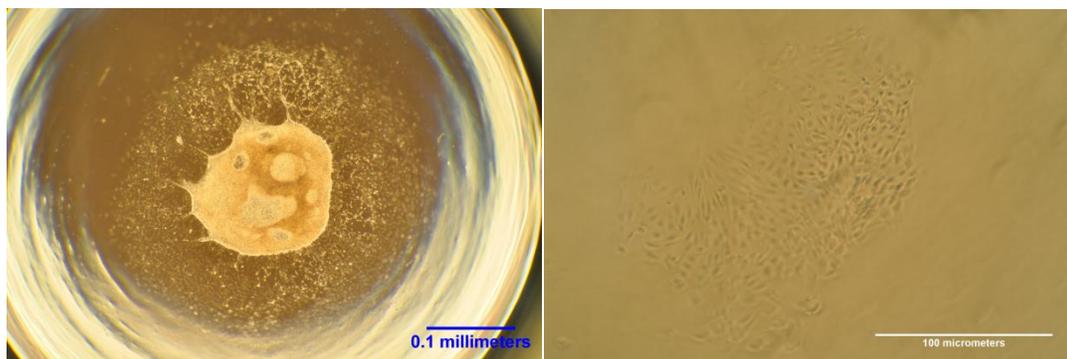
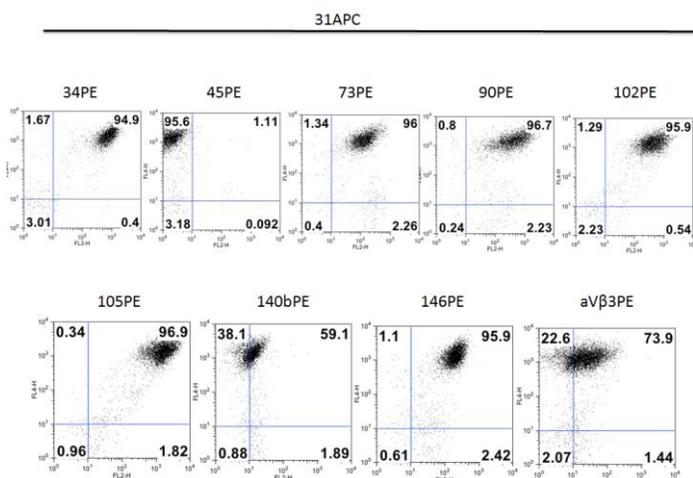
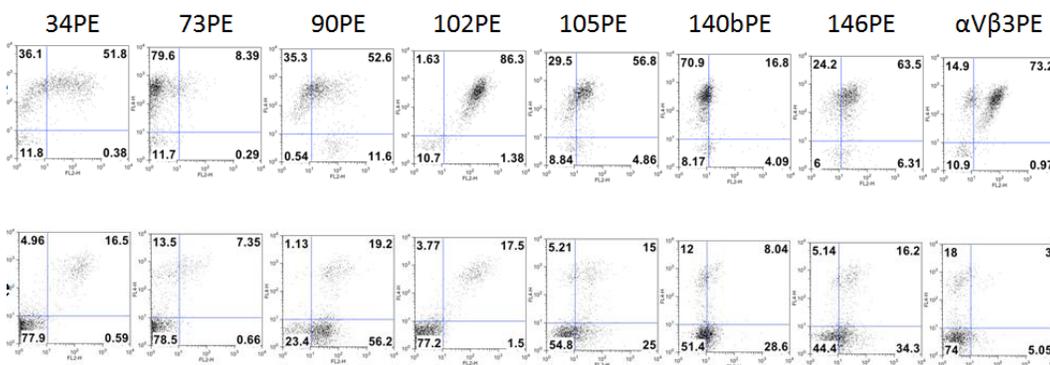
Figure 3**A****B****C**

Figure 3. Endothelial cells expand outward from EBs but do not differentiate well on fibronectin. (A) Spin EB at D7 of differentiation in VBS-VBF media. The EB is at the center with an outgrowth of EC-like cells surrounding it (*left*). Adherent cells that remained after mechanical removal of the EB for sorting following one week of being fed with EGM-2MV (*right*). **(B)** Flow cytometry analysis of the adherent cells shown above. **(C)** Flow cytometry analysis at D7 of Spin EB differentiation both without (*top*) and with (*bottom*) fibronectin coating on the wells.

Spin EB Defined Media Differentiation

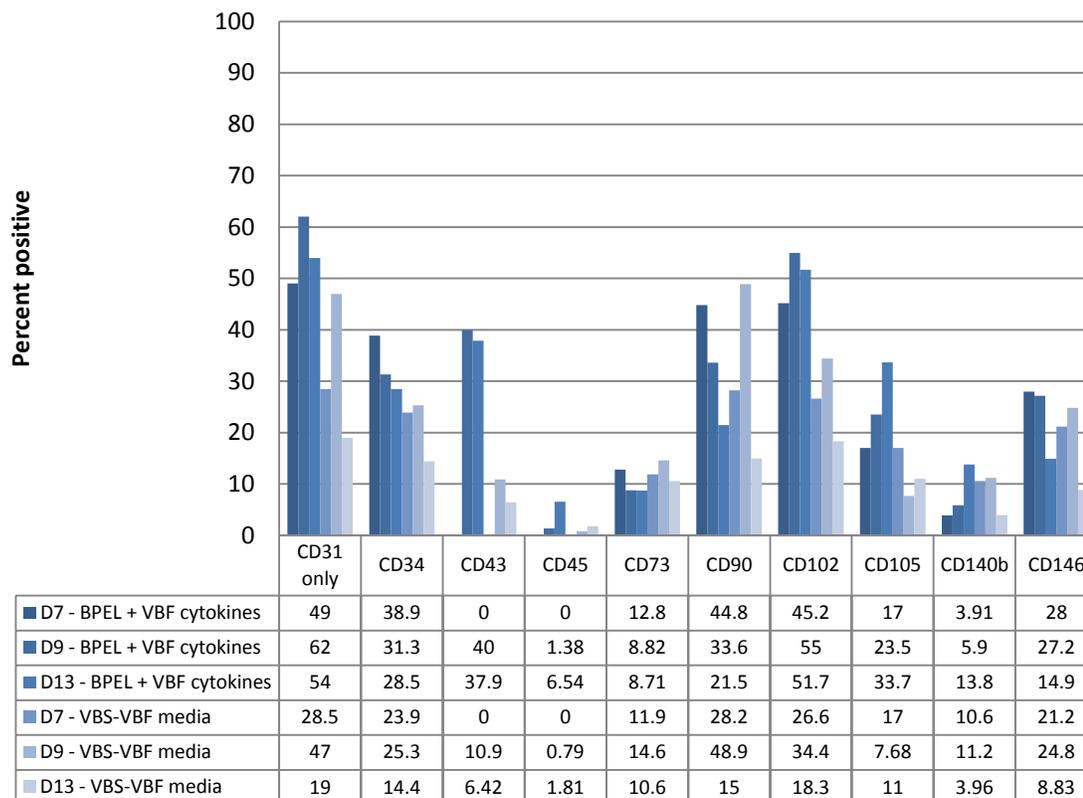


Table 1. Comparison of endothelial and hematopoietic marker expression between BPEL + VBF and VBS-VBF media. Analysis based on flow cytometry of hematopoietic and endothelial surface markers following differentiation in BPEL + VBF cytokines or VBS-VBF differentiation media. Cells were analyzed at days 7, 9, and 13. Each set of data except "CD31 only" represents the percentage of cells double positive for CD31 and the specific marker indicated.

(VF) at day 6, we saw an even further increase in endothelial marker expression the following day with roughly 72% of the pre-sort cells being CD31+ (**Fig. 4A**). We also saw large increases in double positive populations for CD31 and other markers except for a minimal increase in CD31+/CD73+ cells. Also promising was the efficiency of the CD31+ sorting as less than 0.1% of the post-sort analyzed cells were CD31-. After a week in endothelial growth media, we saw that there was still nearly 100% CD31+ cells and that the double positive populations had increased with the exception of CD31+/CD146+. The CD31+/CD73+ fraction had greatly increased from 12.4% to 82.8% over the course of 7 days. These cells were analyzed and passed each week when they reached confluency and continued to show robust EC marker expression for more than 6 weeks as well as no evidence of expansion of the minimal CD31- population that was present (**Fig. 4B**). We noticed that CD90 (also known as thy1), which is only present on activated endothelial cells⁷⁷, was present at high levels at early time points and slowly diminished to near undetectable levels at later analyses. The reasons for this are unknown at this point but may be evidence for inflammatory cytokine release in the post-sort cell population.

Characterization of differentiated endothelial cells

In order to further characterize the endothelial cells that we were able to isolate and maintain in long term culture, we used immunocytochemistry (ICC) to evaluate the expression of both extracellular and intracellular proteins (**Fig. 4C**). While the expression of CD31 and CD144 that was shown in flow cytometry analysis was confirmed, we also saw expression of the endothelial-specific intracellular proteins Von Willebrand factor (VWF) and endothelial nitric oxide synthase (eNOS). Von Willebrand factor is a critical component of the clotting process following injury to vascularized tissue and eNOS, as its name implies, generates nitric oxide which is required for vasodilation and reduce constriction of vessels. The presence of both of these components is strong evidence for the development of mature endothelial cells in our culture based on comparison to HUVEC protein expression. In order to analyze the functional capacity of these cells we performed a tube forming assay by plating the cells on matrigel-coated plates. Mature endothelial cells grown on matrigel will form rudimentary two-dimensional tube-like structures within a few hours, a process that resembles *in vivo* vessel formation. We observed that the ESC-derived ECs showed similar tube structures compared to HUVECs with multiple branch points from the "hubs" and extensive networks being formed (**Fig. 4D**). Based on the endothelial protein expression and functional similarity to primary cells in our ESC-derived ECs, we determined that our differentiation protocol is able to generate mature endothelial cells reliably and effectively.

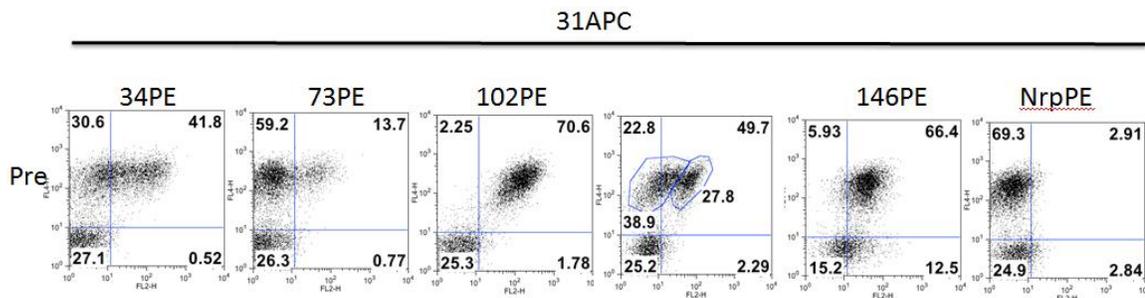
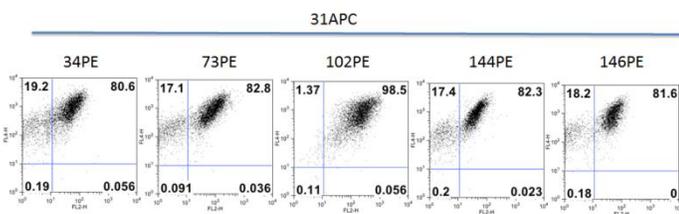
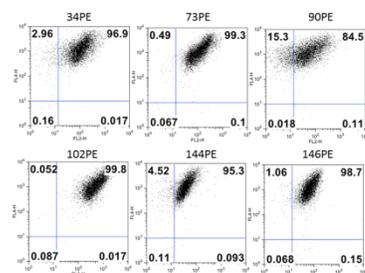
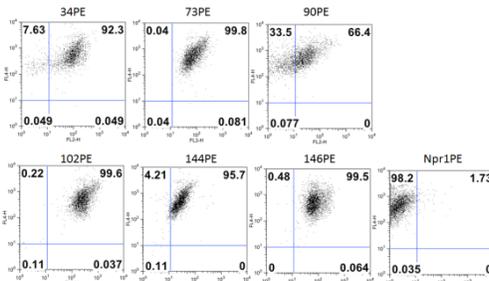
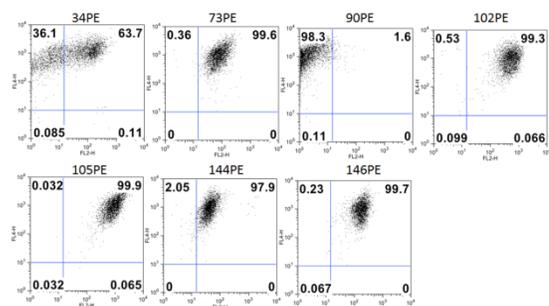
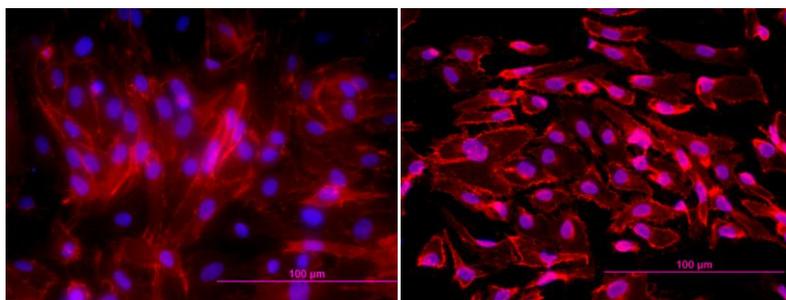
Figure 4**A****B****Day 7 post-sort****Day 14 post-sort****Day 21 post-sort****Day 40 post-sort**

Figure 4. hESC-derived ECs express endothelial markers and are functionally similar. (A) Surface marker expression of differentiating EBs at D7 after switching from VBS-VBF to media without BMP-4 at D6. (B) Long term analysis of CD31⁺ cells in standard endothelial culture. The cells were passage 6 at the D40 time point.

C

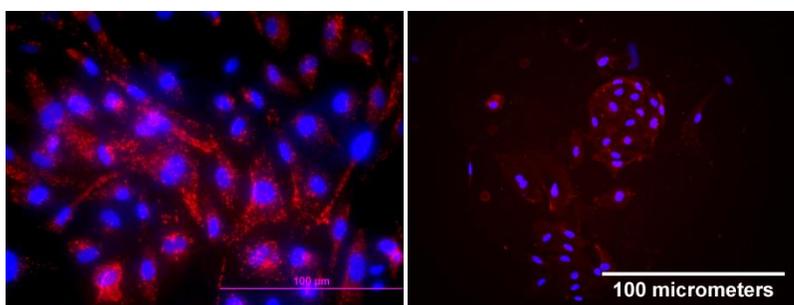
CD31

CD144



Von Willebrand Factor

eNOS

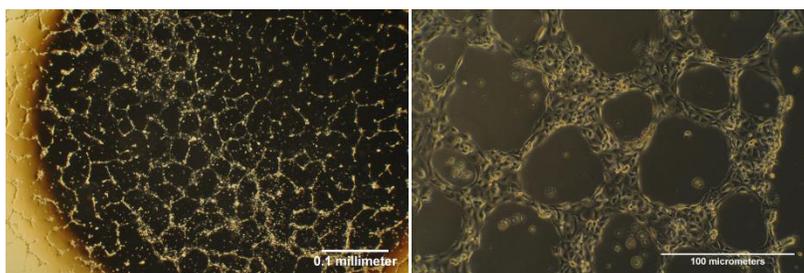
**Figure 4. (C)**

Immunocytochemistry images of ES-derived ECs for typical EC markers show positive expression of both intracellular and extracellular proteins CD31, CD144, VWF, and eNOS. **(D)** Matrigel tube formation assays with HUVECs (*top*) and H9 ECs (*bottom*). Images were taken after 18 hours of culture.

D

HUVEC – 40x

HUVEC – 100x



H9 EC – 40x

H9 EC – 100x



Smooth muscle cell differentiation from sorted endothelial cells

While endothelial cells are the primary component of blood vessels, they are also complemented by perivascular smooth muscle cells and pericytes which provide stability and modulate vessel dilation. Previous studies, as well as experiments in our lab, have shown that vascular progenitors are capable of differentiating into smooth muscle cells in the presence of the cytokines PDGF-BB and TGF- β ^{78,79}. By switching the endothelial growth media to media containing these cytokines relatively shortly after sorting, a small portion of these cells would further differentiate into SMCs. The efficiency of this process was seemingly dependent on how many of the sorted cells were committed to the endothelial lineage as the majority of cells became non-adherent following introduction of the SMC differentiation media. Cells that remained adherent assumed a SMC morphology including an increase in size, loss of cell to cell contact, and development of intracellular filaments (**Fig. 5A**). We used ICC to examine the expression of SMC specific proteins smooth muscle actin, SM22- α , and calponin (**Fig. 5B**). We also saw that these cells lost the ability to form tubes on matrigel and instead formed large clumps instead of tubes (**Fig. 5C**). However, by co-culturing ECs with SMCs on matrigel we saw that not only was the ability to form tubes recovered, but also that the tubes were thicker and more robust which mimic the ability of these cells to create thicker vessels *in vivo*. By using ECs derived from a GFP-expressing ESC line as well as SMCs from a mCherry-expressing ESC line, we could see that both cell types were evenly distributed throughout these tubes and contributed equally to their formation (**Fig. 5D**). This process began within an hour after introduction of the cells to the matrigel and tube formation was mostly complete by 3 hours (**Fig. 5E**). Combined with our characterization by flow cytometry and ICC, this assay showed we had derived functional ECs and SMCs from ES cells.

Apelin enhances endothelial differentiation

In an additional effort to further optimize our differentiation protocol for developing vascular progenitors and subsequent EC and SMC generation, we looked at the use of known enhancers of vascular development. We analyzed the effect of introducing APLN to our VBF media at days 1 and 3 of EB differentiation in both ES and iPS cells. We saw that at day 4 there was little difference in CD31+ populations as well as double positive CD31+/CD34+, CD31+/CD73+, and CD31+/CD102+ populations (**Fig. 6A**). By day 6 however, we saw that APLN added at D3 caused a noticeable increase in the total CD31+ cells (**Fig. 6B**). Additionally, the CD31+/CD102+ population showed a similar increase while the CD31+/CD34+ increase was

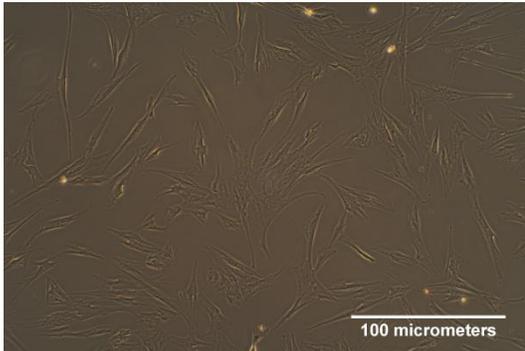
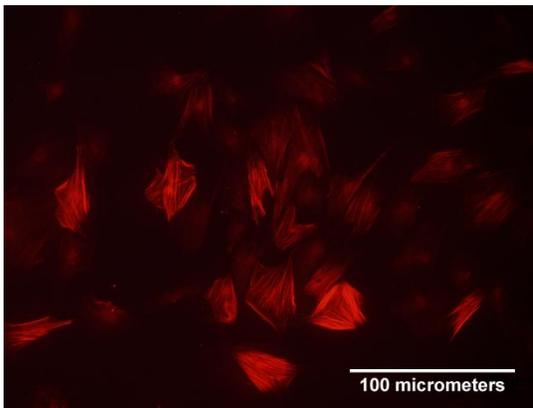
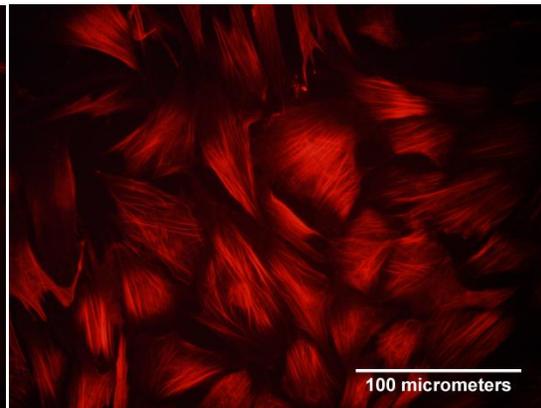
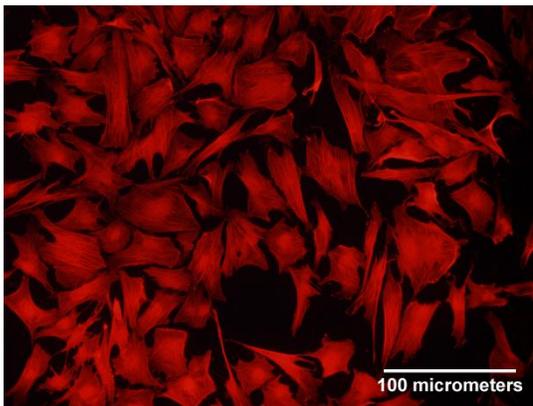
Figure 5**A****B** **α -SMA – 40x****Calponin – 40x****SM22 – 40x**

Figure 5. Smooth muscle cells from hESCs express SMC-specific markers and can form tubes with ECs *in vitro*. (A) Phase image of cells with a smooth muscle cell phenotype following differentiation from CD31⁺ sorted cells. (B) Immunocytochemistry images for H9-derived SMCs showing α -SMA, calponin, and SM22 expression.

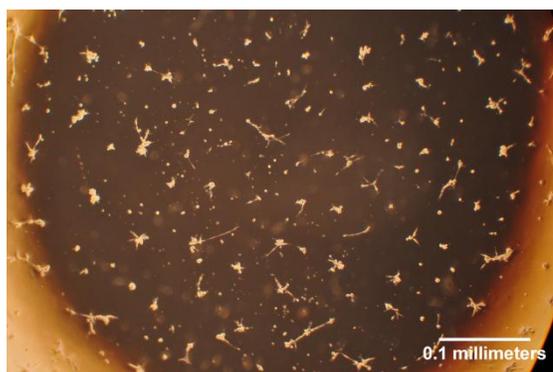
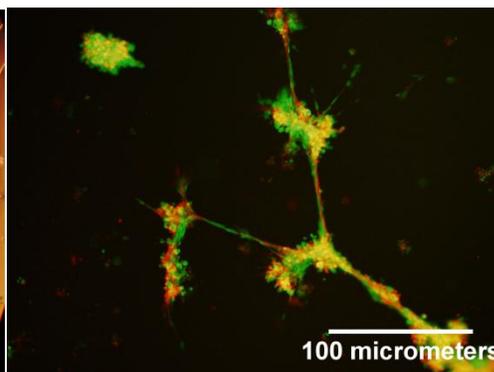
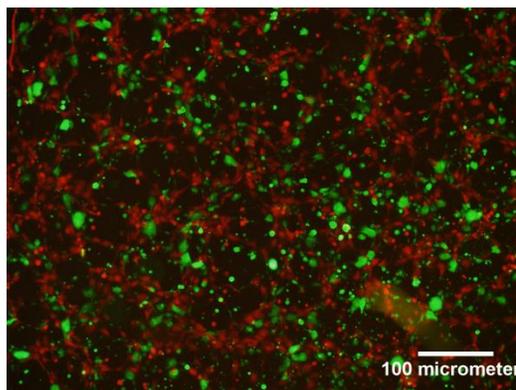
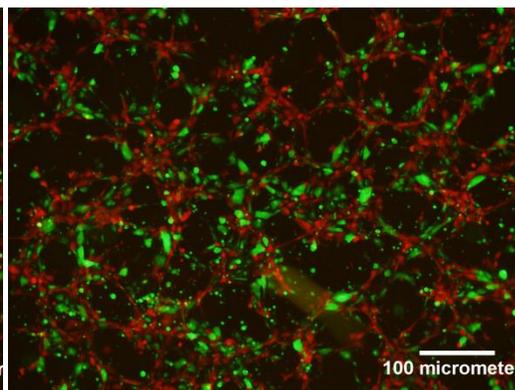
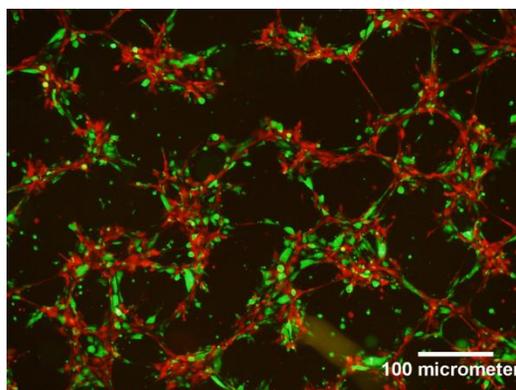
C**D****E****1 Hour****2 Hours****3 Hours**

Figure 5. (C) Image taken following 18 hours of ESC-derived SMCs in a matrigel tube formation assay. (D) Co-culture of GFP-ECs and mCherry-SMCs show equal contribution to the tubes that form. (E) Time lapse assay of GFP-ECs and mCherry-SMCs. Migration of the green ECs can be seen to contribute to the tube formation which is robust by 3 hours in culture. Images are not from the same area at each time point.

less pronounced and CD31+/CD73+ populations were diminished when APLN was added at both days 1 and 3 compared to the control group. By day 8 we saw much higher increases in CD31+ cells in both D1 and D3 APLN treatments as well as CD31+/CD102+ cells. Consistent with D4 and D6 analyses, the CD31+/CD34+ populations were similar among both APLN treatments and the control and CD31+/CD73+ development was diminished in the presence of APLN compared to the control (**Fig. 6C**). Subjecting umbilical cord blood-derived iPS cells to the same treatment resulted in similar expression patterns overall but with slightly diminished percentages across the board which may be due to the reduced capability of those iPS cells to undergo hematovascular differentiation (**Fig. 6D**). These moderate increases in surface markers did not necessarily translate to improved endothelial long-term viability as sorted cells from these differentiation conditions did not retain an EC phenotype any better after multiple passages than without APLN added.

DAPT enhances expression of endothelial markers

Another method of encouraging vascular development we investigated was to utilize DAPT, a small molecule inhibitor of notch signaling, in VBF media at various time points during differentiation. Recent studies have shown that inhibition of notch signaling after commitment to the hemangioblast lineage can encourage endothelial differentiation at the expense of hematopoietic commitment¹⁹. We expanded upon our treatment schedule compared to APLN by adding DAPT at days 1, 4, 7, and 12 in an effort to find the optimal time point at which the most cells would be considered committed to the hemangioblast lineage. We found that the most significant increases in EC markers was found on day 7 of analysis when DAPT was added at day 4 of differentiation (**Fig. 6E**). Experiments involving this treatment are ongoing so we have not been able to characterize the ability of DAPT-treated EC differentiation for long term viability, but these early results are encouraging.

Characterization of arterial, venous, and lymphatic endothelial cell phenotypes

Once we established a reliable protocol for development of phenotypically and functionally similar ECs from pluripotent cell sources, we next sought to compare these cells to primary arterial, venous, and lymphatic endothelial cells in order to determine whether our derived ECs possessed characteristics of any of these lineages. When comparing the primary cells of these lineages, extracellular markers are not especially useful as all three EC types share similar expression patterns of most markers. However, we did find that some specific markers have slight variations between the three lineages (**Fig. 7A**). Apelin receptor (APLNR), the

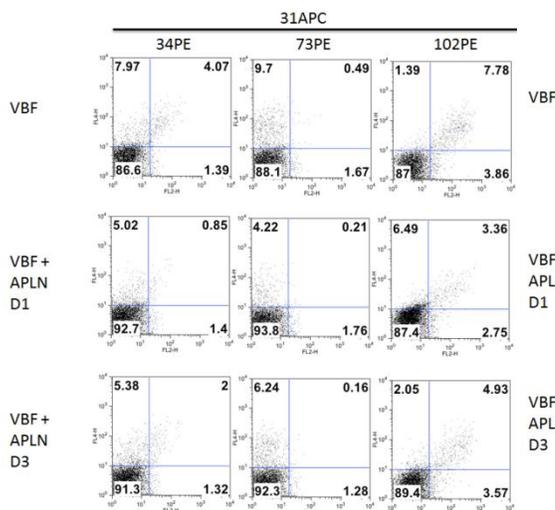
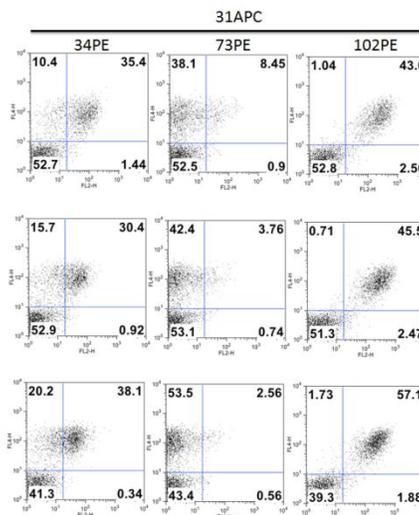
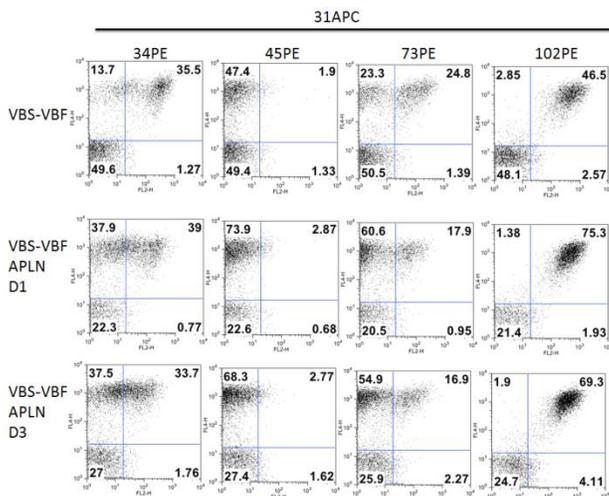
Figure 6**A****B****C**

Figure 6. Apelin and DAPT promote higher expression of hemato-endothelial markers. (A) Day 4 phenotype analysis of cells differentiated in either VBF media alone, VBF+APLN at D1, or VBF+APLN at D3. **(B)** Day 6 phenotype analysis of the same cells and **(C)** D8 analysis. APLN greatly increased the expression of CD31 and CD102 while decreasing CD73.

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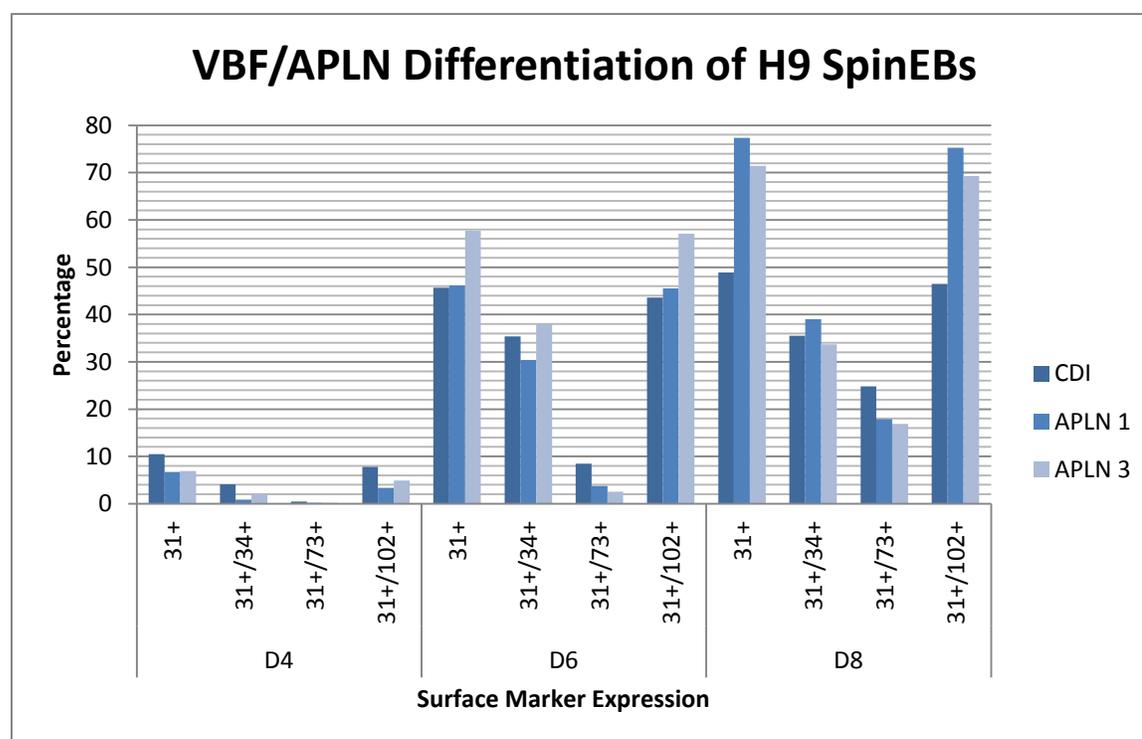
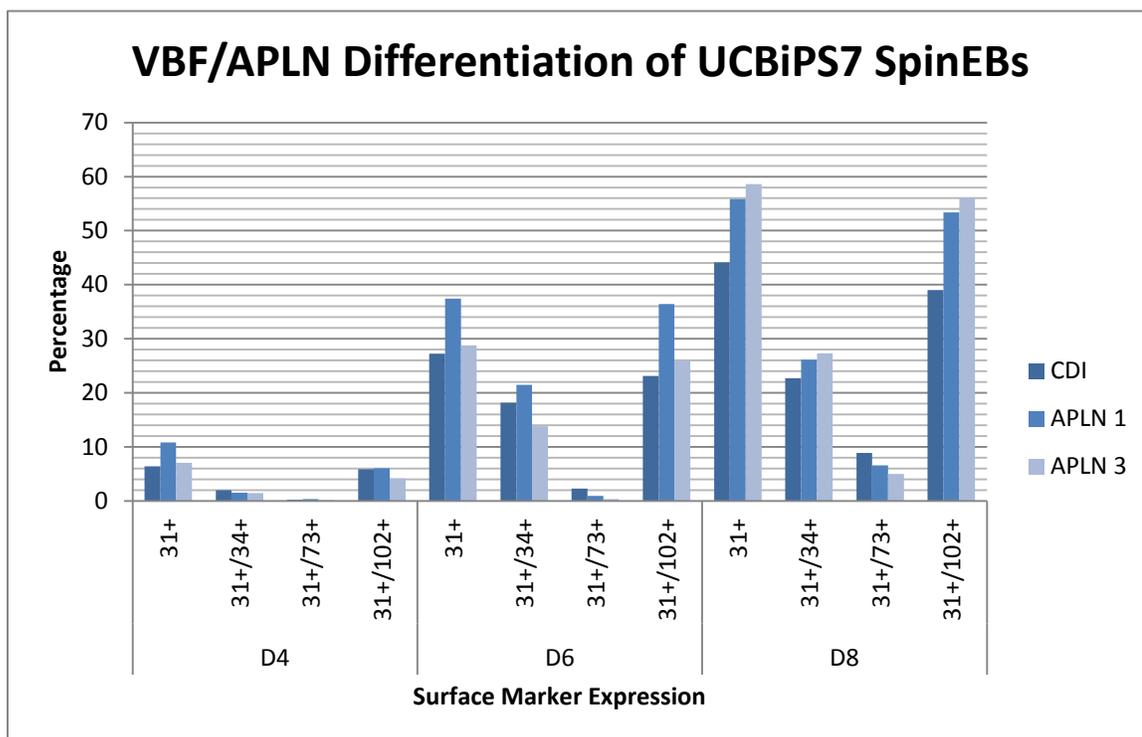


Figure 6. (D) Expression patterns of differentiating UCBiPS7 cells at days 4, 6, and 8 (*top*). Each color represents either standard VBF media, VBF with APLN added at D1, or VBF with APLN added at D3 of differentiation. Data represents percentage of total cells positive for the indicated markers analyzed with flow cytometry. The bottom chart shows the same analysis done during differentiation of H9 ESCs.

E

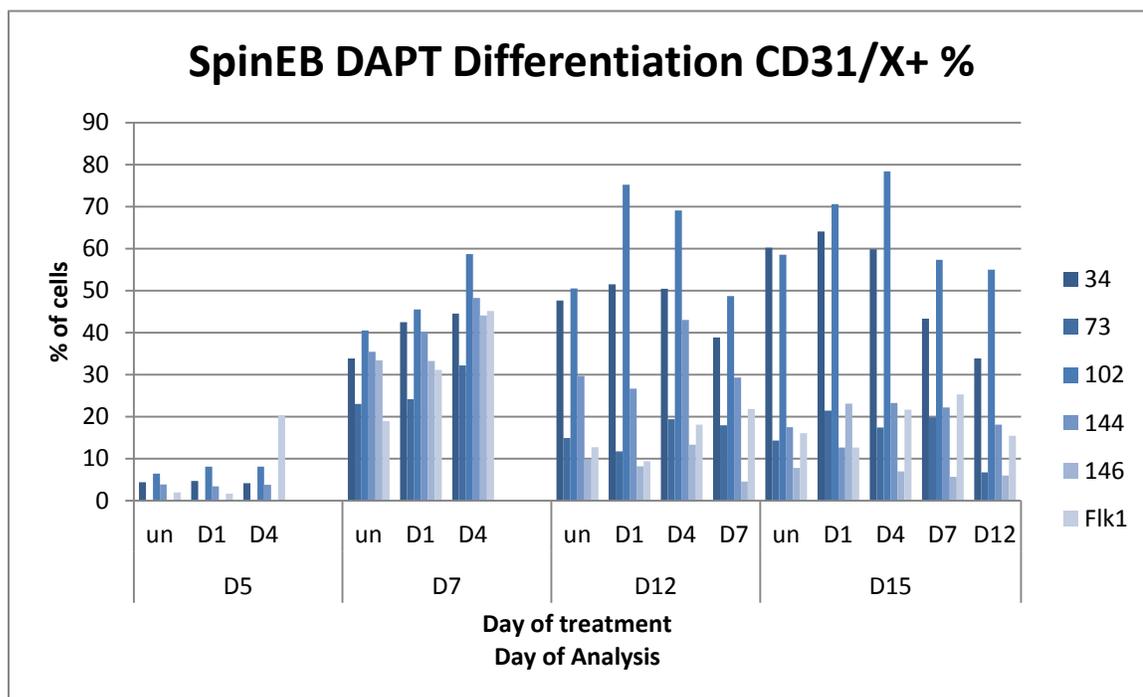


Figure 6. (E) Expression patterns of cells double positive for CD31 and other endothelial markers in VBF differentiation with DAPT added. The top categories on the x-axis describe media with DAPT added at days 1, 4, 7, 12, or with no DAPT added (un). The bottom categories show the day of differentiation on which the cells were analyzed. Each color represents the extracellular marker being analyzed for co-expression with CD31.

receptor for the aforementioned apelin protein, was found to be expressed slightly higher on HUVECs than on HUAECs or HLECs. Neuropilin-1 (NRP1) also had a slightly higher expression on venous cells than lymphatic or arterial cells, but expression was reasonably high for all three lineages. We saw a more significant difference in the VEGF receptor 3 (VEGFR3) expression with both HUVECs and HUAECs less than that in HLECs. While VEGFR2 is expressed at consistent levels across all three lineages, VEGFR3 is more specific to lymphatic ECs which was confirmed by our analysis⁸⁰. Considering the minimal differences in extracellular protein expression, we next used quantitative rt-PCR to examine the differences in gene expression profiles. We identified upstream and downstream effectors of the notch signaling pathway (*JAG1*, *JAG2*, *DLL4*, *HEY2* and *HES1*) as identifiers of an arterial endothelial phenotype. Venous and lymphatic phenotypes were both identified with increased *COUP-TFII* expression while lymphatic ECs were further characterized by increased *PROX1* expression. We indeed found that *JAG1*, *JAG2*, and *DLL4*, ligands for notch receptors, were upregulated in primary arterial cells compared to both venous and lymphatic ECs (**Fig. 7B**). *NOTCH4* gene expression was also elevated in HUAECs compared to HUVECs and HLECs but *NOTCH1* was found to be more highly expressed in HLECs. *Hes1* and *Hey2*, which are downstream products of notch-mediated transcriptional activation, were also found to be upregulated in arterial cells compared to the other two with *Hey2* being significantly expressed (**Fig. 7C**). *COUP-TFII* was found to be more highly expressed in both HUVECs and HLECs as we had expected and *PROX1* showed significant expression in HLECs compared to HUVECs and HUAECs. Knowing that our primary cells showed the characteristic gene expression profiles of their lineage as well as that our primers were effective for qrt-PCR, we next compared H9-derived ECs to the three lineages. *DLL4* was expressed more highly in all three primary cells compared to the H9 ECs while *JAG1* and *JAG2* were upregulated compared to HUVECs and HLECs but not as much as HUAECs (**Fig. 7D**). Interestingly, H9 ECs showed greater expression of *COUP-TFII* than all three primary cell lines but showed about equal *PROX1* expression compared to HUAECs which is much lower than HLEC *PROX1* expression.

PI3K inhibition along with cAMP increases CXCR4 expression

In order to try and manipulate our pluripotent cell-derived ECs to adopt a more arterial phenotype, we examined using small molecule inhibitors and messengers of various signaling pathways in an effort to influence gene expression profiles. Specifically, we analyzed the expression of CXCR4, which is more highly expressed on arterial ECs as compared to venous and lymphatic ECs, following inhibition of PI3K in conjunction with addition of cAMP.

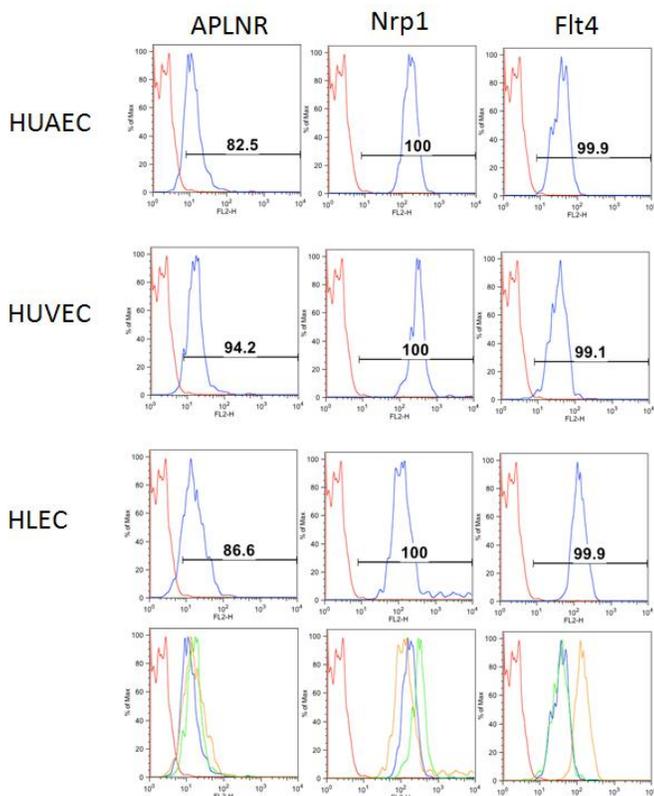
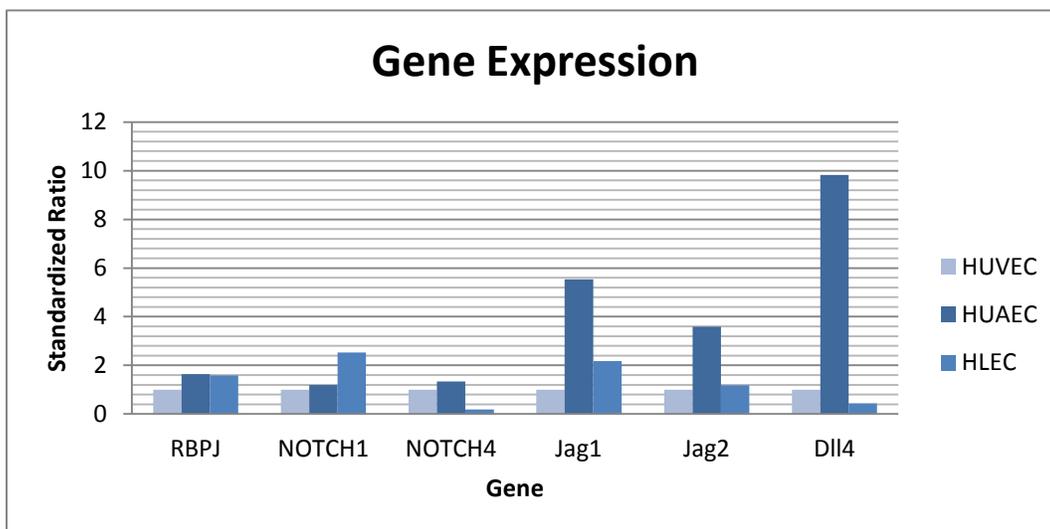
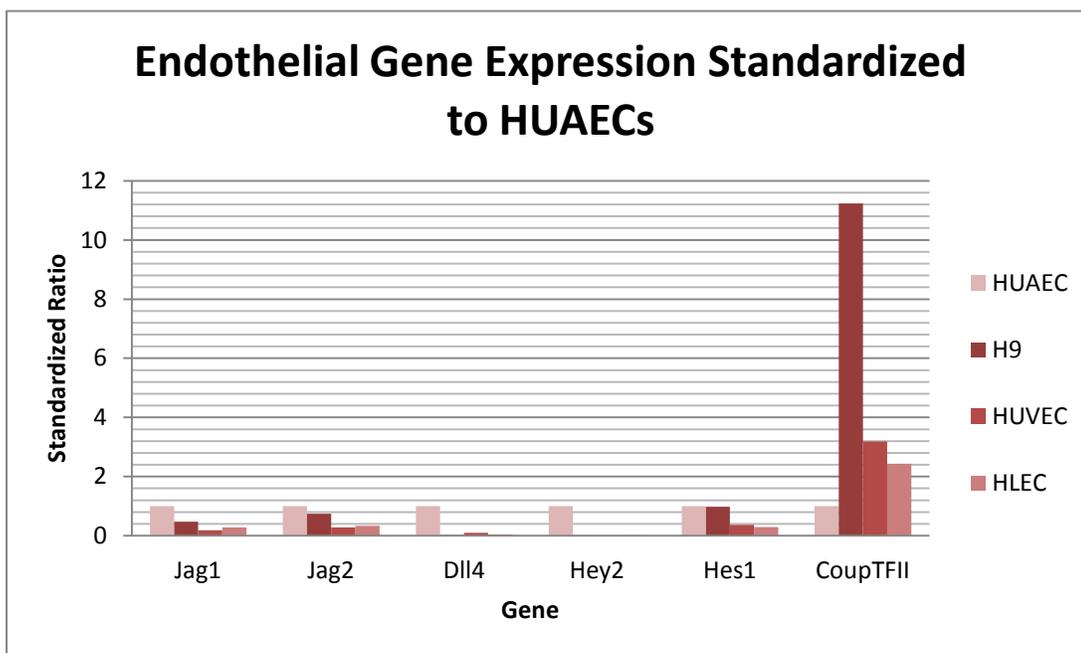
Figure 7**A****B**

Figure 7. Comparison of expression of lineage-specific genes between H9-ECs, HUAECs, HUVECs, and HLECs. (A) Flow cytometry analysis of expression of APLNR, Neuropilin-1, and Flt-4 in primary arterial, venous, and lymphatic endothelial cells. Percentages are the number of cells bound by the PE-conjugated antibodies of each protein compared to IgG controls. **(B)** qrt-PCR analysis of gene expression in primary ECs. Ratios are standardized to HUVECs for HUAECs and HLECs using the $\Delta\Delta C_t$ method of analysis.

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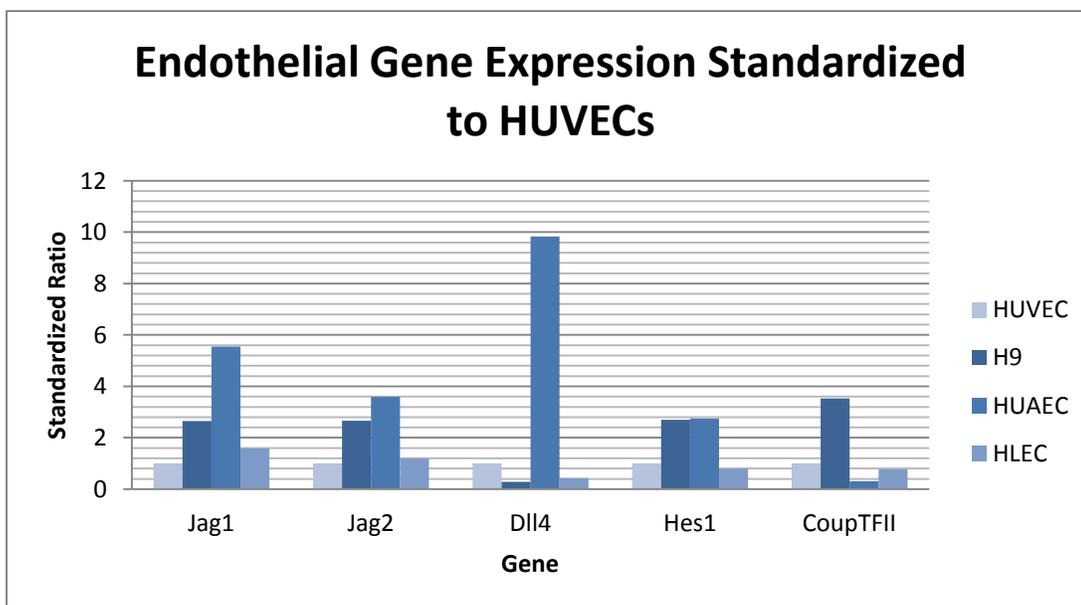


Figure 7. (C) qrt-PCR gene analysis of HUVEC, HLEC, and H9-ECs standardized to HUAEC gene expression. The ratio of DLL4 and Hey2 expression in these three cell types compared to HUAECs is less than 0.1 for DLL4 and 0.0005 for Hey2. (D) Similar analysis with HUAEC, HLEC, and H9-EC standardized to HUVECs. The HUAEC ratio of Hey2 expression compared to HUVECs was over 8600 fold (not shown).

Treatment of HUAECs with LY294002, an inhibitor of PI3K, showed a large increase of *CXCR4* surface expression as measured by flow cytometry in a dose-dependent manner (**Fig. 8A**). This increase was further amplified by the addition of 0.5mM cAMP both with and without 1 μ M or 5 μ M LY294002. Similar increases were seen in HUVECs treated under the same conditions, but with a lesser overall percentage of increase as compared to HUAECs considering the normal expression of the protein. Measurement of *Cxcr4* gene expression via qRT-PCR showed similar increases in both cell types while venous and lymphatic-specific genes, *Coup-tfII* and *Prox1* respectively, remained unchanged (**Fig. 8B**). Applying these treatments to ESC-derived ECs resulted in similar increases of *CXCR4* measured by flow cytometry (**Fig. 8C**). Normal *CXCR4* expression in the untreated cells appeared to be less than HUAECs but more than HUVECs. Gene analysis also showed an increase in *Cxcr4* with the fold change and pattern being similar to that which was seen in HUAECs (**Fig. 8D**). These assays were performed for 24 hours, so we next looked at the expression of *CXCR4* in ESC-derived ECs following treatment for 72 hours (**Fig. 8E**). Treatment with 5 μ M LY294002 and 0.5mM cAMP for 24 hours followed by standard media for the next 48 hours showed a return to relatively normal protein levels by the end of the assay. Addition of LY294002/cAMP every 24 hours showed an increase over the first 48 hours followed by a drop in the final day which may be a result of the toxicity of inhibiting PI3K activity for that period of time. LY294002 has been shown to be slightly promiscuous in its binding targets and does not exclusively interact with members of the PI3K family⁸¹. We tested the effects of PI-103, a more effective inhibitor of PI3K which also inhibits the mTOR signaling pathway⁸². We saw tremendous increases in both extracellular protein expression as well as gene expression in ESC-derived ECs following treatment with the same concentration as LY294002 as well as cAMP (**Fig. 8F**).

While inhibition of PI3K along with cAMP increased *CXCR4* gene and surface protein expression, we next wanted to analyze other arterial-specific genes to see if there was a similar effect. The gene expression profiles of *Notch1*, *Dll1*, and *Jag1* were analyzed given the importance of the notch signaling pathway in arterial cells. Treatment of HUAECs with LY294002 showed increased transcription in all three genes at 1 μ M but only *Notch1* at 5 μ M (**Fig. 9A**). Treatment with cAMP only showed the greatest increase in *Notch1* across all conditions but increases similar to 1 μ M LY294002 treatment for *Dll1* and *Jag1*. Interestingly, the combination of both concentrations of LY294002 with cAMP showed decreases in expression of all three genes. When HUVECs were subjected to these conditions, we found similar increases in *Notch1* transcription albeit not as drastically as HUAECs (**Fig. 9B**). Also, treatment with 1 μ M

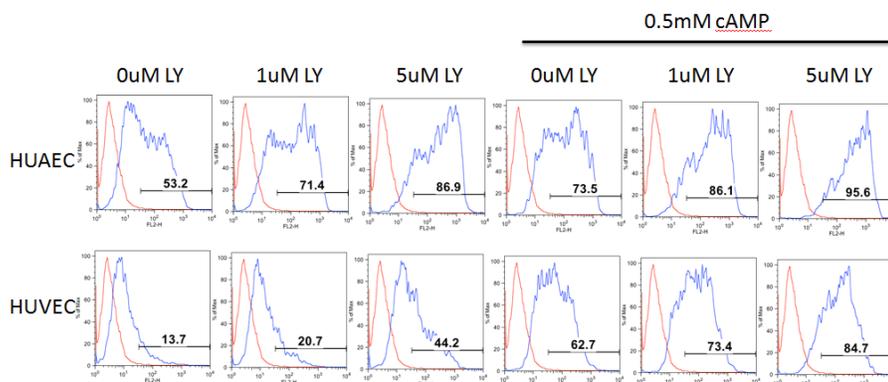
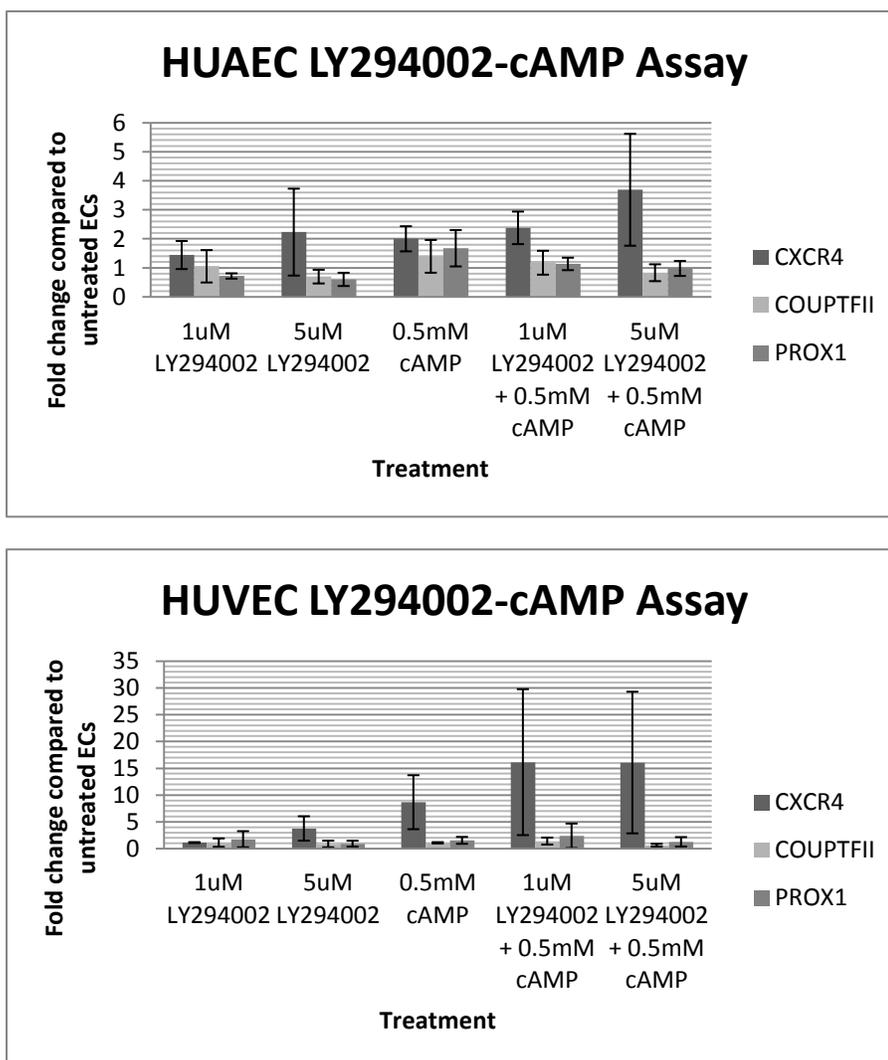
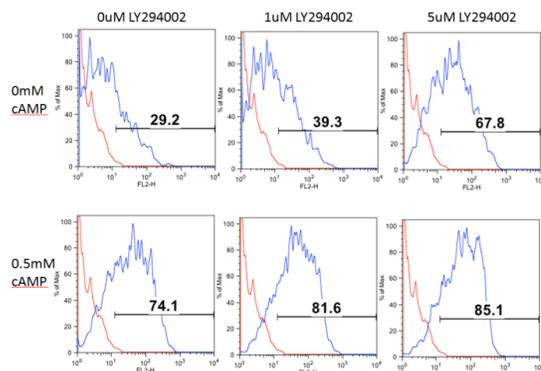
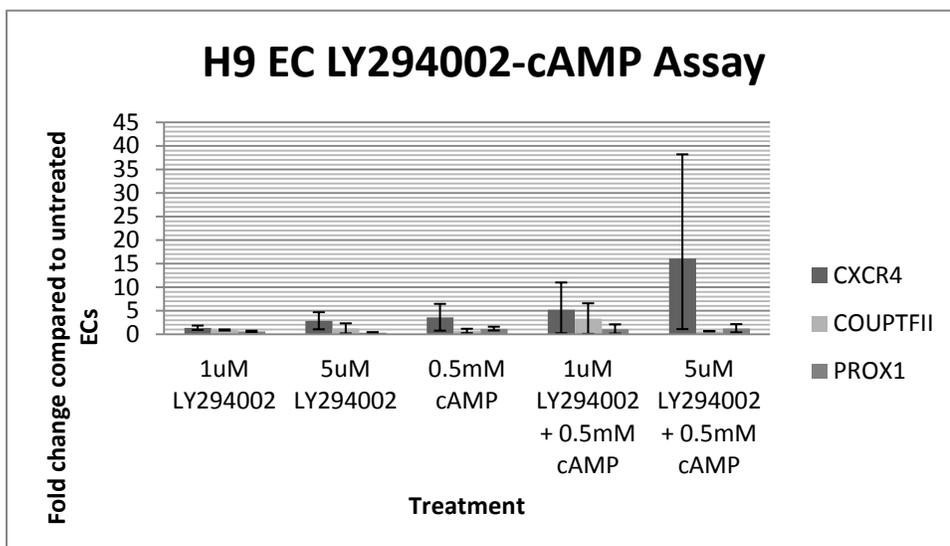
Figure 8**A****B**

Figure 8. PI3K inhibitors and cAMP increase CXCR4 expression after 24 hours. (A) Analysis of CXCR4 expression following treatment for 24 hours with 0 μ M, 1 μ M, or 5 μ M LY294002 as well as with the addition of 0.5mM 8-Br-cAMP to each. Percentages are cells positive compared to streptavidin-PE only controls. (B) qrt-PCR analysis of HUAECs and HUVECs following treatment LY294002 and/or 8-Br-cAMP. Each set of data represents three separate cell replicates. Fold change was calculated compared to untreated cells.

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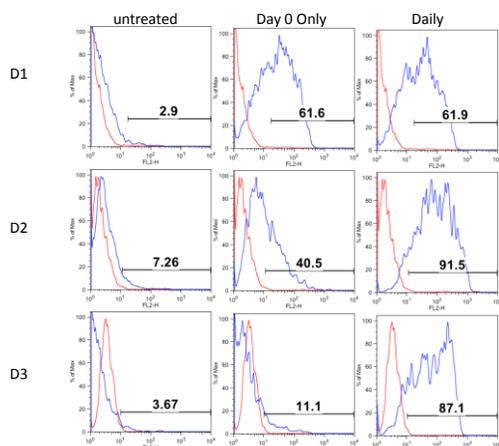


Figure 8. (C) Analysis of H9-ECs following treatment with LY294002 and/or 8-Br-cAMP for 24 hours as described in Fig. 8A. (D) Gene expression analysis using qrt-PCR of H9-ECs. (E) Longer term analysis of H9-EC CXCR4 expression treated with 5 μ M LY294002 at day 0, daily for three days, or untreated. Cells were analyzed at daily for three days. Percentages represent the comparison to streptavidin-PE only controls.

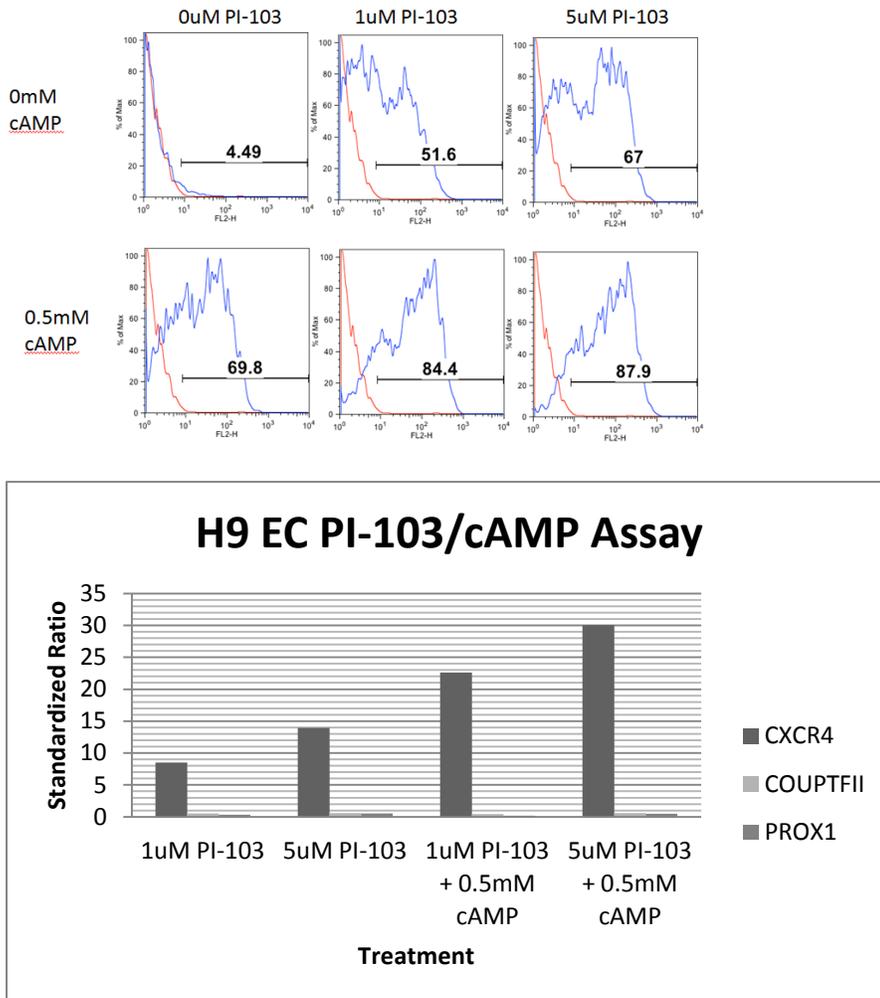
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Figure 8. (F) Flow cytometry (*top*) and qrt-PCR (*bottom*) analysis of H9-ECs treated with 0 μ M, 1 μ M, or 5 μ M PI-103 alone or with 0.5mM 8-Br-cAMP. Flow cytometry data shows comparison of CXCR4 expression on treated cells compared to untreated controls. Cells were analyzed 24 hours after treatment.

LY294002/cAMP did not produce the same reduction in transcription as seen with HUAECs. ESC-derived ECs showed a similar dose-dependent response to LY294002 in regards to *Notch1* but slight decreases in transcription of *Dll1* and *Jag1* (**Fig. 9C**). The combination of 5 μ M LY294002 and 0.5mM cAMP also tended to decrease *Notch1* expression compared to each individual treatment while 1 μ M LY294002/cAMP still showed more robust expression, similar to HUVECs.

Cyclic AMP can counteract alterations to gene expression caused by NOTCH inhibition

We next sought to establish the effects on CXCR4 expression as well as arterial gene transcription in the presence of DAPT. Given the ability of this compound to inhibit notch signaling, we theorized that this would diminish the expression of key arterial components that were being analyzed. HUAECs and H9-derived ECs showed similar decreases in *CXCR4* surface expression compared to untreated controls whereas HUVECs actually showed a slight increase that was dose-dependent (**Fig. 10A**). Treatment with cAMP alone showed the expected increase in expression but concurrent treatment with DAPT again showed dose-dependent decreases in HUAECs and H9-derived ECs alongside increases in HUVECs. Gene expression data shows that HUAEC *Cxcr4* transcription is indeed slightly diminished in the presence of DAPT without cAMP while DAPT and cAMP treatments show increased transcription with dose-dependent decreases (**Fig. 10B**). H9-EC gene expression data shows a similar trend albeit with a lower increase in magnitude (**Fig. 10C**). HUVEC results show slight increases in the DAPT only treatments, but show dose-dependent decreases to *Cxcr4* transcription that are similar to HUAEC and H9-EC which does not follow the trend seen in the flow cytometry data (**Fig. 10D**). Interestingly, HUAECs treated with 1 μ M and 5 μ M DAPT resulted in significant increases in *Notch1* expression along with less pronounced increases in *Dll1* and *Jag1* (**Fig. 10E**). Even further increases in expression were seen in the 1 μ M DAPT/cAMP samples but the 5 μ M DAPT/cAMP showed a significant decrease in *Notch1* transcription although the levels were still above the untreated control. HUVECs showed a similar trend of increased transcription of *Notch1* in the presence of DAPT only with diminished expression when cAMP was included (**Fig. 10F**). H9-ECs did not show increased transcription of *Notch1* when treated with 5 μ M DAPT which we saw in HUVECs and HUAECs and also did not respond similarly in the presence of cAMP (**Fig. 10E**).

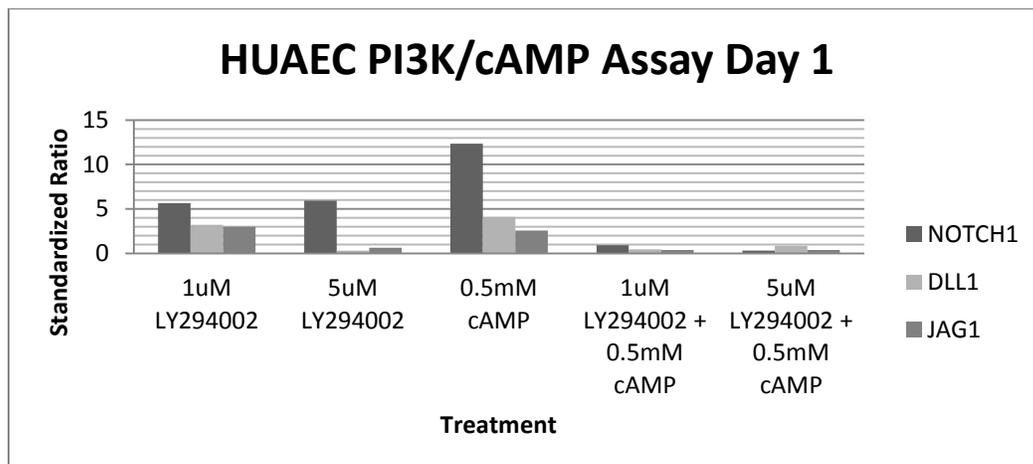
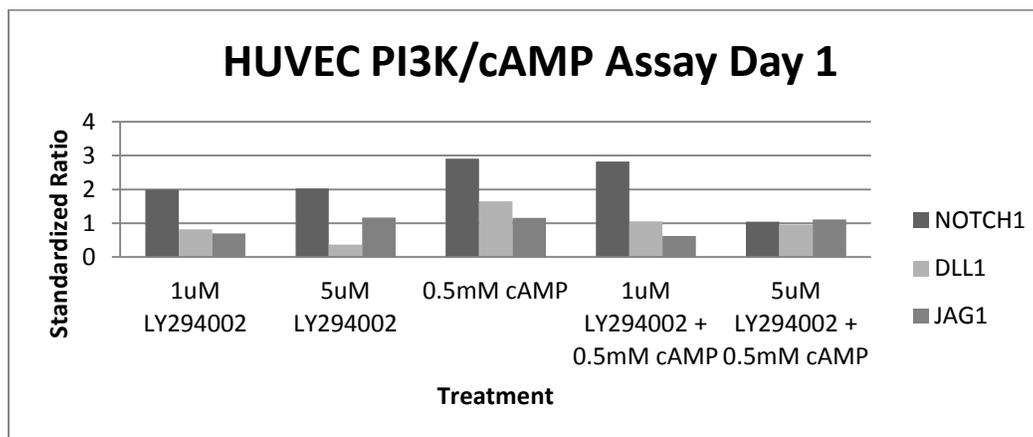
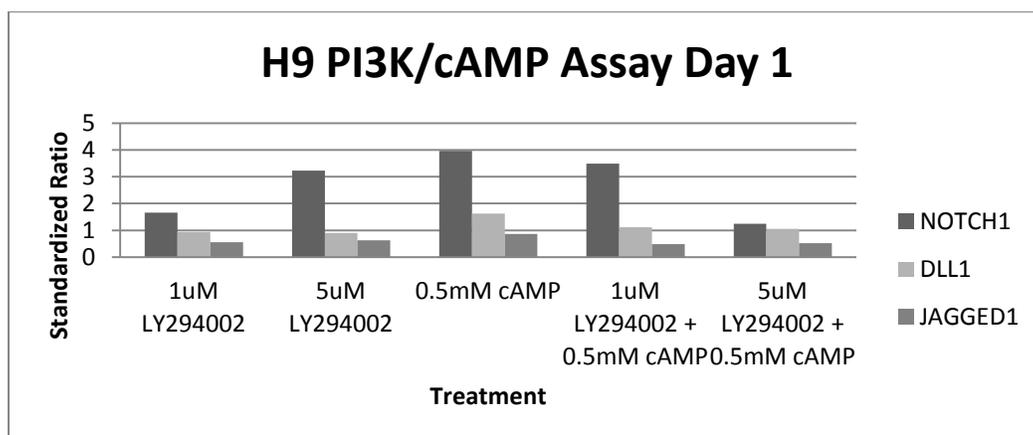
Figure 9**A****B****C**

Figure 9. Arterial gene expression varies following treatment with LY294002 and/or cAMP. Gene expression analysis of arterial genes Notch1, Dll1, and Jagged1 in (A) HUAECs, (B) HUVECs, and (C) H9-ECs following LY294002/cAMP treatment for 24 hours. Each set of data is standardized to

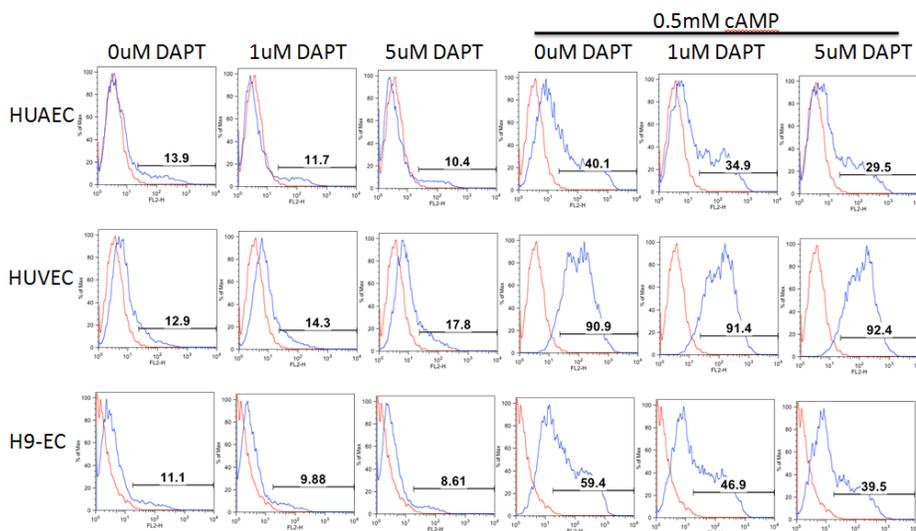
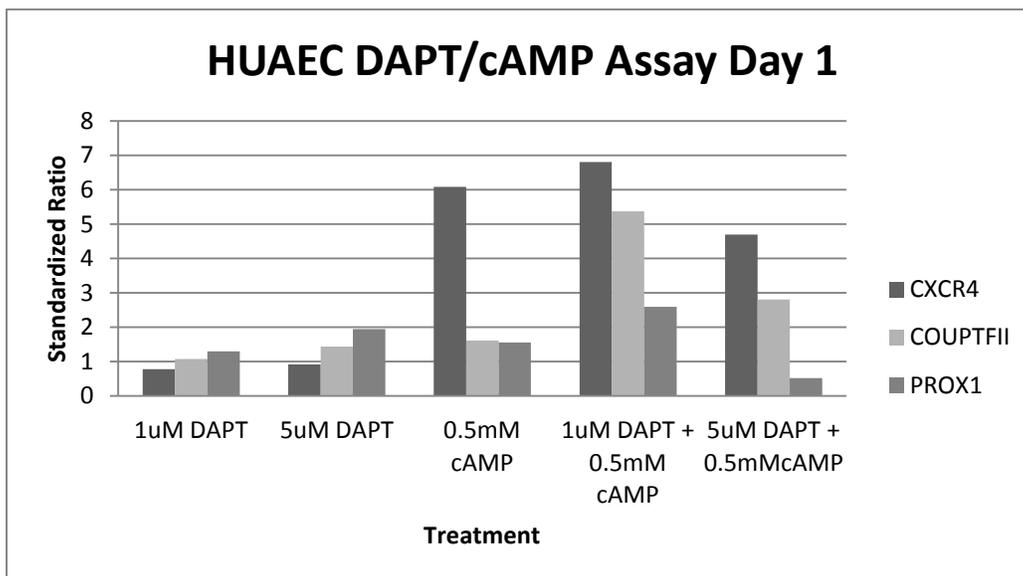
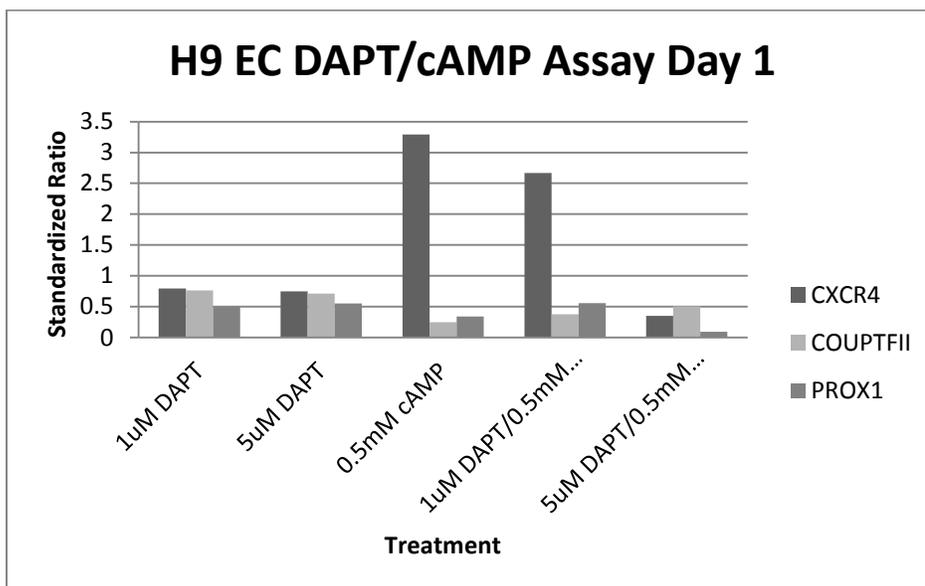
Figure 10**A****B**

Figure 10. Notch inhibition limits CXCR4 expression but can increase *Notch1* transcription. (A) Analysis of CXCR4 expression following treatment for 24 hours with 0 μ M, 1 μ M, or 5 μ M DAPT as well as with the addition of 0.5mM 8-Br-cAMP to each. (B) qRT-PCR analysis of *Cxcr4*, *CoupT-FII*, and *Prox1* expression in HUAECs after DAPT/cAMP treatment compared to untreated controls.

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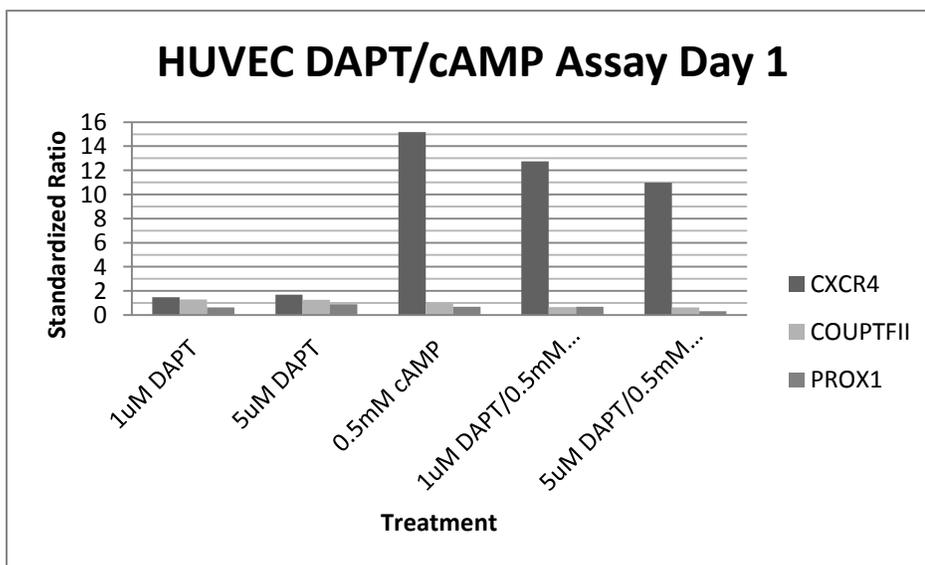
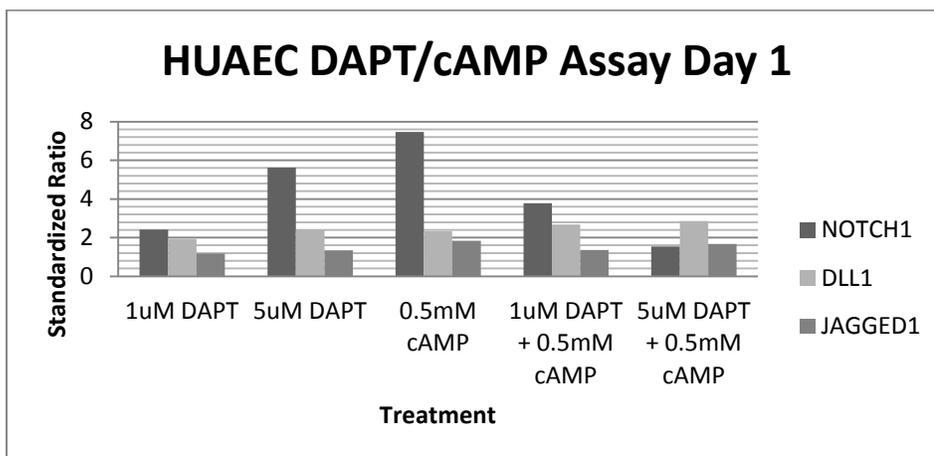
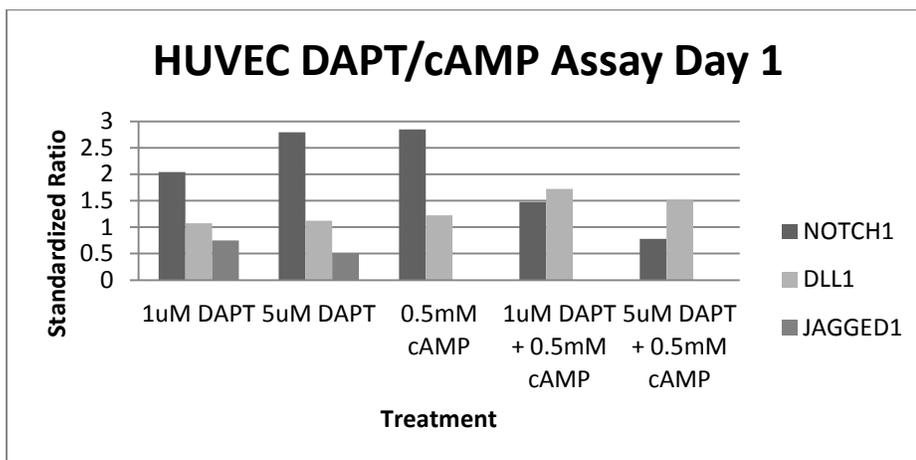


Figure 10. Gene expression of Cxcr4, CoupT-FII, and Prox1 in (C) H9-ECs and (D) HUVECs following DAPT/cAMP treatment for 24 hours.

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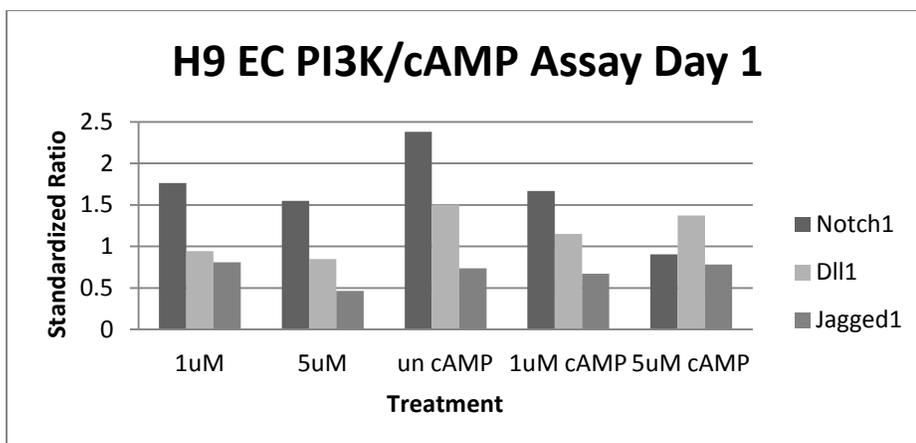


Figure 10. Gene analysis of arterial genes Notch-1, Dll-1, and Jagged-1 in (E) HUAEC, (F) HUVEC, and (G) H9-ECs following DAPT/cAMP treatment.

Discussion

As mentioned, the goal of this study was twofold: first, to enhance and optimize our differentiation protocol by identifying a defined media that would increase the development of mature endothelial cells and secondly to analyze the type of EC which resulted from this process and identify ways to increase arterial commitment. Our initial methods of differentiation suffered from ambiguity in that the variety of cytokines present in the serum-containing media caused proliferation of multiple cell types of various lineages. While we were still able to identify and isolate ECs from this mixture, it was difficult, inefficient, and time consuming. Separation of the desired cells from the culture as a whole was also made difficult by the limitations of the MACS systems we used as the overwhelmingly large population of pre-sort cells presumably decreased the ability of the magnetic antibodies to isolate single cells from any aggregates that would form during collection. We made efforts to only sort cell concentrations that fell within the manufacturer's specified ranges but the nature of the cell to cell adhesion of the cultures made truly single cell suspensions difficult to achieve. These cultures were characterized by large swaths of fibroblast/mesenchymal-like tissue which also seemingly contained a high concentration of extracellular matrix proteins making enzymatic dissociation complicated. The switch to a media containing defined cytokines helped to limit the overgrowth of these types of cells and the hematopoietic/vascular cells present were seemingly not as dependent on them for growth and expansion. Our initial analyses involved using the defined media with pluripotent cultures in a feeder-free system on various substrates such as matrigel or fibronectin (data not shown). While we saw better development of cells expressing EC markers, these cultures were still characterized by some overgrowth of the fibroblast/mesenchymal cell types which continued to limit the efficiency of isolating the desired cells. By switching to the spin EB system for differentiation, we saw a dramatic decrease in these types of cells which not only made sorting easier, but also reduced the amount of time needed for differentiation.

Having greatly enhanced the efficiency of our differentiation protocol, we needed to then ensure that we were not only developing cells that expressed the endothelial markers we desired but also that they would maintain endothelial phenotype and function for extended periods of time. The largest and most persisting difficulty of this project was that sorted cell cultures that exhibited a genuine endothelial phenotype initially would "transform" into a more mesenchymal phenotype after a few passages. We are still unsure of the exact nature of this event, but we suspect that there are a few potential reasons for it. The process of endothelial-to-mesenchymal transition has been only recently identified and is still not entirely understood. Studies have

shown, however, that it is an event that occurs during cardiac development as endothelial cells migrate and transform into mesenchymal cells which form the mitral and tricuspid valves in the heart as well as the septum^{83,84}. Another recent study by Igor Sluvkin's group at the University of Wisconsin found that mesenchymal cells differentiated in culture originated from an APLNR⁺ precursor that was also characterized by angiogenic capabilities⁸⁵. To this end, we also theorize that the cells we sort for may not be fully committed to a mature endothelial phenotype and are undergoing additional differentiation when they are cultured in our standard endothelial conditions. One way of addressing this would be to sort for multiple markers through use of fluorescent-activated cell sorting (FACS). Analysis of cells sorted only for CD31 following spin EB differentiation reveal that there are distinct populations with varied expression of secondary markers such as CD43, CD144, and Flk1. Even up to two weeks post-sort, non-adherent cells that are collected and analyzed express early hematopoietic markers such as CD43 and CD45 (data not shown). It is unknown at this point what effect these differing populations have on the long-term viability of the ECs in culture. Considering that our differentiation protocol is capable of inducing hematopoietic differentiation, it stands to reason that a portion of our CD31⁺ sorted cells are committed to or are in the process of committing to this lineage. One final reason for deficiency in proliferation and maintenance of an endothelial phenotype in sorted cells may be the lack of cell to cell interactions. This situation mostly applies to our earlier studies with stromal cell co-culture methods as low yields of sorted cells would result in sparse density in the standard endothelial conditions culture. EC cultures are defined by tight intercellular junctions so the lack of these may have detrimental effects on the ability of these cells to proliferate and adhere to an endothelial phenotype. Whatever the case, we continue to try and understand this process and, in doing so, hope to find ways to limit this transition in order to maintain mature endothelial cells.

In our efforts to induce further endothelial commitment and differentiation, we have examined and continue to assess the effects of various growth factors and small molecule inhibitors. The addition of APLN to differentiation cultures has shown a clear increase in certain endothelial markers, but has not provided any clearly evident benefit to long-term endothelial maintenance. It is also unclear what effect this polypeptide has on commitment to a venous endothelial sub-type as opposed to arterial commitment. As mentioned, activation of the apelin receptor, APJ, has been shown to be involved in not only vascular development as a whole but also commitment to the venous phenotype. We do not know the true extent of this process in our *in vitro* system and further experiments are needed to determine whether the benefits to

endothelial development outweigh any decrease in ability to adopt an arterial phenotype. The same is true for our use of the small molecule notch inhibitor DAPT as we are unsure of the long term effects on endothelial identity following treatment during differentiation. While notch signaling has been shown to be essential for arterial development, these conclusions were drawn from studies using knockout mouse models. Our use of DAPT in *in vitro* differentiation cultures may represent a more transient inhibition of notch signaling as it is only introduced into the media on day four. It is unknown how persistent and effective it is at signal inhibition during the remaining three days of the differentiation. Also, our use of DAPT treatment in primary cells and sorted ECs was only analyzed following treatment for 24 hours so it would be of interest to examine the effects of longer exposure to the inhibitor. There is no known *in vitro* assay to assess functional differences between venous and arterial cells so it is difficult to determine the effects of this treatment outside of phenotypic differences. The use of cAMP and PI3K inhibitor assays was also limited to more mature cells so we are unsure of the effects these would have on differentiating cells and their expression of arterial markers and genes. More extensive analysis of the effects of these assays would be beneficial considering the variety of signaling processes that these two pathways interact with. Also of interest would be the long term gene expression modification that these assays may or may not affect.

While inhibition of PI3K did not show robust increases across the board of arterial-specific genes, there is clearly a positive dose-dependent response in CXCR4 expression which can be further enhanced in the presence of cAMP. We have not yet identified the exact nature of this mechanism or the potential benefits it may have for use in the cardiac ischemia models which leaves room for future examinations. We also continue to experiment with alternative PI3K inhibitors, such as PI-103, which possess different capacities to bind and inhibit not only PI3K but also related signaling pathway kinases such as mTOR. Long-term inhibition of these signaling kinases is not ideal as they are involved in important pathways that are responsible for cell survival and proliferation. It would be of interest to compare the results of these assays with other forms of expression augmentation such as small interfering RNAs (siRNA) in order to determine the efficiency of each system. Also of interest would be a more thorough analysis of the downstream effects of cAMP and PI3K inhibition. This would be beneficial in determining whether the effects we see are the result of gene expression adjustment of CXCR4 or from some other process such as post-translational modification. As we further elucidate the effects of this inhibition and the exact mechanisms of its action we hope to be able to further augment it in order to achieve effective conversion or enhancement of an arterial phenotype in differentiated ECs.

Conversely, we have yet to identify or experiment with methods of inhibiting COUP-TFII or other effectors which are critical for development of the venous endothelial phenotype. We are unaware of any small molecule inhibitors like DAPT that are capable of efficiently targeting and disrupting COUP-TFII signaling so inhibition assays at this point would require genetic knockout or knockdown of COUP-TFII transcripts. Gene analysis of differentiating cells in our spin EB system could be used to identify the temporal expression of those genes which are involved in the arterial-venous specification and would be essential for determining the most ideal time points for inhibition/activation in order to drive arterial commitment.

Given the difficulties of isolating and maintaining endothelial cells derived from pluripotent stem cells in culture, it has been encouraging to see the results from our feeder-free system using a defined differentiation media. It is still not perfect but has allowed us to grow large numbers of these cells for experimental use compared to the stromal cell co-culture method that was originally used. We continue to refine this process in order to develop a more reliable system that can be faithfully replicated by us and others who may desire to use it. We are also encouraged by the results obtained from our experiments regarding arterial commitment and persist in optimizing them in order to truly understand the cellular processes involved. The hope is that this research can be used to further the understanding of endothelial development and potentially for future cellular therapies that would be beneficial for treatment of cardiovascular disorders and disease.

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