DERIVATION OF ENDOTHELIAL COLONY FORMING CELLS FROM HUMAN CORD BLOOD AND EMBRYONIC STEM CELLS

J. Luke Meador

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Master of Science
in the Department of Biochemistry and Molecular Biology,
Indiana University
August 2013

Accepted by the Faculty Indiana University, in partial
fulfillment of the requirements for the degree of Master of Science.

_____

Mervin C. Yoder MD, Chair

_____

Reuben Kapur PhD

Master's Thesis
Committee

_____

Timothy W. Corson PhD

_____

Jason S. Meyer PhD

Dedication

To my wife Kaitlin

Acknowledgements

I would like to express my gratitude to my mentor Dr. Mervin C. Yoder for his commitment to my education. He not only provided me with expert advice and guidance, but always managed to do so in a positive way which helped me to excel in the program. His commitment to graduate student education is unparalleled due to his commitment to the balance of work and life.

I would like to thank Monica Henry and everyone in the Interdisciplinary Biomedical Gateway program for their assistance in finding the right laboratory during our first year in the program and setting me up for success in the subsequent years.
I would like to thank the members of my advisory committee: Tim Corson, Reuben Kapur, and Jason Meyer, for there insight and guidance as I formulated my project and learned the skills necessary to be successful.

I would also like to thank my colleagues in the IBMG program and the Biochemistry Department for their friendship and willingness to study late into the night. I have formed many lifelong friendships during my time at IUSM.

I am continually grateful to my parents, Ramona Meador and Bruce Meador, who helped shape me into person I am today. I would like to thank my in-laws, Samuel Vogt and Adina Vogt, who have helped my wife and me in every aspect as we started our life together. And most importantly, I would like to thank the love of my life, Kate Meador, who has done everything for me and who I am eternally grateful for.

J. Luke Meador

DERIVATION OF ENDOTHELIAL COLONY FORMING CELLS FROM HUMAN CORD BLOOD AND EMBRYONIC STEM CELLS

Endothelial Colony Forming Cells (ECFCs) are highly proliferative endothelial progenitor cells with clonal proliferative potential and *in vivo* vessel forming ability. While endothelial cells have been derived from human induced pluripotent stem cells (hiPS) or human embryonic stem cells (hES), they are not highly proliferative and require ectopic expression of a TGFβ inhibitor to restrict plasticity. Neuropilin-1 (NRP-1) has been reported to identify the emergence of endothelial precursor cells from human and mouse ES cells undergoing endothelial differentiation. However, the protocol used in that study was not well defined, used uncharacterized neuronal induction reagents in the culture medium, and failed to fully characterize the endothelial cells derived. We hypothesize that NRP-1 expression is critical for the emergence of stable endothelial cells with ECFC properties from hES cells. We developed a novel serum and feeder free defined endothelial differentiation protocol to induce stable endothelial cells possessing cells with cord blood ECFC-like properties from hES cells. We have shown that Day 12 hES cell-derived endothelial cells express the endothelial markers $CD31^+$ $NRP\text{-}1^+$, exhibit high proliferative potential at a single cell level, and display robust *in vivo* vessel forming ability similar to that of cord blood-derived ECFCs. The efficient production of the ECFCs from hES cells is 6 logs higher with this protocol than any previously published method. These results demonstrate progress towards differentiating ECFC from

hES and may provide patients with stable autologous cells capable of repairing injured, dysfunctional, or senescent vasculature if these findings can be repeated with hiPS.

Mervin C. Yoder MD, Chair

Table of Contents

List of Tables

List of Figures

Abbreviations

APC ..... Allophycocyanin

BMP ..... Bone Morphogenetic Factor

BMP4 ..... Bone Morphogenetic Factor 4

CD31 ..... Platelet Endothelial Cell Adhesion Molecule-1

CD144 ..... Vascular Endothelial Cadherin

CD146 ..... Melanoma Cell Adhesion Molecule

CLI ..... Critical Limb Ischemia

EB ..... Embryoid Body

EC ..... Endothelial Cell

ECFC ..... Endothelial Colony Forming Cell

EMT ..... Epithelial to Mesenchymal Transition

ES ..... Embryonic Stem Cell

FACS ..... Fluorescence Activated Cell Sorting

FBS ..... Fetal Bovine Serum

FITC ..... Fluorescein Isothiocyanate

FGF ..... Fibroblast Growth Factor

hES ..... Human Embryonic Stem Cells

hPS ..... Human Pluripotent Stem Cells

IHC ..... Immunohistochemistry

IPS ..... Induced Pluripotent Stem Cell

MHC ..... Major Histocompatability Cell Antigen

NRP-1 .....Neuropilin-1

PAD..... Peripheral Arterial Disease

PE.....Phycoerythrin

Pen/Strep.....Penicillin/Streptomycin

SMAD..... Portmanteau for genes (MAD) and (SMA)

TGFβ.....Transforming Growth Factor Beta

VEGF ..... Vascular Endothelial Growth Factor

INTRODUCTION

Endothelial colony forming cells (ECFCs) are rare circulating endothelial cells with robust clonal proliferative potential that display intrinsic *in vivo* vessel forming ability[1-7]. ECFCs, also called blood outgrowth endothelial cells (BOEC)[8], have been shown to be directly transplantable in sex-mismatched human bone marrow transplant patients with the most proliferative circulating BOEC displaying genetic marking of the donor marrow[8, 9]. While this study undoubtedly proves the direct transplantable nature of these cells (without any intervening period of *in vitro* culture), it does not fully explain the cell of origin within the transplanted donor marrow that gives rise to the BOEC, the engraftment site of the BOEC in the host, or the mechanisms with which the cells are mobilized into the systemic circulation. Given the known residence of ECFC within the vascular endothelial intima[3], it is plausible that blood vessel endothelial cells may be the resident niche for the circulating ECFC. ECFC rapidly mobilize into the blood stream following an experimental myocardial ischemic event[10] and in human subjects, the concentration of circulating ECFC correlates with the severity of coronary artery occlusion and chronic myocardial ischemia[11, 12]. When cultured ECFCs are injected intravenously into rodent vascular injury models, they are rapidly recruited into the site of vascular injury or tissue ischemia to orchestrate the initiation of a vasculogenic response[13-17]. In numerous animal models of human disease, ECFCs have been reported to enhance vascular repair and improve blood flow following myocardial infarction[18, 19], stroke[13], ischemic retinopathy[20, 21], ischemic limb injury[14-17], and to engraft and re-endothelialize denuded vascular segments or implanted grafts. The ability to increase the microvascular density within these regenerating tissues heralds ECFC as one of the most promising cell-based strategies for therapeutic

revascularization. While cultured and implanted ECFCs form durable and functional human blood vessels *in vivo*, they are rare in number in peripheral blood ($1/10^7$-10^8 peripheral blood mononuclear cells)[1, 2, 8, 12, 22, 23] and their number tends to decline with age and disease in human[11, 24, 25] and non-human primates[26]. In elderly patients and subjects with peripheral arterial disease (PAD) and critical limb ischemia (CLI), circulating or resident ECFC may become prone to replicative senescence lacking proliferative potential, thus, rendering them impotent for autologous vascular repair. Thus, it is necessary to find an alternate source of healthy ECFCs that may be used for vascular repair in these subjects.

Human pluripotent stem cells (human embryonic stem [hES] and induced pluripotent stem [hiPS] cells) display virtually unlimited self-renewal capacity and ability to differentiate into any cell types in our body[27-29] and these cells offer the opportunity to derive ECFC for vascular repair. Both hES and hiPS cells have been reported to differentiate into cells of the endothelial lineage[30-42], however the derived endothelial cells are in some cases, unstable and are reported to drift to various non-endothelial phenotypes[34, 43] or exhibit low proliferative potential with a proclivity to reach replicative senescence within 5-7 passages[43-45]. Indeed, there is no published evidence for derivation of ECFC that display the properties of high clonal proliferative potential with in vivo vessel forming capacity from hES or hiPS cells.

Here we describe a novel hES cell differentiation protocol that can reproducibly generate a homogenous and stable population of endothelial cells with ECFC properties that are similar to cord blood ECFC. With our simple one step 2D serum- and stromal cell-free differentiation protocol, we were able to consistently generate over a trillion

endothelial cells in less than 3 months of culture; an efficiency that has not been approached by any of the previously published endothelial differentiation protocols. This method involves initiation of endothelial lineage differentiation by growing hPS cells in serum-free endothelial differentiation media supplemented with growth factors for 12 days on Matrigel coated dishes. On day 12, cells were harvested and sorted using antibodies that recognize several well-established endothelial antigens. Only the $NRP^{-}1^{+}CD31^{+}$ subset of cells exhibited ECFC properties, including robust *in vivo* vessel formation when implanted in immune-deficient mice. These ECFCs were not transformed and could be implanted for >3 months without evidence of teratoma formation. We have also determined that KDR activation via addition of a NRP-1-Fc dimer and $VEGF_{165}$ is required for the derivation and emergence of ECFCs from hES cells. Overall, our studies reveal a novel paradigm for derivation of endothelial cells with ECFC properties from hES cells via activation of a specific molecular pathway. Our innovative strategy to produce clinically relevant numbers of ECFC with *in vivo* vessel forming cell potential should be tested in hiPS cells and if successful, may soon permit trials to regenerate the deficient microvasculature that may underlie the most prominent deficit for vascular repair in patients with cardiovascular disease.

Novel Endothelial Colony Forming Cell Marker

Vascular endothelial cadherin (CD144) was at one time thought to be a specific marker for vascular endothelial cells. However it has since been discovered that CD144 is present on other cell types as well, such as hematopoietic cells[46]. Previous publications on the derivation of endothelial cells employed the use of this marker to isolate the

endothelial cell types. Possibly extraneous cell types may have been isolated using this process and restricted the ability of cells to reinforce cellular signaling and fully commit the endothelial lineage. Because of their use of this marker inhibitors were necessary to maintain the desired phenotype. Thus we set out to use a novel marker for the isolation of ECFCs.

Neuropilin1 (NRP-1) is a VEGF coreceptor which is able to synergistically upregulate the downstream signaling of some VEGF receptors[47]. NRP-1 homozygous deficient mice demonstrate greatly diminished yolk sac vasculature, disorganized somatic blood vessels, and this defect results in embryonic lethality by E12.5-13.5. In 2009 Cimato et al reported that NRP-1 was an early marker for endothelial cells predating CD144 expression during endothelial cell differentiation from hES cells[33]. It has also been shown that NRP-1 is crucial for the development of vascular endothelial cells. We set out to use NRP-1 antibodies in concert with other known endothelial antigens to develop a system to specifically isolate endothelial cells. While we show NRP-1 to be essential for the specification of ECFCs it does not appear to be necessary for the maintenance of the ECFC lineage and is not correlated with proliferative capacity in later passages.

Aim of this Study

Our primary goal was to obtain highly proliferative endothelial cells with all of the ECFC hallmarks. This goal required that I verify the techniques in which ECFCs are obtained and characterized from human umbilical cord blood. I was fortunate to be involved with writing a review protocol on the isolation, expansion, and characterization

of ECFCs during my first few months in the lab. This review was published in the *Journal of Visualized Experiments*. This publication allowed me to perfect the techniques necessary to perform my work on our current project, which will be submitted soon.

Chapter I discusses the isolation, derivation, and characterization of cord blood derived ECFCs. We report optimized procedures for extracting ECFCs from Cord blood, methods to culture and purify a homogenous population of ECFCs, and provide protocols to characterize ECFC functions *in vitro* and *in vivo*. This work provides the foundation and basis of comparison for all subsequent work on deriving ECFCs from hPS cells.

Chapter II discusses the major focus of my work on developing and optimizing a novel protocol for isolating, expanding, and characterizing hES derived ECFCs. By modifying several existing protocols we developed a novel robust protocol to efficiently isolate ECFCs with stable phenotypes through many passages without loss of functions including *in vivo* vessel forming potential. To date this is the only protocol for deriving stable highly proliferative ECFCs from pluripotent stem cells.

Chapter I: Phenotypic and Functional Characterization of Endothelial Colony Forming Cells Derived from Human Umbilical Cord Blood

Abstract

Longstanding views of new blood vessel formation via angiogenesis, vasculogenesis, and arteriogenesis have been recently reviewed[48]. The presence of circulating endothelial progenitor cells (EPCs) were first identified in adult human peripheral blood by Asahara et al. in 1997[49] bringing an infusion of new hypotheses and strategies for vascular regeneration and repair. EPCs are rare but normal components of circulating blood that serve as sites of blood vessel formation or vascular remodeling, and facilitate either postnatal vasculogeneis, angiogenesis, or arteriogenesis largely *via* paracrine stimulation of existing vessel wall derived cells[50]. No specific marker to identify an EPC has been identified, and at present the focus of the field is to understand that numerous cell types including proangiogenic hematopoietic stem and progenitor cells, circulating angiogenic cells, $Tie2^{+}$ monocytes, myeloid progenitor cells, tumor associated macrophages, and M2 activated macrophages participate in stimulating the angiogenic process in a variety of preclinical animal model systems and in human subjects in numerous disease states[51, 52]. Endothelial colony forming cells (ECFCs) are rare, circulating viable endothelial cells characterized by robust clonal proliferative potential, secondary and tertiary colony forming ability upon replating, the ability to form intrinsic *in vivo* vessels upon transplantation into immunodeficient mice[1-3]. While ECFCs have been successfully isolated from the peripheral blood of healthy adult subjects, umbilical cord blood (CB) of healthy newborn infants, and vessel wall of numerous human arterial and venous vessels [1-3, 53], CB possesses the highest frequency of ECFCs[1] that display the most robust clonal proliferative potential and form durable and functional blood vessels *in vivo*[2, 4-7]. While the derivation of ECFCs

from adult peripheral blood has been presented[8, 54], we describe the methodologies for the derivation, cloning, expansion, and *in vitro* as well as *in vivo* characterization of ECFCs from human umbilical CB.

Reagents and Solutions

EGM-2 media (Lonza, Cat. No. cc-3162 containing EBM-2 basal medium and EGM-2 SingleQuot kit Supplements, and growth factors)

EBM-2 (Lonza, Cat. No. cc-3156) supplemented with the entire singlequot kit supplements and growth factors (Lonza, Cat. No. cc-4176), 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin (10,000 U/ml)/streptomycin (10,000 µg/ml)/amphotericin (25 µg/ml). Store up to 1 month at 4˚C. Recommended EGM-2 volumes to use for ECFC culture are 500 µl/well for 24-well plates, 2 ml/well for 6-well plates, 5 ml/25-cm^2 flasks, and 10 ml/75-cm^2 flasks, unless otherwise specified in the protocol.

Collagen I solution

Dilute 0.575 ml of glacial acetic acid (17.4 N) in 495 ml of sterile distilled water (0.02 N final concentration). Sterile filter the dilute acetic acid with a 0.22-µm vacuum filtration system. Add 25 mg rat tail collagen I to the dilute acetic acid to a final concentration of 50 µg/ml. The amount of collagen added will vary depending on the collagen stock concentration. Store up to a month at 4˚C.

Preparation of collagen I-coated tissue culture surfaces

Place 1 ml of collagen I solution in each well of a 6-well tissue culture plate (use 300 µl/well for 24-well plates, 3 ml/25-cm^2 flasks, and 8 ml/75-cm^2 flasks). Incubate 1 hour to overnight at 37˚C. Remove the collagen I solution and

wash surface two times, each time with PBS (use 500 µl/well for 24-well plates, 2 ml/well for 6-well plates, 5 ml/25-cm^2 flasks, and 10 ml/75-cm^2 flasks). Use plates immediately for cell cultures.

FACS staining buffer

Phosphate-buffered saline (PBS) supplemented with 2% (v/v) fetal bovine serum (FBS). Store up to 2 weeks at 4˚C.

Name of the reagent	Company	Catalogue Number
Heparin Sodium Injection, USP	APP Pharmaceuticals	504031
Ficoll-Pague	Amersham Biosciences	17-1440-03
Mixing cannula	Maersk Medical	500.11.012
EGM-2	Lonza	CC-3162
Defined FBS	Hyclone	SH30070.03
TrypLE express	Gibco	12605
Rat type I collagen	BD Biosciences	354236
Matrigel	BD Biosciences	356234
FcR Block	Miltenyi Biotech	130-059-901
hCD31, FITC conjugated	BD Pharmingen	555445
hCD45, FITC conjugated	BD Pharmingen	555482
hCD14, FITC conjugated	BD Pharmingen	555397
hCD144, PE conjugated	eBioscience	12-1449-80
hCD146, PE conjugated	BD Pharmingen	550315
hCD105, PE conjugated	Invitrogen	MHCD10504
Ms $IgG_{1,k}$ antibody, FITC conjugated	BD Pharmingen	555748
Ms $IgG_{1,k}$ antibody, PE conjugated	BD Pharmingen	559320
Ms $IgG_{2a,k}$ antibody, FITC con.	BD Pharmingen	555573
Anti-human CD31	Dako	clone JC70/A
Anti-mouse CD31	BD Pharmingen	553370
0.22-µm vacuum filtration system	Millipore	SCGPU05RE
Glacial acetic acid, 17.4N	Fisher	A38-500
Antibiotic-Antimycotic	Invitrogen	15240-062
Fetal bovine serum (FBS)	Hyclone	SH30070.03
IHC Zinc Fixative	BD Biosciences	550523
Sytox green reagent	Invitrogen	S33025
Cloning cylinders, sterile	Fisher Scientific	07-907-10

Table 1. Reagent List

Protocol Text

A.) ECFC Outgrowth, Cloning and Expansion

A.1.1) Collect CB with heparin (10 U heparin/ml blood) or EDTA as an anticoagulant and transport to the laboratory at room temperature and immediately (within 2 hours of infant's delivery) process the sample for mononuclear cell (MNC) isolation.

A.1.2) Aliquot 15 ml of CB into each 50-ml conical centrifuge tube and add 20 ml of PBS to each tube of CB and pipet several times to mix. Draw up 15 ml Ficoll-Paque into a 20-ml syringe and attach a 20G needle or mixing cannula. Place the tip of the mixing cannula at the bottom of the tube of diluted blood and carefully underlay 15 ml Ficoll-Paque. Centrifuge the tubes 30 min at 740 x g, at room temperature, without the deceleration brake setting.

A.1.3) Using a transfer pipet, remove the hazy layer of low-density MNCs located at the interface between the Ficoll-Paque and diluted plasma. Dispense the MNCs into a 50-ml conical tube containing 10 ml EGM-2 medium. Centrifuge the MNCs 10 min at 515 x g, at room temperature, with a high deceleration brake setting.

A.1.4) Carefully aspirate and discard the supernatant. Wash the pelleted cells with EGM-2 twice (10 min at 515 x g) and re-suspend the MNCs in EGM-2 at 1.25 x 10^7 cells/ml. Prepare collagen I coated 6-well tissue culture plates by adding 1 ml rat-tail collagen type I (50 µg/ml) per well and incubating the plate overnight. Pipet 4 ml of this MNC suspension into each well and place the cells in 37˚C, 5% CO_2 humidified incubator.

A.1.5) After 24 hours (day 1), slowly remove the spent medium (do not disturb the loosely adherent cells) from the well with a pipet, slowly add 4 ml EGM-2 to the well,

and return plate to the incubator. On day 2, refresh the medium by slowly removing the medium (do not disturb the loosely adherent cells) from the well with a pipet and adding 4 ml EGM-2 to each well. Repeat the medium change daily until day 7 and every other day thereafter until ECFC colonies appear for cloning. Typically colonies appear between day 5 and day 14[1] **(Fig. 1)**.

A.2.1) Visualize typical ECFC colonies with cobblestone morphology using inverted microscope (magnification 10x) and mark the colony boundaries with a marker on the underside of the well to indicate the position of each colony.

A.2.2) On day 14, wash these primary colonies 2 times with PBS and harvest individual colonies using cloning cylinders and just enough TrypLE express to cover the cells inside the cylinders. After aspirating the final wash of PBS, place a sterile gel coated cloning cylinder around each colony and press firmly against the plate using sterile forceps. Add 2-3 drops of warm TrypLE express into each cloning cylinder and incubate for 3-5 min until cells within the cylinder begin to detach. Add approximately 250 µl EGM-2 medium into the center of the cylinder and pipet up and down to generate single cell suspension. Transfer the single cell suspension from each cloning cylinder separately into an individual micro-centrifuge tube.

A.2.3)Wash the area within the cylinder 2-3 times with approximately 250 µl EGM-2 medium to collect all remaining cells and transfer the wash from each cylinder into the respective micro-centrifuge tube. Centrifuge the cells at high speed (≤300 x g) on tabletop centrifuge for 5 min.

A.2.4) Remove the supernatant and re-suspend the cell pellet in 1.5 ml fresh EGM-2 medium. Transfer the cell suspension (containing all the cells from individual colonies)

from each tube into one well of a 24-well tissue culture plate pre-coated with 500 µL rat-tail collagen I (50 µg/ml).

A.2.5) Place inside the incubator for expansion with media change performed every other day.

A.3.1) When cells approach 80-90% confluency, remove the spent medium then wash the cells 2 times with PBS and add 500 µl TrypLE express medium to each well of 24-well tissue culture plate. Place the plate inside the incubator for 3-5 min until cells begin to round up and detach. Add 1 ml of EGM-2 (with 10% defined FBS) to each well to collect the cells by washing and transfer them to a 15 ml tube. Wash the well with 1 ml EGM-2 medium to collect all remaining cells and transfer the wash from each well into respective 15 ml tube. Centrifuge the cells at 300 x g for 5 min.

A.3.2) Remove the medium and re-suspend the cell pellet in 1 ml fresh EGM-2 medium (with 10% defined FBS). Obtain the viable cell count of an aliquot using a hemacytometer and trypan blue exclusion.

A.3.3) Expand the cells by seeding about 5000 cells/cm^2 onto a collagen I pre-coated tissue culture surfaces in EGM-2 media with media change every other day until cells approach 80-90% confluency before sub-culturing again.

B.) *In vitro* phenotypic characterization of ECFCs: Endothelial cell surface antigen expression and single cell assay for clonal proliferative potential

B.1.1) For endothelial cell surface antigen expression, detach cells with TrypLE express to collect cells by using methods described for ECFC cloning and expansion.

B.1.2) Re-suspend cells in FACS staining buffer (10 X 10^6 cells/ 1 ml FACS staining buffer) then add FcR blocking reagent (20 µl/ 10 X 10^6 cells), mix gently and place on ice for 10 min.

B.1.3) Aliquot 100 µl of this cell suspension in micro-centrifuge tube for each endothelial surface antigen and isotype controls. Add appropriate amount of fluorochrome labeled human monoclonal antibodies that recognize endothelial antigens (CD31, CD144, CD146, and CD105) or hematopoietic antigens (CD45, and CD14) and leave on ice for 30 min protected from light. *Caution: 1 X 10^6 cells are not required for each test. The authors typically prepare 0.5 to 1 X 10^5 cells/tube to obtain 2 X 10^4 analyzed events. Also, follow the manufacturer's recommendation for the amount of antibody to be used for each test. Some antibodies may require titrating to determine the optimal antibody concentration. All staining that the authors have performed are single color staining.*

B.1.4) After 30 min of incubation on ice, centrifuge each tube at high speed on a tabletop centrifuge for 5 min then remove the supernatant and re-suspend the cell pellet in FACS buffer.

B.1.5) Analyze the samples on a flow cytometer to determine the percentage of cells that stain positively or negatively for each antigen. ECFCs stain uniformly positive for CD31, CD144, and CD146, but negative for CD45 and CD14 compared to the corresponding isotype controls.

B.2.1) For single cell assay to examine the clonal proliferative potential, detach early passage (2-3) cells with TrypLE express to collect cells using methods similar to that was described for ECFC cloning and expansion.

B.2.2) Infect these cells with enhanced green fluorescent protein (EGFP) expressing lentiviruses[55] and collect cells expressing EGFP by fluorescence cytometry. *Caution: Working with lentvirus is considered a biohazard and all bio-safety precautions should be obeyed.*

B.2.3) Culture them as described for culturing ECFC in the section for ECFC expansion.

B.2.4) Prepare Collagen I coated 96-well plates by adding 50 µl rat-tail collagen type I (50 µg/ml) per well and incubate the plate for overnight. Collect the $EGFP^+$ ECFCs by using TrypLE express and resuspend them in EGM-2 medium with final concentration ~10^6 cells /ml.

B.2.5) Use FACS Vantage (Becton Dickson) or other comparable sorter with low flow rate of 20 cells/second to sort one single $EGFP^+$ ECFC per well of a 96-well plate and adjust the final volume to 200 µl per well with EGM-2. Use an inverted fluorescence microscope to ensure that each well received only one cell. *Alternatively, single cells can be sorted based on FSC and SSC and Sytox nuclear dye staining the colonies can be used for cell scoring and quantification.*

B.2.6) Incubate the plate over a period of two weeks with two media changes (200 µl EGM-2 using multichannel pipetter) performed on day 5 and day 10. On day 14 of culture, wash each well with 100 µl PBS before fixing the cells with 100 µl of 4% paraformaldehyde for 30 min. For ECFCs that are not expressing EGFP, add Sytox reagent, a green fluorescent nuclear dye, following paraformaldehyde fixation and incubate at 4 ˚C overnight.

B.2.7) Use a fluorescence microscope to examine each well to quantitate the number of endothelial cells that expanded from a single cell cultured for 14 days. For quantitative

analysis, score wells with 2 or more endothelial cells as positive (for proliferation) and examine them further for total endothelial cell count by visual counting with the fluorescence microscope.

B.2.8) To display the obtained data, wells with an endothelial cell number of: 2 to 50, 51-2000, and 2001 or more, are considered as: endothelial cell clusters, low proliferative potential (LPP)-ECFCs, and high proliferative potential (HPP)-ECFCs respectively. ECFCs exhibit complete hierarchy of clonal proliferative potential by giving rise to endothelial clusters, LPPs, and HPPs when cultured at a single cell level for 14 days[1].

C.) *In vivo* functional characterization of ECFCs: *in vivo* vessel formation assay to examine the ECFC's potential for vasculogenesis

C.1.1) Prepare cellularized gel implants by calculating the total volume (ml) of each of the following gel materials (with the final concentration in the parenthesis) needed to cast 1-ml gel (which will be later bisected to generate 2 implants): FBS (10%), EBM-2 10:1 (adjust the final volume to 1 ml), sodium bicarbonate (1.5 mg/ml), NaOH (adjust pH to 7.4), HEPES (25 mM), fibronectin (100 µg/ml), and collagen I (1.5 mg/ml).

C.1.2) After gel material calculation, detach cells with TrypLE express to collect cells using methods similar to that was described for ECFC cloning and expansion. Obtain a viable cell count of an aliquot using a hemacytometer and trypan blue. Transfer 2.4 million viable cells into a 50 ml-conical tube and pellet the cells by centrifugation at 515 x g, room temperature. Simultaneously, prepare gel matrix solution by adding calculated volumes of HEPES, sodium bicarbonate, EBM-2 10:1, FBS, fibronectin, and collagen to

an ice-cold 50-ml conical tube (in the same order as they are listed). Mix thoroughly and adjust the pH to 7.4 with NaOH while keeping solution on ice.

C.1.3) Discard the supernatant from the cells after centrifugation and re-suspend the pellet in 360 µl warm EGM-2 and to it add pH adjusted 840 µl of gel matrix solution, making the final volume of gel implant 1 ml. Mix the cells into gel matrix solution slowly until cells are thoroughly suspended in the gel solution.

C.1.4) Transfer this 1 ml cellularized gel suspension to one well of a 12-well tissue culture plate and incubate for 20-30 min until the gel polymerizes. Gently cover the gel with 2 ml warm EGM-2 and incubate overnight.

C.1.5) Immediately before the implantation, bisect the overnight incubated gel into two equal pieces using iris scissors and return the gel pieces to the same culture well containing EGM-2 medium.

C.1.6) Use the animal surgical facility to sedate the animal (5-6 week old NOD/SCID mice) by administrating isoflurane anesthesia. Shave the lower part of abdomen and thoroughly clean the surgical site with alcohol pads.

C.1.7) Using sterile sharp iris scissors to make an approximately 5 mm incision in the lower quadrant of the abdomen, exposing the subcutaneous space between the skin and abdominal muscle. Perform blunt-dissection through the dermal layer from the abdominal muscle to create a 15-20 mm wide pocket leading superior into the upper abdominal quadrant. Repeat similar procedure to create similar open pocket in another side of the same mouse abdomen.

C.1.8) Insert one half piece of gel into one side of abdominal pocket and another half piece of gel into another side of the abdominal pocket by lifting the dermal layer just caudal to the incision.

C.1.9) After insertion of gels, close each incision with 2-3 stitches using 5-0 polypropylene suture on a cutting needle. Appropriately label the cage cards and perform post-surgical monitoring and analgesia as per the institutional and protocol requirements and allow 14 days for cells in these implanted gels to generate *de novo* vasculature.

C.1.10) Finally, harvesting of gel implants is typically performed 14 days after implantation after euthanizing the mouse.

C.1.11) Swab the abdominal area with alcohol pads and cut the abdominal skin caudal to the original incision line. Carefully, dissect out the implant by excising a flap of skin caudal to the probable location of the gel. Excise the implant by cutting circumferentially around the gel then place in zinc fixative (BD Biosciences, follow manufacturer's recommendation for optimal results) and allow them to fix for 1-2 hours at room temperature.

C.1.12) Prepare the paraffin-embedded gels using standard histochemical protocols and prepare 5 μm sections on glass slides to perform staining with hematoxylin and eosin, anti-human CD31 or anti-mouse CD31 to visualize the vasculature within the gel. ECFCs undergo de novo vasculogenesis to generate humanized vessels in cellularized gel implants inserted into immunodeficient mice for 14 days[2, 5, 51].

Representative Results

Using this ECFC derivation technique we have observed out-growth of primary colony formation as early as day 5 **(Fig. 1)**. The out-growth ECFC colonies exhibited typical cobblestone appearance and gave rise to >40 population doublings upon long term expansion after colony pickup by cloning. Expanded colonies expressed endothelial antigens, but did not express hematopoietic antigens **(Fig. 2)**. Importantly, they displayed a complete hierarchy of clonal proliferative potential at a single cell level **(Fig. 3)**. Moreover, ECFCs formed humanized blood vessels that are perfused with host RBCs when implanted into immunodeficient mice[2, 3, 5, 51] **(Fig. 4)**.

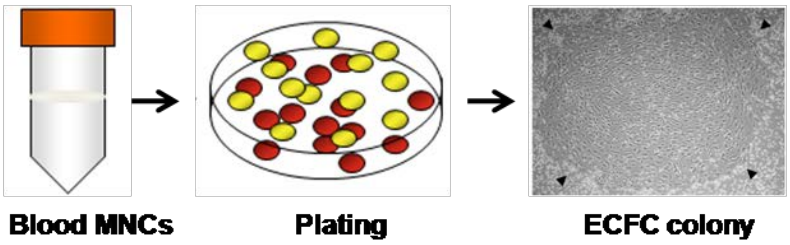

Figure 1. Isolation of mononuclear cells (MNCs) from cord blood and outgrowth of endothelial colony forming cells (ECFCs) from cultured MNCs. MNCs isolated from the buffy coat layer during Ficoll-Pague density gradient separation of cord blood cells. Isolation of buffy coat layer to culture MNCs on rat-tail collagen I (BD Biosciences) coated plates results in outgrowth of ECFC colony in 5 to 14 days. The outgrowth ECFC colony (indicated by arrow heads) displayed a cobblestone morphology[1].

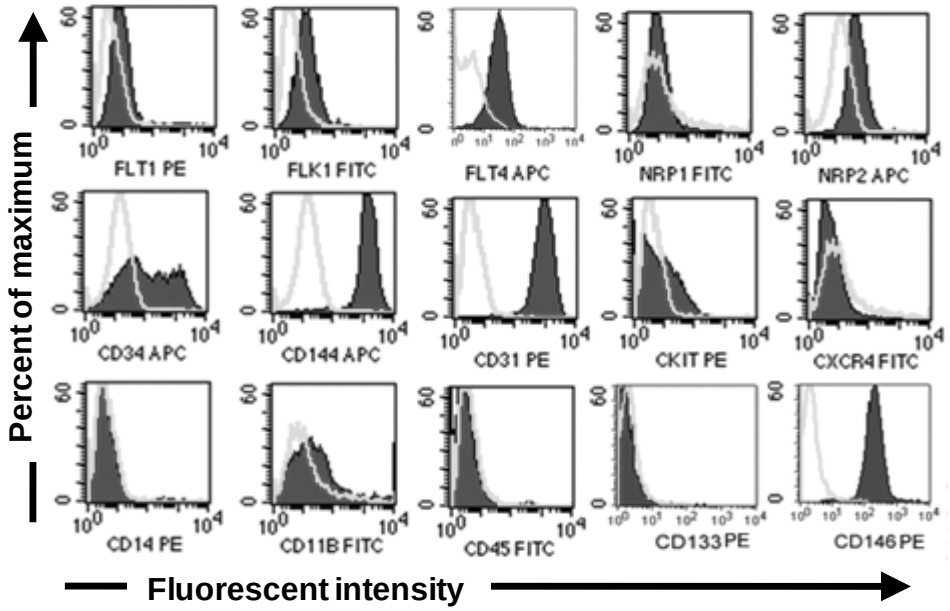

Figure 2. Representative *in vitro* phenotypic assessment of endothelial and hematopoietic cell surface antigen expression. Immunophenotyping of cord blood-derived ECFCs revealed that ECFCs expressed typical endothelial antigens CD31, CD34, CD144, CD146, Flt-1, Flk-1, Flt-4, and Nrp2 but did not express typical hematopoietic antigens CD45, CD14, CD11b, cKit, CXCR4 or AC133[1, 2].

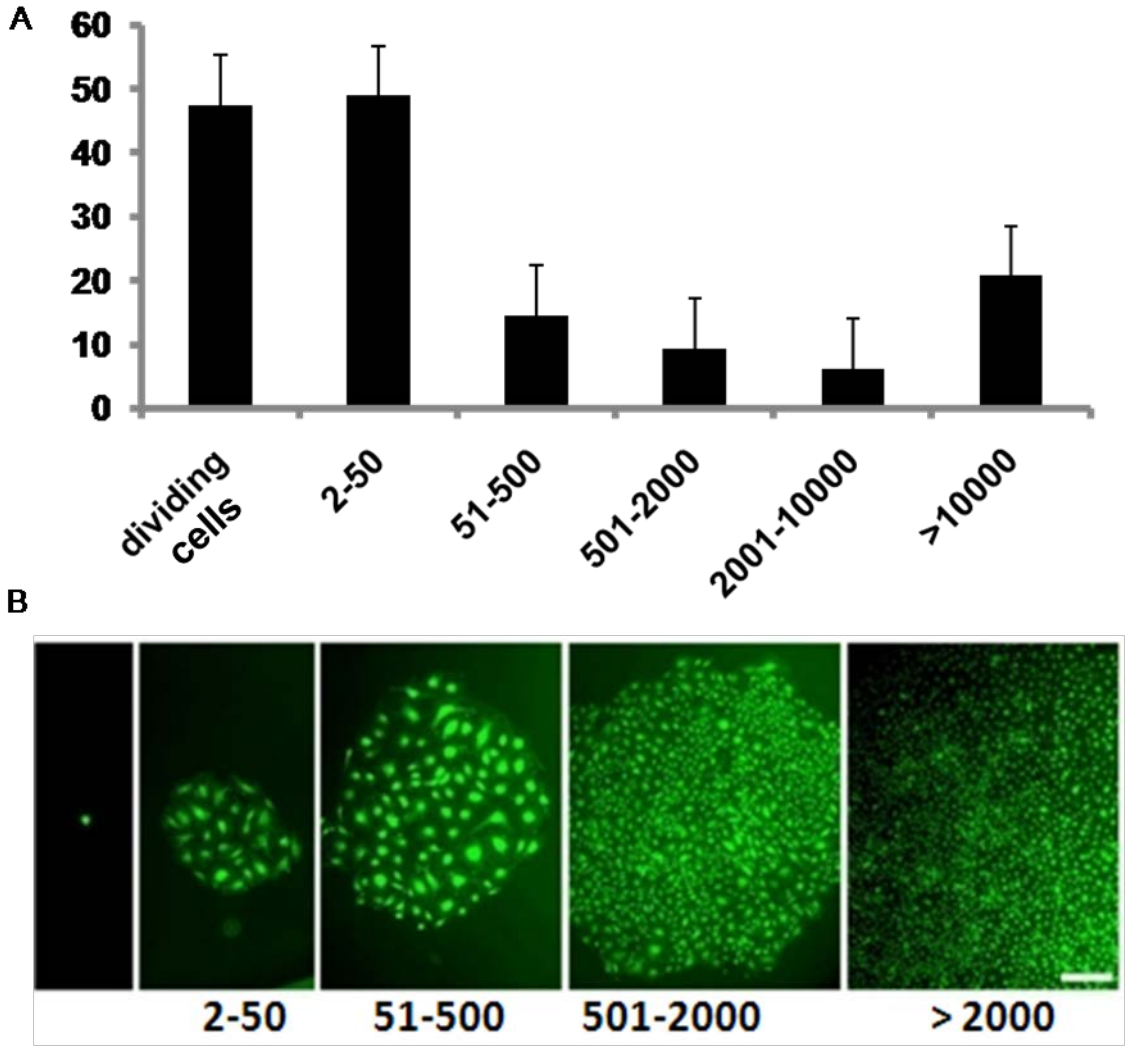

Figure 3. Representative *in vitro* quantitation of the clonogenic and proliferative potential of CB derived ECFCs. (A) cord blood-derived ECFCs display clonal proliferative potential with a hierarchy of colonies ranging from clusters of 2-50 cells up to colonies of >2001. (B) Micrographs of hierarchy of colonies (colonies stained with, Sytox, a green fluorescent nuclear dye) obtained after cord blood-derived ECFCs were cultured at a single cell level for 14 days. Scale bar represents 100µm[1, 2].

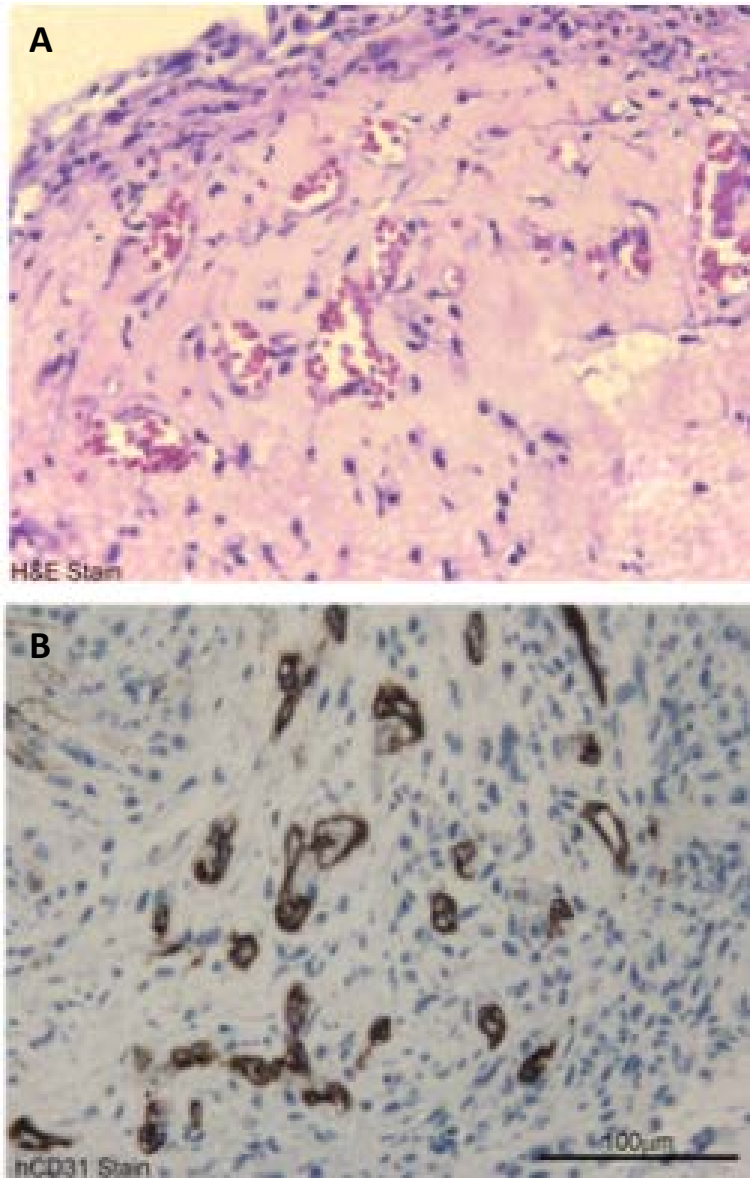

Figure 4. Representative *in vivo* functional characterization of cord blood-derived ECFCs. (A) H&E staining of cord blood-derived ECFC containing cellularized gel implants indicated microvessel (filled with host RBCs) formation in collagen-fibronectin gel after 14 days of implantation. (B) Anti-human CD31 staining (brown staining) further confirms the human origin of these vessels.

Discussion:

Phenotypic and functional characterization of putative endothelial progenitor cells is important to identify the *bona fide* ECFCs that are capable of clonally and serially re-plating in culture and give rise to durable and functional implantable blood vessels *in vivo*. Human umbilical cord blood is enriched with ECFCs and the concentration of these circulating cells declines with aging or disease. Recent studies suggest that ECFC may play important roles in vascular repair or regeneration in situations of vascular injury, myocardial infarction, or retinopathy[18, 21, 56].

Here, we have described simple and efficient methodologies for the derivation, cloning, expansion, and *in vitro* as well as *in vivo* characterization of ECFCs from human umbilical CB. These approaches enable researchers to identify and isolate *bona fide* EPCs from CB which possess clonal proliferative potential and *in vivo* vessel forming ability.

Chapter II: Derivation of Endothelial Colony Forming Cells from Human Embryonic Stem Cells

Introduction

Endothelial dysfunction with resulting atherosclerosis is a fundamental cause of peripheral arterial disease (PAD) in a large percentage of elderly individuals. Development of critical limb ischemia (CLI) in patients with PAD is associated with adverse events such as stroke and myocardial infarction[57-60]. Circulating endothelial progenitor cells (EPCs) are rare but normal components of circulating blood[49] that are implicated in the repair of damaged blood vessels such as those found in patients with PAD. Endothelial colony forming cells (ECFCs) represent those rare circulating EPCs with robust clonal proliferative potential that display intrinsic *in vivo* vessel forming ability[1-3]. While ECFCs form durable and functional blood vessels *in vivo*, they are rare in peripheral blood ($1/10^7$-10^8 peripheral blood mononuclear cells) and their number tends to decline with age and disease[1]. In elderly patients and subjects with PAD, these cells may become prone to replicative senescence lacking proliferative potential, thus, rendering them impotent for vascular repair. Developing a protocol for the efficient derivation and expansion of ECFCs is crucial for their implementation in regenerative medicine.

While human umbilical cord blood is enriched with ECFCs, use of these cells in patients with PAD as a cell therapy to repair endothelial dysfunction would constitute an allogeneic transplant. This predisposes ECFCs for immune-rejection by the host. Human embryonic stem (hES) cell differentiation into the endothelial lineage has been reported to generate endothelial cells with mature endothelial phenotypes but low proliferative potential[44]; indeed there is no published evidence for derivation of ECFC (clonal proliferative endothelium) from hES cells. While human ES derived cells would also be

predisposed for host immune-rejection if not matched for MHC compatibility, identifying a differentiation pathway for ECFC from the well-studied hES lines is an appropriate starting point for future application to hiPS cells. While directed differentiation of hES cells into the endothelial lineage has been reported to generate endothelial cells with a mature endothelial phenotype possessing low proliferative potential, derivation of endothelial cells with cord blood ECFC-like properties (progenitor cells with clonal proliferative potential and *in vivo* vessel forming ability) from hES cells has not yet been reported. Papers have even reported that hES and IPS cells may be incapable of generating proliferative vascular cells[38, 61, 62].

In 2010, Rafii et al. reported that ECs could be derived from hES cells using a protocol which first involved the formation of EB bodies and isolation by CD144 expression[62]. While this has been the gold standard protocol to date, reports also indicate that the TGFβ inhibitor had to be present throughout the culture period in order to restrict the EC from expressing alpha-smooth muscle actin (a protein more commonly seen in mesenchymal cells). Since CD144 has been reported to be expressed by mesoderm precursors, hematopoietic cells, and some cultured mesenchymal cells, we questioned whether the use of CD144 as a marker to enrich for hES-derived EC may lead to isolation of cells of many lineages.

In 2009 a report by Cimato et al. reported that NRP-1 may be useful in identifying the emergence of endothelial cells during development from hES[33]. NRP1 is important in endothelial cell development and acts as a co-receptor for VEGF which helps to increase downstream signaling and enrich the commitment of cells to endothelial cell

fates[63]. Thus, we hypothesized that ECFCs may emerge from mesoderm precursors co-expressing NRP-1 and CD31.

Here we report that by modifying earlier reported protocols, with addition of a novel cell surface antigen selection, we have isolated endothelial cells with stable cord blood ECFC-like properties from hES. Compared to other sub-sets of sorted cells, only NRP-1^{+}CD31^{+} cells exhibited ECFC properties. Our results show that NRP-1^{+}CD31^{+} isolated cells formed a homogenous cell monolayer with characteristic cobblestone morphology, exhibited clonal proliferative potential, generated capillary like structures when cultured on Matrigel, and formed robust *in vivo* vessels when in implanted in immune deficient mice. We speculate that this method of hES differentiation may also be effective in isolating ECFC from hiPS cells.

Materials and Methods

Culturing of hES and IPS cells

H9 human embryonic stem cells were kindly provided by the Dr. Hal Broxmeyer lab (IUSM) and were maintained as previously published [64]. Cells were maintained in mTeSR1 complete media (Stem Cell Technologies, Vancouver, Canada) on Matrigel (BD Biosciences) in 10 cm^2 tissue culture dishes at 37˚C and 5% CO_2. After the plating of cells, media was changed on days 2, 3, and 4. Cells were routinely passaged on day 5.

Passaging of hES and IPS cells

Prior to passaging the hES cells, 10 cm^2 tissue culture dishes were pre-coated with Matrigel for 1 hr. Tissue culture media from the hES cultures was aspirated and 4-5 mL of 2 mg/mL Dispase (Gibco, Grand Island, New York) was added to the plate. Cells were then incubated at 37˚C for 3-5 minutes until the edges of the colonies had lifted from the plate. The Dispase was aspirated from the plate and cells were gently washed with DMEM-F12 (Gibco) 3 times to remove any lingering Dispase. Fresh media was then used to harvest the cells from the plate using a forceful wash and scraping with a 5mL pipette taking care to avoid generation of excessive bubbles. Collected colonies were then centrifuged at 300xG for 5 min. During centrifugation, 7 mL of mTeSR1 complete media was added to Matrigel coated dishes to serve as culture medium for the pelleted colonies. The media was aspirated off of the centrifuged cells and then cells were resuspended in 10 mL of mTeSR1 complete media and added to the Matrigel coated dishes. Cultures were checked for hES colony morphology quality on day 2.

Directed Differentiation

After 2 days of culture in mTeSR1 media, hES cell cultures were directed toward the mesodermal lineage by pulsing with Activin A (10 ng/mL) under constant signaling of FGF-2 (Stemgent, Cambridge, Massachusetts), VEGF-165 (R&D, Minneapolis, Minnesota) and BMP4 (R&D) (10 ng/mL each). On day 1 Activin A containing media was removed and replaced with 8mL of Stemline II Media (Sigma, San Antonio, Texas) containing only FGF-2 and BMP4. Media was subsequently changed on days 3, 5, 7, and 8 with 8 mL of differentiation media. On day 9 and thereafter media was changed with 10 mL of differentiation media. This system was interrupted at any time for removal of cells for FACS analysis.

Flow Cytometry

To release the cells from the plate and the secreted ECM, TryplE (Life Technologies, Inc., Grand Island, New York) was added until the cells were just covered (5mL). The plates were then incubated at 37˚C for 5 minutes. In the presence of TryplE, cells were triturated using a 5 mL pipette and then deposited through a 0.4 micron filter into a 50 mL conical tube containing 10 mL of EGM2 (Lonza, Walkersville, Maryland). Tubes were then centrifuged at 300xG for 5 minutes. The supernatant was aspirated leaving behind the cell pellet. The pellet of cells was resuspended in 1 mL of EGM2.

The cells were then counted and the appropriate amount of FCR block (Miltenyi Biotech, Auburn, California) was then added to the tube and incubated for 15 min as per manufacturer guidelines. A portion of the cell mixture was then withheld for an unstained control while another portion was stained with the appropriate antibodies as per their

recommended protocol. Antibodies used included anti-human CD31 (CD31-FITC, clone WM59 from BD Pharmigen, San Jose, California, CAT # 555445), CD144 (CD144-PE, clone 16B1 from ebioscience, San Diego, California, CAT# 12-1449-82), and NRP-1 (NRP1-APC, clone AD5176 from Miltenyi Biotech, CAT # 130-090-900). The cells and antibody were centrifuged at high speed for 30 sec. and the supernatant was removed. The pelleted cells were resuspended in EGM2 containing propidium iodide (3 ng/mL). The sample would then be immediately ready for analysis and remain on ice until analyzed. Samples were analyzed using a FACS Vantage (Becton Dixon) flow cytometer at a flow rate of 1/min. Compensation was set using cord blood derived ECFC color controls. Samples were analyzed using flow minus one (FMO) control analysis and the FloJo analysis software.

Cell Culture of Sorted Cells

Sorted cells were centrifuged at 300xG for 5 minutes then resuspended in 50% EGM2 and 50% complete Stemline II differentiation media. A 12 well plate was then coated with rat tail type I collagen (BD Biosciences) and plated with 2500 cells/well. After 2 days the media was changed to 75% EGM2 and 25% differentiation media. Then again after 2 days the media was changed to 100% EGM2. After approximately 1 week, ECFC colonies appear as tightly adherent cells with cobblestone morphology. Cloning of endothelial cell clusters was performed to isolate pure populations of highly proliferative endothelial cells. Cells were then split and replated at about 80% confluency and plated at 10,000 cells/ cm^2. Cells were then maintained in an endothelial culture system of cEGM-2 media on collagen coated plates with media changes every other day.

In Vitro Network Formation Assay

Matrigel coated 96 well plates (50 µl of Matrigel to each well) were prepared in triplicate. 10,000 ECFCs were prepared in 50 µl of EGM2 per well. 50 µl of EGM2 containing cells were placed on top of the Matrigel coated surface. Next the plate was incubated overnight in 37˚C 5% CO_2 (12-16 hours). On the next day the plate was removed and the network formation could be observed under phase contrast microscope.

Immunohistochemistry and Western Blot Analysis

Twelve well plates with sterile glass coverslips on the bottom of the well were prepared and coated with type 1 rat tail collagen (BD Biosciences). 50,000 ECFC were added and cultured overnight. When cells formed a subconfluent monolayer they were processed for fixation using 4% (w/v) paraformaldehyde for 30 min and permeabilized with 0.1% (v/v) TritonX-100 in PBS for 5 min. After blocking with 10% (v/v) goat serum for 30 min, cells were incubated with primary antibodies; anti-CD31 (Santa Cruz), anti-CD144 (ebioscience), anti-NRP-1 (Santa Cruz) and anti-a-SMA, (Chemicon) overnight at 4˚C. Cells were washed with PBS, then incubated with secondary antibodies conjugated with Alexa-488 or Alexa-565 (Molecular Probe) and visualized by confocal microscopy after counterstaining with 2 g/ml DAPI (Sigma-Aldrich). The confocal images were viewed with Olympus FV1000 mpE confocal microscope using as objective 2-photon and Olympus uplanSapo 60xW/1.2NA/eus. All the images were taken at room temperature.

In Vivo Vessel Formation Assay

Type 1 pig skin collagen master mix for gel implant was prepared by dissolving lyophilized collagens in 0.01 *N*HCL and neutralized with 10x PBS and 0.1 *N* sodium hydroxide to achieve neutral pH (7.4) and final collagen concentrations ranging from 0.5 to 2.75 mg/ml[65]. The master mix was then mixed with the desired cell number (500,000 cells/ 250 microliter gel) in a 48 well tissue culture plate. The gel was allowed to solidify for 30 minutes at 37˚C and 5% CO_2 and then 500µl of EGM2 was added. The cells then incubated overnight in 37˚C 5% CO_2 (16-18 hours). The plate was taken the next day for implantation into NOD/SCID mice flanks. Detailed information on preparing, implanting, and removing gels after 14 days can be found in JOVE [66]. After removing gel after 14 days the gel was fixed with 4% paraformaldehyde and samples were processed for immunohistochemistry by a pathology laboratory for H&E staining and rat antihuman-CD31 monoclonal staining antibodies which do not cross react with host murine cells.

Biostatistics

All experiments were performed 3 times in triplicate and data are represented as mean $\pm$ standard deviation for statistical comparison. Significance of differences was assessed by an unpaired t-test at $p < 0.05$.

Results

Endothelial cells derived from hES cells using existing published protocols exhibit low proliferative potential and lack properties of cord blood ECFC

Human endothelial cells have been derived from hPS cells using a variety of methods. One approach is to promote differentiation of hPS through co-culture with OP9 stromal cells[32, 37-39, 41] or mouse embryonic fibroblasts[61]. Another more recent approach seeks differentiation of hPS through embryoid body (EB) formation[30, 31, 33-36, 40, 42, 44, 45, 67, 68] followed by application of various growth factors and/or receptor signaling pathway inhibitors to promote endothelial cell differentiation. While each method provides emergence of endothelial cells from the differentiated hPS cells, many of these studies failed to examine or discuss the proliferative potential of the endothelial cells produced or if mentioned, the endothelial cells were reported to possess very low proliferative potential[34, 43, 44] and to undergo cell senescence after only a few passages[45]. Indeed, none of the prior reports for the differentiation of endothelial cells from hPS described cells that appeared to display the clonal proliferative potential, replating capacity, or in vivo vessel forming ability of human cord blood ECFCs. Therefore, we sought to determine whether endothelial cells derived via the EB formation method of hES differentiation produces endothelial cells that possess cord blood ECFC properties. After 7 days of differentiation through EB formation, $KDR^{+}NRP\text{-}1^{+}$ cells were sorted and cultured in endothelial media as described[33]. When sorted cells were expanded, we found early passage cells (P.1) gave rise to a heterogeneous population of cells (**Fig. 5**), where only a portion of cells appeared to display an endothelial cell morphology. When these cells were further expanded (p.4), the cultures became

predominantly comprised of cells with a fibroblastic-like appearance and little endothelial cobblestone formation (**Fig. 5**). When these cells were characterized for endothelial surface antigen expression using flow cytometric analysis, a heterogeneous pattern of CD31, CD144, and CD146 expression was exhibited (**Fig. 5**) with only a portion of the cells expressing each of these antigens. Similarly when these cells were analyzed for ability to form vascular like networks on Matrigel they formed vascular like networks with numerous smaller incomplete branches that were sprout-like in appearance (**Fig. 5**). When we analyzed these cells (passage 3 to 4) for clonal proliferative potential we scored the outcomes as single cells that did not divide or divided to form colonies of 2-50 (ECFC clusters), 51-500 (low proliferative potential ECFC; LPP-ECFC), 501-2000 (LPP-ECFC), or >2000 cells (high proliferative potential-ECFC; HPP-ECFC). Less than 2% of the EB-derived cells gave rise to HPP-ECFC (**Fig. 6**). More of the EB-derived endothelial cells gave rise to LPP-ECFC (**Fig. 6**). Most of the EB-derived endothelial cells did not divide or give rise to ECFC clusters (**Fig. 6**). These patterns of ECFC colony formation were significantly different from the pattern displayed by single endothelial cells derived from CB-ECFC colonies (**Fig. 6**). We were unable to expand the EB-derived endothelial cells beyond passage 7 without them becoming senescent or drifting into a non-endothelial fibroblastic phenotype and the endothelial cells at passage 5 failed to give rise to human blood vessels in vivo upon implantation (**Fig. 5**).

Endothelial lineage differentiation of human embryonic stem (hES) cells results in generation of subsets of cells that differ in phenotype and proliferative potential

Using a more recently published 2 step endothelial differentiation protocol that involves initial EB formation and then 2D adherent cell culture (with added growth factors)[66], we next sought to determine whether endothelial lineage differentiation of hES cells would give rise to different subsets of endothelial cells, including a subset displaying ECFC properties. Based upon the known importance of vascular endothelial growth factor (VEGF) signaling pathway in the emergence of endothelial cells both during development[69-71] and during endothelial lineage differentiation of hES cells[33], we utilized neuropilin-1 (NRP-1) as one of the endothelial markers for identifying emergence of ECFCs from hES cells. NRP-1 is a VEGF co-receptor and Semaphorin 3A binding multifunctional protein expressed in various tissues including endothelial cells, vascular smooth muscle cells and lymphocytes[72]. While the role of NRP-1 in vasculogenesis is unknown, a double knock out of NRP-1 and NRP-2 in mice leads to an embryonic lethal phenotype similar to that of the VEGFR-2 knockout[73, 74]. NRP-1 and NRP-2 double knockout mice die at day 8.5 and display an avascular yolk sac[73]. Conversely, exogenous over-expression of NRP-1 has previously been shown to form excess capillaries and hemorrhages in the developing cardiovascular, brain, and limb regions of the chimeric mouse embryos[75]. Since NRP-1 has been suggested as a potential early marker for the endothelial lineage during hES cell differentiation[33], we hypothesized that NRP-1 expression early in hES differentiating down the endothelial lineage may identify the emergence of ECFCs. We generated hES (H9 line) EBs in suspension culture for 4 days, and seeded them on Matrigel coated dishes for 10 days as described[34]. Cells were harvested and analyzed using antibodies that recognize typical endothelial antigens (CD31, CD144, and CD146) and NRP-1 on days 0, 3, 6, 9 and 14, to

determine the kinetics of endothelial antigen expression during the differentiation protocol (**Fig. 7**). Cells co-expressing NRP-1 and CD31 (NRP-1^{+}CD31^{+} cells) appeared on day 3 (0.17%) and increased overtime to peak at day 14 (1.6%) (**Fig. 7**). The different subsets of day 14 sorted cells were cultured in endothelial growth (EGM-2) media supplemented with TGFβ inhibitor (10 μM SB431542) for 2 weeks, as TGFβ inhibition has been reported to promote endothelial lineage differentiation from hES cells and to prevent the cells from transitioning to a mesenchymal fate[34]. All of these subsets survived in endothelial growth media and formed confluent monolayers of cells. While most hES-derived subsets formed incomplete capillary-like networks upon plating in Matrigel, NRP-1^{+}CD31^{+} cells formed complete structures similar to those exhibited by CB derived ECFCs, (**Fig. 8**). When the endothelial subsets were seeded at a clonal level, more than 55% of NRP-1^{+}CD31^{-} cells and more than 70% of NRP-1^{-}CD31^{+} (**Fig. 7**) cells divided and displayed a near complete hierarchy of clonal proliferative potential. However, few clones from each of the sorted hES derived endothelial subsets gave rise to HPP-ECFC (the most proliferative population derived from a single input cell). In contrast, all of the NRP-1^{+}CD31^{+} plated cells divided and many clones (37%) formed HPP-ECFC, indicating that the NRP-1^{+}CD31^{+} cell subset possesses ECFC properties with the highest clonal proliferative potential (**Fig. 7**). Thus co-expression of NRP-1 and CD31 in hES-derived cells undergoing endothelial differentiation (EB plus 2D protocol) identifies a progenitor subset that gives rise to endothelial cells with high clonal proliferative potential and angiogenic activity, but only if cultured in the continual presence of TGF-β inhibition (upon removal of the TGF-β inhibitor, cells ceased to proliferate, changed morphology from cobblestone to spindle elongated shapes, and

upregulated alpha-smooth muscle actin [α-SMA] a marker of mesenchymal differentiation similar to previously published data)[66].

Novel ECFC protocol generates NRP-1^{+}CD31^{+} cells from hES cells at higher frequency and does not require TGFβ inhibition

While a two-step, EB plus 2 dimensional endothelial differentiation protocol was able to give rise to NRP-1^{+}CD31^{+} cells with clonal high proliferative potential, this was an inefficient process that generated heterogeneous EBs containing cells from all germ layers, required persistent presence of TGF-β inhibition to maintain the endothelial fate, and yielded a low percentage of NRP-1^{+}CD31^{+} cells. Therefore, we sought to develop an endothelial lineage differentiation protocol that did not require EB formation and TGF-β inhibition but ensured early preferential differentiation of hES cells towards the mesodermal lineage to give rise to an increased yield of the NRP-1^{+}CD31^{+} cells possessing ECFC properties. Human PS cells were cultured on Matrigel coated plates in mTeSR1 media for two days as described[76]. To induce endothelial lineage differentiation, mTeSR1 media was replaced with Stemline II media supplemented with 10 ng/mL Activin-A, BMP4, $VEGF_{165}$ and FGF2 on day 0 of differentiation. The tissue culture media was replaced the following day with fresh Stemline II media supplemented with 10 ng/mL BMP4, $VEGF_{165}$ and FGF2. Cells were harvested at day12 and analyzed for cells co-expressing CD31, CD144 and NRP-1 antigens (**Fig. 9**). Using this protocol, we were able to harvest an average 2% NRP-1^{+}CD31^{+} cells from hES cells (**Fig. 10**). We directly compared NRP-1^{+}CD31^{+} and NRP-1^{-}CD31^{+} cells for their ability to give rise to endothelial colonies and total cell expansion after 7 days of plating in an endothelial

transitioning media. When we plated 2500 NRP-1^{+}CD31^{+} and NRP^{-}CD31^{+} sorted cells in a well of a 12 well collagen coated plate, NRP-1^{+}CD31^{-} cells gave rise to significantly fewer endothelial colonies compared to colonies generated from plated NRP-1^{+}CD31^{+} cells (**Fig. 11**). Also, the morphology of NRP-1^{+}CD31^{+} progeny was homogenous and cobblestone in appearance (**Fig. 12**; top right panel), whereas, we found a heterogeneous cell population within the colonies obtained from NRP-1^{-}CD31^{+} cells. Finally, we counted the total number of cells in these colonies after 7 days of culture and found a significant (15 fold) increase in cultures initiated with NRP-1^{+}CD31^{+} cells compared to CD31^{+}NRP^{-} cells (**Fig. 11**). Further we performed immunocytochemistry analysis of both NRP-1^{+}CD31^{+} and NRP^{-}CD31^{+} sorted cells using antibodies recognizing the endothelial antigens CD31, CD144 and NRP-1 and the non-endothelial antigen α-SMA. Cells grown from the NRP-1^{+}CD31^{+} sorted fraction exhibited surface co-expression of CD31 and NRP-1 (yellow arrows, **Fig. 12**; top left panel) and uniform expression of CD144 but completely lacked expression of α-SMA (white arrows, **Fig. 12**; top middle panel). However, in cells produced from the CD31^{+}NRP^{-} sorted fraction, few cells expressed CD144 and almost the entire cell subset expressed α-SMA (arrows, **Fig. 12**; top right panel), indicating a drift away from an endothelial phenotype. Finally we examined hES derived NRP-1^{+}CD31^{+} and NRP^{-}CD31^{+} cells to determine the clonal proliferative behavior of the cells and found that both cell subsets displayed some evidence of clonal proliferative potential (**Fig. 12**). While more than 27% of cells derived from the CD31^{+}NRP^{-} subset failed to divide, 45% formed endothelial clusters, 25% formed LPP-ECFC and a mere 1% formed HPP-ECFC colonies. In contrast, only 2% of the NRP-1^{+}CD31^{+} cell subset failed to divide, 4% formed endothelial clusters, 45% formed LPP-

ECFC, and 48% formed HPP-ECFC (**Fig. 12**). Furthermore, NRP-$1^{+}CD31^{+}$ cells formed highly branching capillary-tube like structures (**Fig. 13**), whereas, $CD31^{+}NRP^{-}$ cells formed incomplete capillary-like networks when plated on Matrigel (**Fig. 13**). Finally, hES derived NRP-$1^{+}CD31^{+}$ cells produced endothelial cells with robust *in vivo* vessel forming ability that inosculated with the host murine vessels (**Fig. 13**) similar to that of CB-ECFCs previously described[2, 5]. Importantly, NRP-$1^{+}CD31^{+}$ cells did not induce teratoma formation after more than 3 months of implantation into immunodeficient mice in more than 24 animals (data not shown). However, $CD31^{+}NRP^{-}$ cells failed to generate functional human blood vessels (documented by the intraluminal presence of murine RBCs), indicating lack of de novo vessel forming and/or inosculating ability (**Fig. 13**). Collectively, ECFC protocol day12 derived NRP-$1^{+}CD31^{+}$ cells exhibited homogenous cobblestone morphology, expressed typical endothelial antigens, formed capillary-like networks on Matrigel in vitro, exhibited high clonal proliferative potential, and produced robust in vivo vessels filled with host RBCs. Therefore, the day 12 hES derived NRP-$1^{+}CD31^{+}$ cell fraction produces endothelial cells that possess properties similar to cord blood ECFC.

NRP-1^{+}CD31^{+} cells emerge as clusters of cells lacking a-SMA expression and upon isolation and culture undergo extensive expansion, maintain stable endothelial phenotype, and ultimately become senescent after long-term culture expansion

While hESD12 differentiated cells possess complete ECFC properties, the earliest emergence of cells co-expressing CD31 and NRP-1 during hES differentiation had not been determined. Cimato et al. reported that NRP-1 is not expressed in undifferentiated

hES cells. Our data also revealed that day 0 undifferentiated hES cells did not co-express CD31 and NRP-1 (**Fig. 10**). We determined that NRP-1^{+}CD31^{+} cells start to appear at day 4 in hES cells undergoing ECFC differentiation (**Fig. 10**). The percentage of NRP-1^{+}CD31^{+} cells progressively increased over time and reached the highest levels at day 12 of differentiation(**Fig. 10**). Morphological analysis of hES cells undergoing ECFC differentiation at day 6, day 9, and day 12 revealed emergence of cobblestone like morphology at day 9 and day 12 (**Fig. 14**). Also, IHC revealed that NRP-1^{+}CD31^{+} cells emerge as a cluster of cells within the mass of differentiating cells (**Fig. 15**). Further examination revealed that while hES-derived NRP-1^{+}CD31^{+} cells emerged as early as day 3 of differentiation, the endothelial cells derived at day 12 gave rise to ECFC that exhibited the highest frequency of co-expression of the typical endothelial antigens without expression of the mesenchymal antigen α-SMA (**Fig. 14,15**).

Next, we sought to examine the ability of hES-ECFCs to undergo long term expansion by continually culturing them in extended endothelial culture until they began to exhibit evidence of replicative senescence. Primary cells do not proliferate indefinitely but instead undergo senescence after long term *in vitro* culture[77]. In extended endothelial culture, we were able to expand both hES-ECFCs and CB-ECFCs up to 18 passages (**Fig. 16**). While the majority of hES-ECFCs and CB-ECFCs at passage 18 were senescent and were exhibiting characteristics of mortal primary cells[77], they still maintained an endothelial cobblestone morphology. We also detected continued homogenous expression of endothelial CD31, NRP-1 and CD144 antigen expression in these cells at passage 18 (**Fig. 16**). Collectively, this suggests that hES-ECFCs maintain a

stable endothelial phenotype throughout long term expansion culture but like any primary cell type, eventually exhibit replicative senescence.

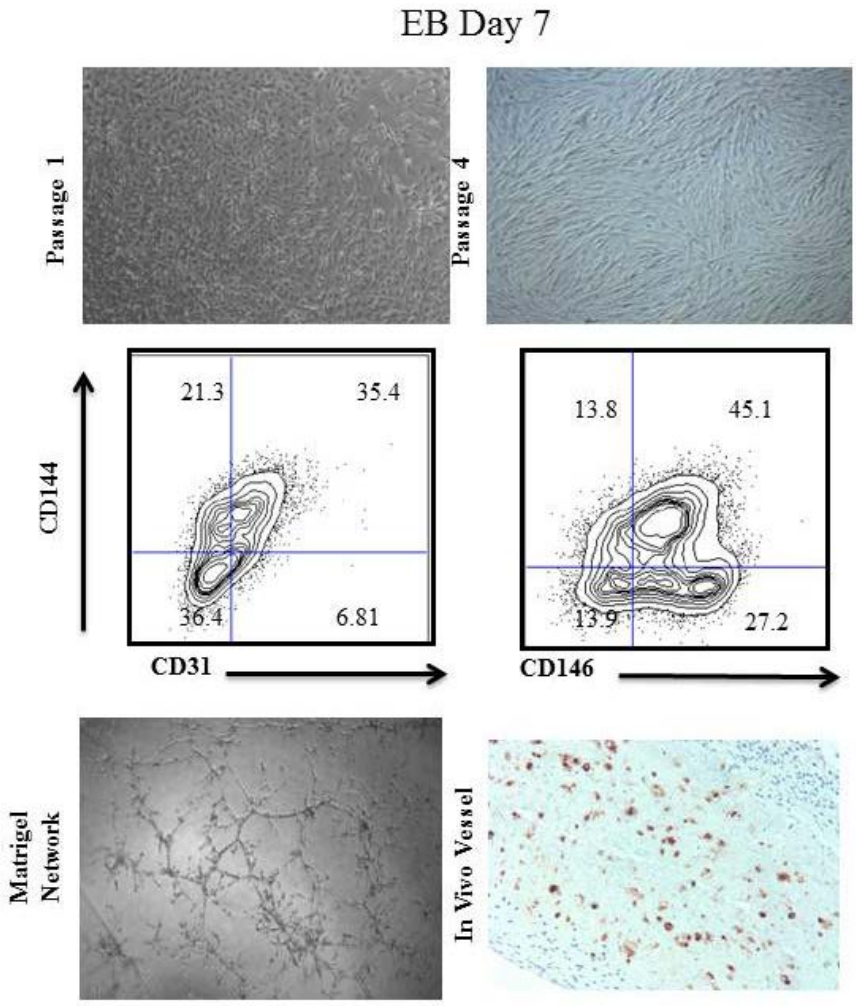

Figure 5. Characterization of EB derived hES-endothelial cells. (A) *top row* Morphology of endothelial cells isolated at day 7 of EB cultured in the endothelial conditions. Cells exhibit heterogeneous morphology. (B) *second row* Endothelial cell surface marker profile of isolated cells at D7 of differentiation. (C) *bottom row, left panel* The day 7 cultured endothelial cells plated on Matrigel exhibit short numerous branches that do not form complete capillary-like networks. (D) *bottom row, right panel* Images of anti-human stained CD31 labeled endothelial cells in sections of implanted gels recovered after two weeks of residence in immunodeficient mice, display the presence of

human endothelial cells but lack vascular lumen formation (no presence of murine red blood cells to indicate inosculation of the human vessels to the mouse circulation).

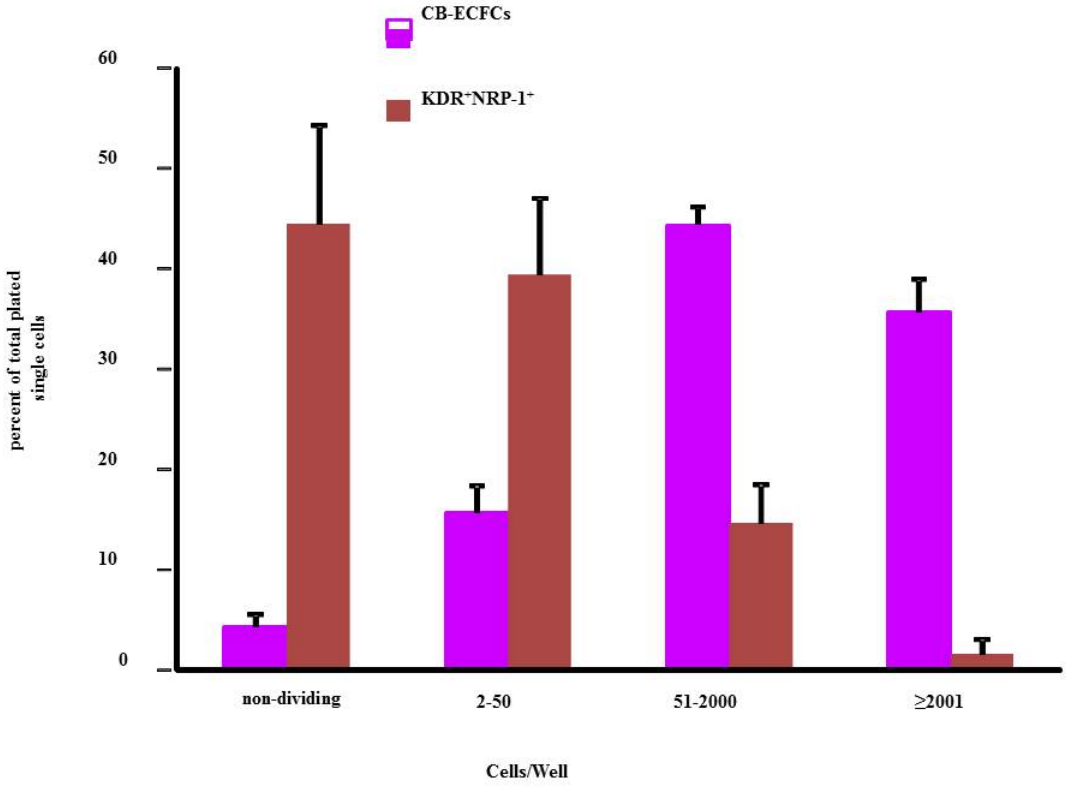

Figure 6. EB derived hES-endothelial cells are largely non-dividing cells and LPP-ECFC. EB derived cells exhibit a largely non-dividing single cell activity with some endothelial clusters, limited LPP-ECFC, and few HPP-ECFC. Human cord blood ECFC display a significantly different pattern with large numbers of LPP-ECFC and HPP-ECFC.

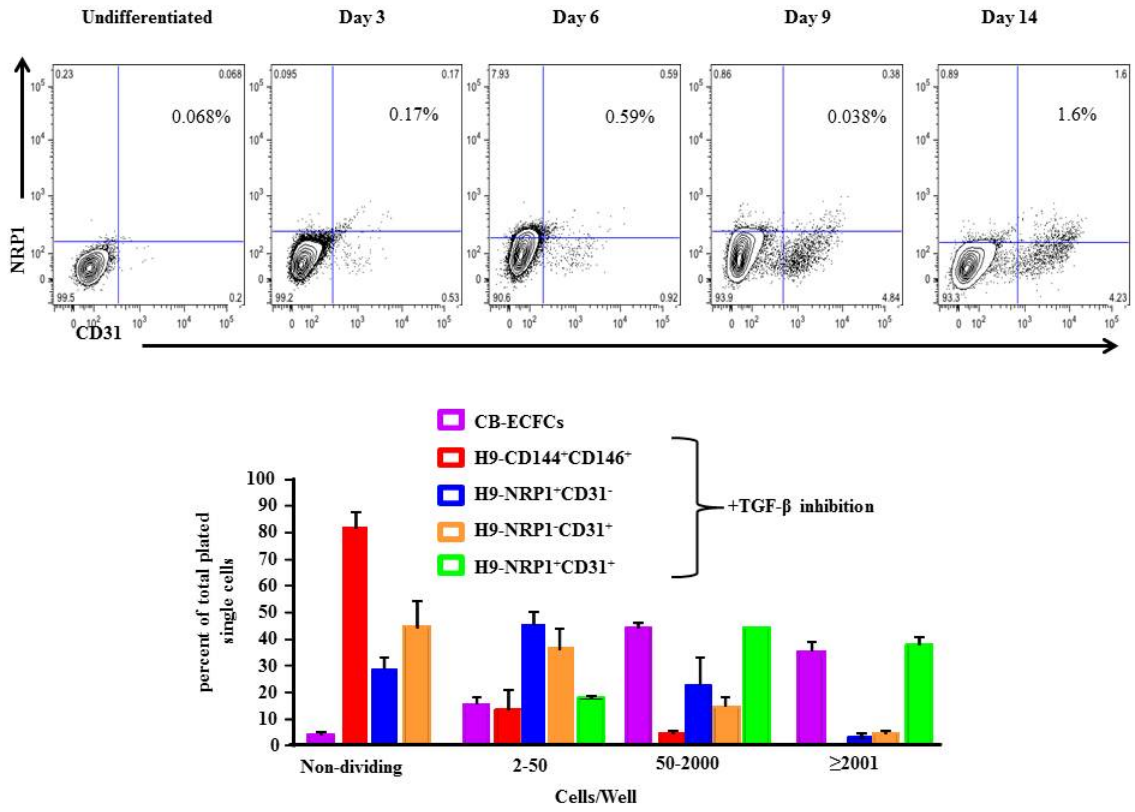

Figure 7. CD31 and NRP-1 expression in EB differentiation of hES-endothelial cells and their proliferative potential. (A) *top row* Day 0 hES cells are negative for CD31 NRP-1 double positive cell expression. Double positive cells begin to emerge at day 3 and the maximum percentage is observed on day 14. (B) *bottom graph* Double positive NRP-1 and CD31 expressing cells generated from the EB protocol exhibit a largely HPP when analyzed in a single cell assay consistent with CB-ECFCs but require the aid of a TGFβ inhibitor.

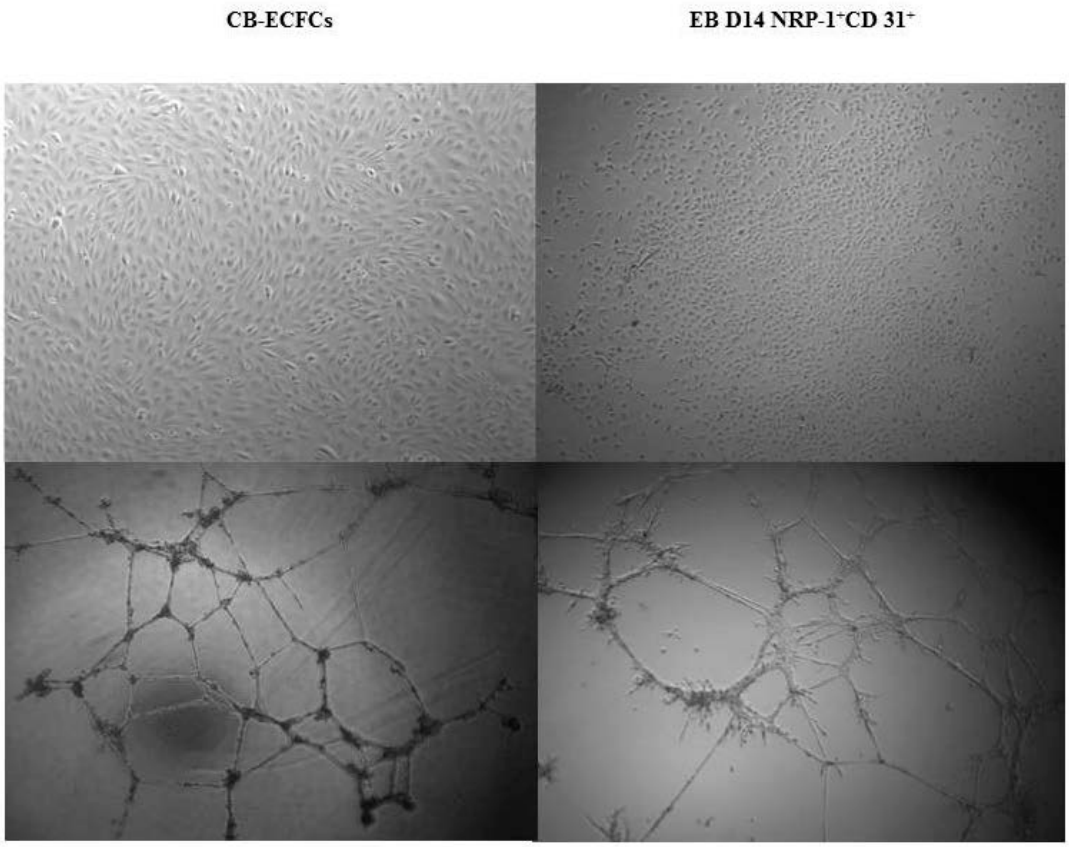

Figure 8. EB derived NRP-1$^+$ CD31$^+$ hES-EC exhibit characteristic CB-ECFC morphology *in vitro* and capillary-like network forming ability on Matrigel coated plates. Cells derived from the EB protocol generate NRP-1$^+$ CD31$^+$ cells that exhibit characteristic EC cobblestone morphology and *in vitro* capillary-like network formation similar to control cord blood ECFC, but in the presence of TGF-□ inhibitor.

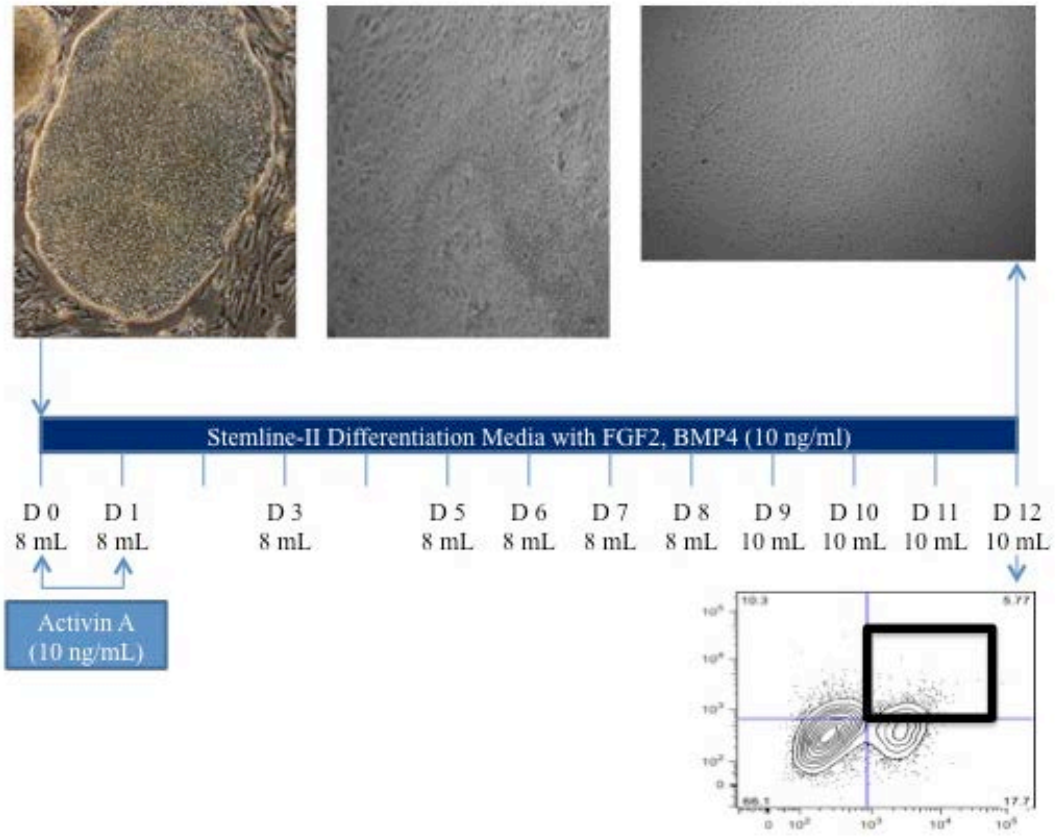

Figure 9. Optimization of hES differentiation into ECFC. Differentiation of hES cells was initiated 2 days after plating and denoted as day 0. Cytokines were distributed in defined media until day 12. On day 12 cells were sorted for $CD31^{+}$ $CD144^{+}$ $NRP\text{-}1^{+}$ phenotype.

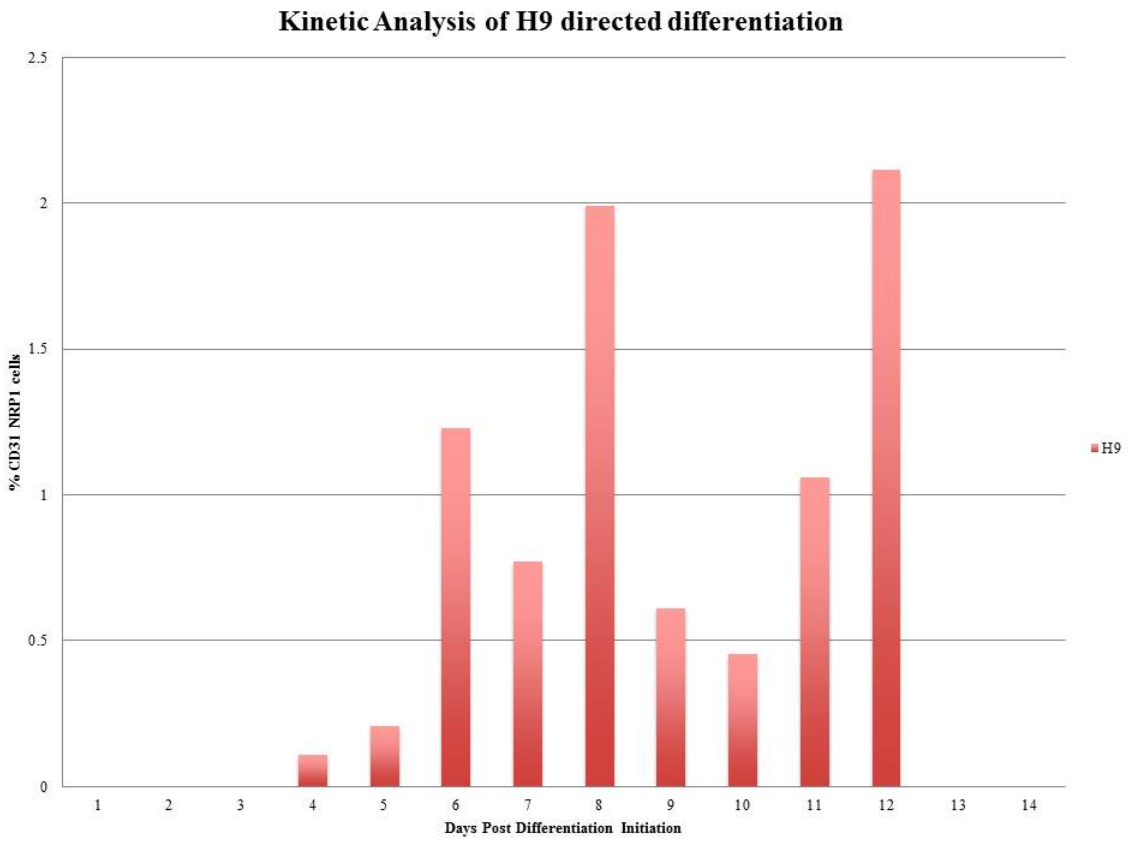

Figure 10. Kinetic Analysis of H9 directed Differentiation. Analysis of $CD31^{+}$ $NRP1^{+}$ $CD144^{+}$ cells present during differentiation. Cells isolated prior to day 10 failed to meet the criteria of an ECFC and did not form vessels *in vivo*. Day 12 shows the optimal time point for efficiently isolating the putative ECFCs based on NRP1 expression. Samples tested at days 6 and 8 failed to generate cells with ECFC-like properties (not shown).

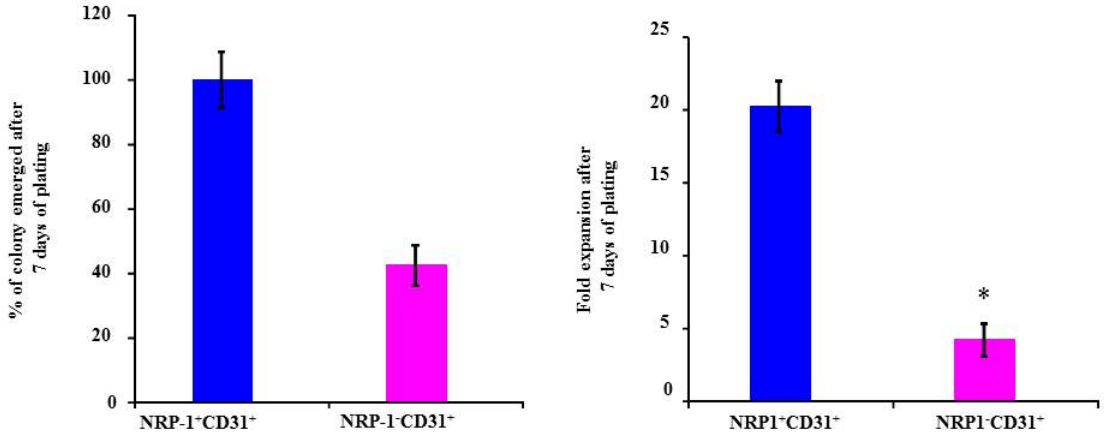

Figure 11. Efficiency of expansion of hES-ECFCs. (A) *left graph* Colonies consistently emerge from isolated CD31 NRP-1 double positive cells. (B) *right graph* Fold expansion is much greater in double positive isolated cells.

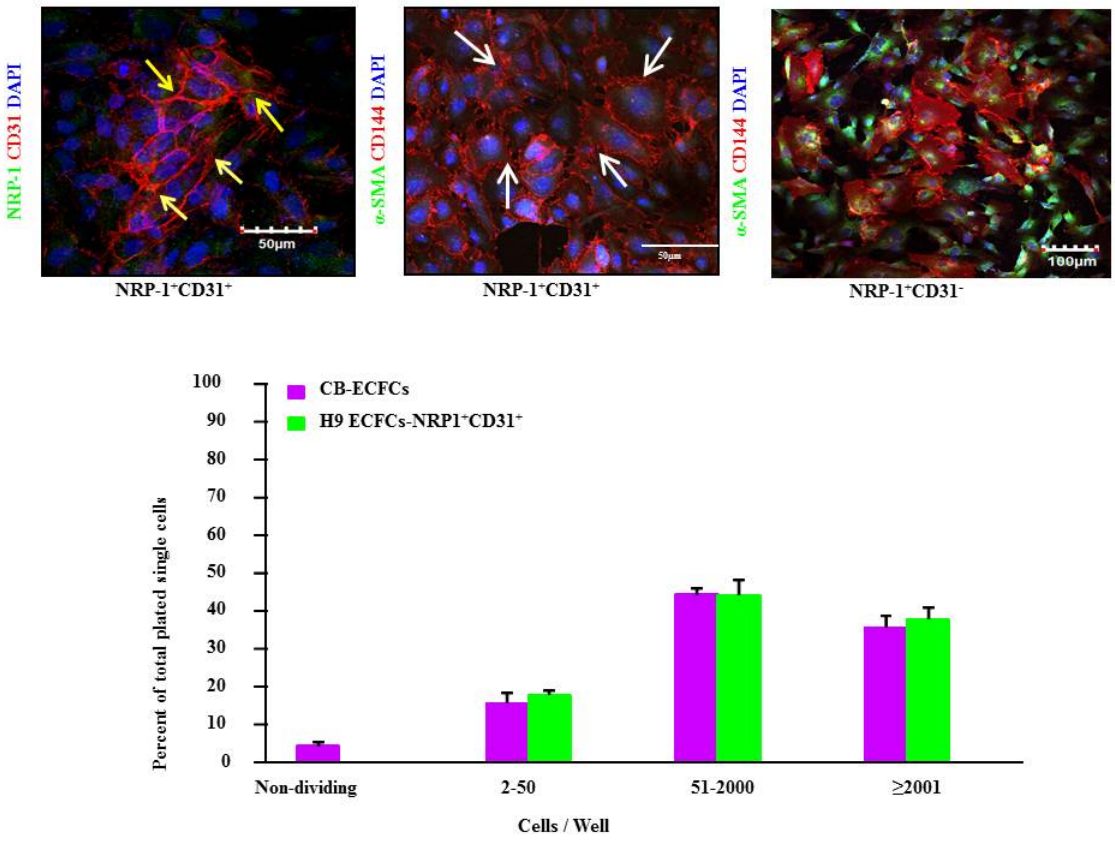

Figure 12. CD31 NRP-1 double positive isolated cells do not express α-SMA and replicate CB-ECFC proliferative potential. (A) *top row* Previous methods had difficulty restricting SMA expression which is not a characteristic of endothelial cells or ECFCs. NRP-1 CD31 double positive cells so not express SMA, however NRP-1^{+}CD31^{-} cells do not. (B) *bottom graph* Double-positive hES-ECFCs exhibit textbook CB-ECFC proliferative hierarchy compared to cord blood ECFC (control).

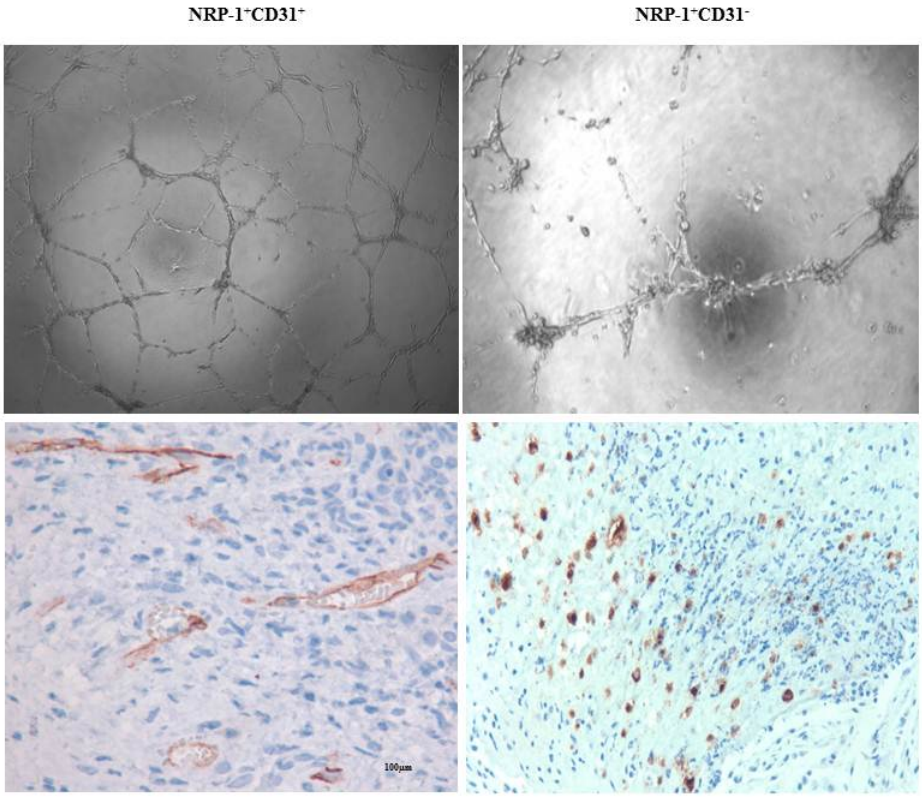

Figure 13. CD31 NRP-1 double positive isolated cells form *in vitro* networks and vessels *in vivo*. (A) While *in vitro* network formation of the Nrp-1+CD31+ cells is robust, NRP-1+CD31- cells fail to form complete networks. (B) Murine red blood cell perfused vessels stained with anti-human CD31 coupled with H&E staining shows the ability of Nrp-$1^{+}$$CD31^{+}$ but not NRP-$1^{+}$$CD31^{-}$ hES-ECFCs to integrate into the host vasculature system

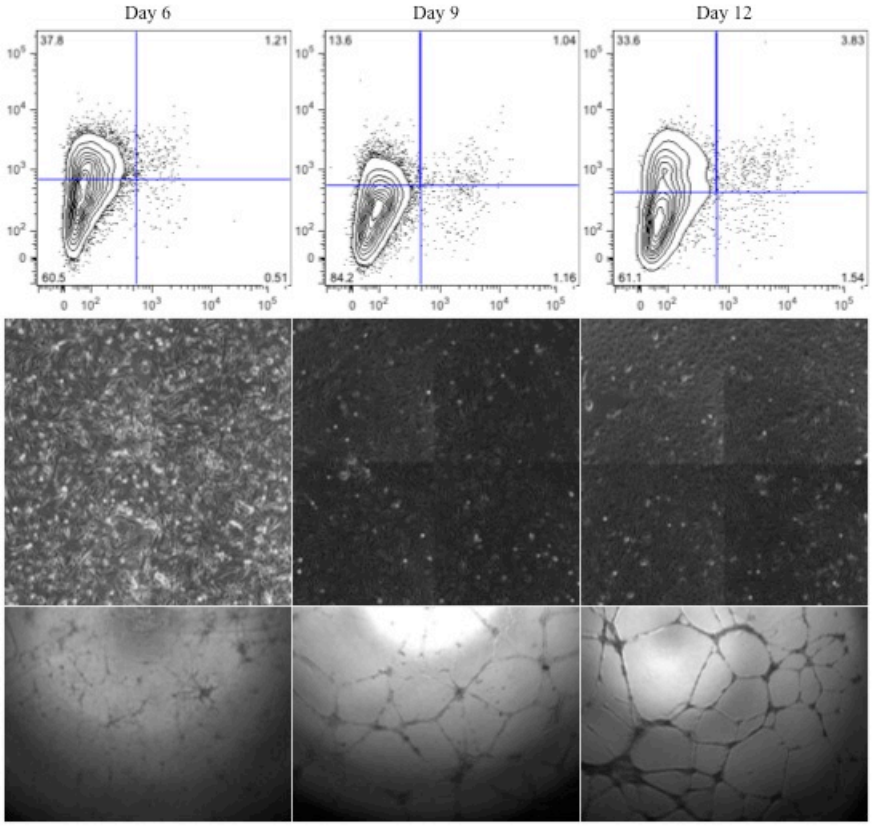

Figure 14. H9 hESC derived ECFC *in vitro* analysis of phenotype and network formation. (A) *Top Row* FACS was used to isolate putative ECFCs at different time points during differentiation using CD31 (x-axis) and NRP1 (y-axis). (B) *Middle Row* Monolayer of isolated putative ECFCs at different time points. (C) *Bottom Row* Matrigel network *in vitro* formation of isolated and expanded putative ECFCs indicate increased ability to form networks towards day 12.

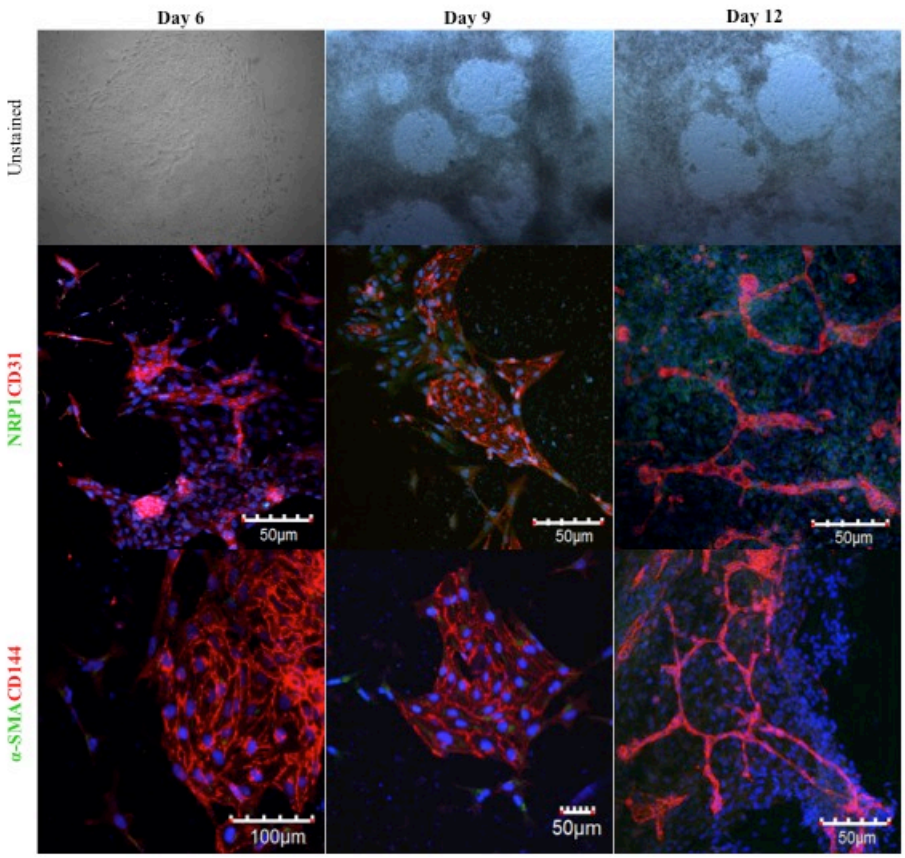

Figure 15. H9 hESC derived ECFC immunohistochemical kinetic analysis of endothelial antigen expression. Differentiation was initiated in 12 well tissue culture dishes and fixed at varying time points. The wells were then stained for endothelial antigens and α-SMA. (A) *top row* unstained control (B) *middle Row* NRP1 staining indicates the increase in expression towards day 12. CD31 staining indicates the increase in expression towards day 12. (C) *bottom Row* αSMA staining indicates the absence of expression at day 12. CD144 staining indicates the increase in expression at day 12 in a pattern reminiscent of capillary-like structures.

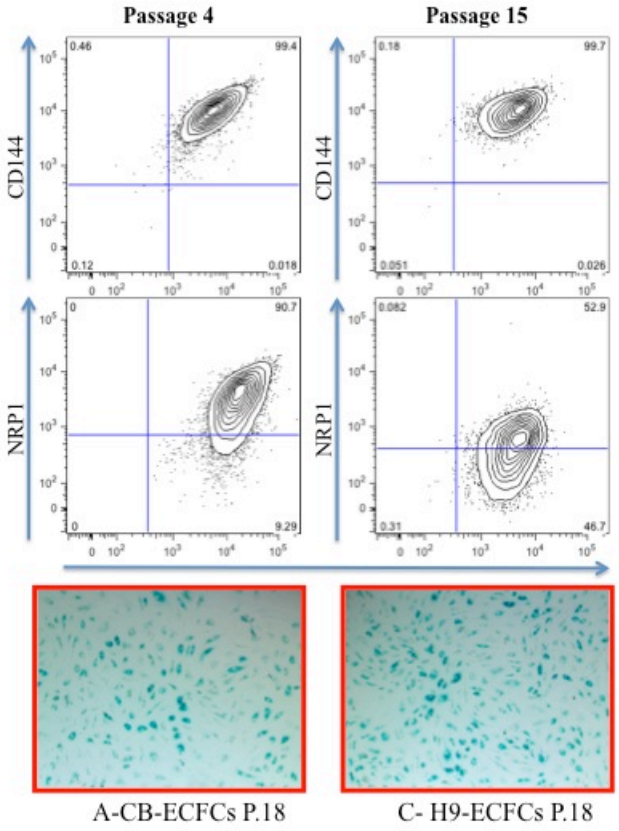

Figure 16. Late Passage Analysis of hESC derived ECFCs. (A) FACS analysis of late passage ECFCs isolated from hESC show decline in NRP1 expression but maintenance of endothelial marker CD31. (B) Late passage isolated ECFCs retain the ability to form vessels *in vivo* in immune compromised mice despite loss of NRP1 expression.

Discussion

We have established a method to reproducibly derive and isolate a homogenous and stable population of endothelial cells possessing umbilical cord blood ECFC-like properties. NRP-$1^{+}$$CD31^{+}$ cells formed a homogenous monolayer with characteristic cobblestone appearance, exhibited high clonal proliferative potential, demonstrated angiogenic behavior by forming complete capillary like structures when cultured on Matrigel, and formed robust *in vivo* inosculated vessels when implanted in immune deficient mice. These hES-derived ECFCs were stable and did not transition to non-endothelial cells over prolonged culture (18 passages) and could expand to over a trillion endothelial cells in less than 3 months of culture.

Timing is essential in the derivation of cell lineages from hES cells. Because of this we developed a detailed protocol and optimized the timing of growth factor delivery to most efficiently isolate ECFCs using the novel marker NRP-1. We found that by optimizing the timing of media alone we were able to substantially increase the efficiency of isolation using this marker. The stepwise differentiation protocol and the use of the double positive expression of NRP-1 and CD31 permitted isolation of cells with ECFC properties for the first time. While endothelial cells have been isolated from human pluripotent cells on many occasions previously, the cells derived failed to display persistent high proliferative potential and were noted to reach senescence after relatively few passages. Some previously reported protocols also required TGF-β inhibition both for the derivation and maintenance of a stable endothelial cell phenotype from differentiated hPS cells[34]. ECFCs generated from our methods exhibit a stable endothelial phenotype and display a complete hierarchy of clonal high proliferative

potential and form robust in vivo functional human vessels that inosculate with host blood vascular system in the complete absence of TGF-β inhibition. Our ECFC differentiation protocol yields one NRP-1^{+}CD31^{+} ECFC per each input hESc after 12 days of differentiation. More importantly, upon expansion, these NRP-1^{+}CD31^{+} cells generate stable highly proliferative potential cells that can undergo long term expansion reaching a ratio of hES/ECFC: 1 to $1.1x10^{8}$ in less than 3 months of culture. This highly efficient output of functional endothelial cells contrasts with reported yields of 0.6[31], 7.4[34] and 11.6[78] endothelial cells from differentiated hPS cells using other published protocols. Thus, our method is highly scalable for potential clinical application and can generate cells sufficient for permitting future clinical trials to regenerate the deficient microvasculature in patients with cardiovascular diseases such as PAD and CLI.

Another key factor in the feasibility of implementation in clinical trials is not only efficacy of the treatments but the potential adverse effects associated with them. The ECFC derived in our protocol are not transformed which is shown by their normal behavior in the characterization of their ECFC qualities. In addition, the cells senesce at comparable passages as CB-ECFCs which indicates that telomere and telomerase activity have not been altered in the directed differentiation of hES-ECFCs. Thus, our culture derived cells are mortal in behavior in vitro and in vivo.

In no instance did these differentiated cells form a teratoma, a benign growth which displays differentiated cell types from all 3 germ layers, when implanted in immunocompromised mice. This proves that these cells are no longer undifferentiated and have assumed a specified state (ECFC state) since all hES cells form teratomas upon

injection as pluripotent cells. This is a key factor which helps to ensure that this cell type when administered to patients would be only positive in nature.

References

1. Ingram, D.A., et al., *Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood.* Blood, 2004. **104**(9): p. 2752-60.
2. Yoder, M.C., et al., *Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals.* Blood, 2007. **109**(5): p. 1801-9.
3. Ingram, D.A., et al., *Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells.* Blood, 2005. **105**(7): p. 2783-6.
4. Au, P., et al., *Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels.* Blood, 2008. **111**(3): p. 1302-5.
5. Critser, P.J., et al., *Collagen matrix physical properties modulate endothelial colony forming cell-derived vessels in vivo.* Microvasc Res. **80**(1): p. 23-30.
6. Melero-Martin, J.M., et al., *Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells.* Circ Res, 2008. **103**(2): p. 194-202.
7. Melero-Martin, J.M., et al., *In vivo vasculogenic potential of human blood-derived endothelial progenitor cells.* Blood, 2007. **109**(11): p. 4761-8.
8. Lin, Y., et al., *Origins of circulating endothelial cells and endothelial outgrowth from blood.* J Clin Invest, 2000. **105**(1): p. 71-7.
9. Ikpeazu, C., et al., *Donor origin of circulating endothelial progenitors after allogeneic bone marrow transplantation.* Biol Blood Marrow Transplant, 2000. **6**(3A): p. 301-8.
10. Huang, L., et al., *Acute myocardial infarction in swine rapidly and selectively releases highly proliferative endothelial colony forming cells (ECFCs) into circulation.* Cell Transplant, 2007. **16**(9): p. 887-97.
11. Meneveau, N., et al., *Presence of endothelial colony-forming cells is associated with reduced microvascular obstruction limiting infarct size and left ventricular remodelling in patients with acute myocardial infarction.* Basic Res Cardiol. **106**(6): p. 1397-410.
12. Massa, M., et al., *Rapid and large increase of the frequency of circulating endothelial colony-forming cells (ECFCs) generating late outgrowth endothelial cells in patients with acute myocardial infarction.* Exp Hematol, 2009. **37**(1): p. 8-9.
13. Moubarik, C., et al., *Transplanted late outgrowth endothelial progenitor cells as cell therapy product for stroke.* Stem Cell Rev. **7**(1): p. 208-20.
14. Lee, J.H., et al., *CD34 Hybrid Cells Promote Endothelial Colony-Forming Cell Bioactivity and Therapeutic Potential for Ischemic Diseases.* Arterioscler Thromb Vasc Biol.
15. Schwarz, T.M., et al., *Vascular incorporation of endothelial colony-forming cells is essential for functional recovery of murine ischemic tissue following cell therapy.* Arterioscler Thromb Vasc Biol. **32**(2): p. e13-21.
16. Bouvard, C., et al., *alpha6-integrin subunit plays a major role in the proangiogenic properties of endothelial progenitor cells.* Arterioscler Thromb Vasc Biol. **30**(8): p. 1569-75.

17. Saif, J., et al., *Combination of injectable multiple growth factor-releasing scaffolds and cell therapy as an advanced modality to enhance tissue neovascularization.* Arterioscler Thromb Vasc Biol. **30**(10): p. 1897-904.
18. Dubois, C., et al., *Differential effects of progenitor cell populations on left ventricular remodeling and myocardial neovascularization after myocardial infarction.* J Am Coll Cardiol. **55**(20): p. 2232-43.
19. Schuh, A., et al., *Transplantation of endothelial progenitor cells improves neovascularization and left ventricular function after myocardial infarction in a rat model.* Basic Res Cardiol, 2008. **103**(1): p. 69-77.
20. Stitt, A.W., et al., *Vascular stem cells and ischaemic retinopathies.* Prog Retin Eye Res. **30**(3): p. 149-66.
21. Medina, R.J., et al., *Outgrowth endothelial cells: characterization and their potential for reversing ischemic retinopathy.* Invest Ophthalmol Vis Sci. **51**(11): p. 5906-13.
22. Solovey, A., et al., *Circulating activated endothelial cells in sickle cell anemia.* N Engl J Med, 1997. **337**(22): p. 1584-90.
23. Reinisch, A., et al., *Humanized large-scale expanded endothelial colony-forming cells function in vitro and in vivo.* Blood, 2009. **113**(26): p. 6716-25.
24. Heiss, C., et al., *Impaired progenitor cell activity in age-related endothelial dysfunction.* J Am Coll Cardiol, 2005. **45**(9): p. 1441-8.
25. Wang, C.H., et al., *Factors associated with purity, biological function, and activation potential of endothelial colony-forming cells.* Am J Physiol Regul Integr Comp Physiol. **300**(3): p. R586-94.
26. Shelley, W.C., et al., *Changes in the frequency and in vivo vessel-forming ability of rhesus monkey circulating endothelial colony-forming cells across the lifespan (birth to aged).* Pediatr Res. **71**(2): p. 156-61.
27. Robbins, R.D., et al., *Inducible pluripotent stem cells: not quite ready for prime time?* Curr Opin Organ Transplant. **15**(1): p. 61-7.
28. Broxmeyer, H.E., et al., *Hematopoietic stem/progenitor cells, generation of induced pluripotent stem cells, and isolation of endothelial progenitors from 21- to 23.5-year cryopreserved cord blood.* Blood. **117**(18): p. 4773-7.
29. Lee, M.R., et al., *Epigenetic regulation of NANOG by miR-302 cluster-MBD2 completes induced pluripotent stem cell reprogramming.* Stem Cells. **31**(4): p. 666-81.
30. Wang, L., et al., *Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties.* Immunity, 2004. **21**(1): p. 31-41.
31. Cho, S.W., et al., *Improvement of postnatal neovascularization by human embryonic stem cell derived endothelial-like cell transplantation in a mouse model of hindlimb ischemia.* Circulation, 2007. **116**(21): p. 2409-19.
32. Choi, K.D., et al., *Hematopoietic and endothelial differentiation of human induced pluripotent stem cells.* Stem Cells, 2009. **27**(3): p. 559-67.
33. Cimato, T., et al., *Neuropilin-1 identifies endothelial precursors in human and murine embryonic stem cells before CD34 expression.* Circulation, 2009. **119**(16): p. 2170-8.

34. James, D., et al., *Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFbeta inhibition is Id1 dependent.* Nat Biotechnol.
35. Levenberg, S., et al., *Endothelial cells derived from human embryonic stem cells.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4391-6.
36. Nakahara, M., et al., *High-efficiency production of subculturable vascular endothelial cells from feeder-free human embryonic stem cells without cell-sorting technique.* Cloning Stem Cells, 2009. **11**(4): p. 509-22.
37. Vodyanik, M.A., et al., *Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential.* Blood, 2005. **105**(2): p. 617-26.
38. Sone, M., et al., *Pathway for differentiation of human embryonic stem cells to vascular cell components and their potential for vascular regeneration.* Arterioscler Thromb Vasc Biol, 2007. **27**(10): p. 2127-34.
39. Taura, D., et al., *Induction and isolation of vascular cells from human induced pluripotent stem cells--brief report.* Arterioscler Thromb Vasc Biol, 2009. **29**(7): p. 1100-3.
40. Rufaihah, A.J., et al., *Endothelial cells derived from human iPSCS increase capillary density and improve perfusion in a mouse model of peripheral arterial disease.* Arterioscler Thromb Vasc Biol. **31**(11): p. e72-9.
41. Yamahara, K., et al., *Augmentation of neovascularization [corrected] in hindlimb ischemia by combined transplantation of human embryonic stem cells-derived endothelial and mural cells.* PLoS One, 2008. **3**(2): p. e1666.
42. Lai, W.H., et al., *Attenuation of hind-limb ischemia in mice with endothelial-like cells derived from different sources of human stem cells.* PLoS One. **8**(3): p. e57876.
43. Ginsberg, M., et al., *Efficient direct reprogramming of mature amniotic cells into endothelial cells by ETS factors and TGFbeta suppression.* Cell. **151**(3): p. 559-75.
44. Goldman, O., et al., *A boost of BMP4 accelerates the commitment of human embryonic stem cells to the endothelial lineage.* Stem Cells, 2009. **27**(8): p. 1750-9.
45. Feng, Q., et al., *Hemangioblastic derivatives from human induced pluripotent stem cells exhibit limited expansion and early senescence.* Stem Cells. **28**(4): p. 704-12.
46. Nishikawa, S.I., et al., *In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos.* Immunity, 1998. **8**(6): p. 761-9.
47. Rossignol, M., J. Pouyssegur, and M. Klagsbrun, *Characterization of the neuropilin-1 promoter; gene expression is mediated by the transcription factor Sp1.* J Cell Biochem, 2003. **88**(4): p. 744-57.
48. Carmeliet, P. and R.K. Jain, *Molecular mechanisms and clinical applications of angiogenesis.* Nature. **473**(7347): p. 298-307.
49. Asahara, T., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. **275**(5302): p. 964-7.
50. Urbich, C. and S. Dimmeler, *Endothelial progenitor cells: characterization and role in vascular biology.* Circ Res, 2004. **95**(4): p. 343-53.

51. Critser, P.J., S.L. Voytik-Harbin, and M.C. Yoder, *Isolating and defining cells to engineer human blood vessels.* Cell Prolif. **44 Suppl 1**: p. 15-21.
52. Matthias, M., N. David, and N. Josef, *From bench to bedside: what physicians need to know about endothelial progenitor cells.* Am J Med. **124**(6): p. 489-97.
53. Reinisch, A. and D. Strunk, *Isolation and animal serum free expansion of human umbilical cord derived mesenchymal stromal cells (MSCs) and endothelial colony forming progenitor cells (ECFCs).* J Vis Exp, 2009(32).
54. Hofmann, N.A., A. Reinisch, and D. Strunk, *Isolation and large scale expansion of adult human endothelial colony forming progenitor cells.* J Vis Exp, 2009(32).
55. Witting, S.R., et al., *Efficient Large Volume Lentiviral Vector Production Using Flow Electroporation.* Hum Gene Ther.
56. Yoon, C.H., et al., *Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases.* Circulation, 2005. **112**(11): p. 1618-27.
57. Norgren, L., et al., *Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II).* Eur J Vasc Endovasc Surg, 2007. **33 Suppl 1**: p. S1-75.
58. Brevetti, G., V. Schiano, and M. Chiariello, *Endothelial dysfunction: a key to the pathophysiology and natural history of peripheral arterial disease?* Atherosclerosis, 2008. **197**(1): p. 1-11.
59. Eberhardt, R.T. and J.D. Coffman, *Cardiovascular morbidity and mortality in peripheral arterial disease.* Curr Drug Targets Cardiovasc Haematol Disord, 2004. **4**(3): p. 209-17.
60. Hirsch, A.T., et al., *Peripheral arterial disease detection, awareness, and treatment in primary care.* JAMA, 2001. **286**(11): p. 1317-24.
61. Wang, Z.Z., et al., *Endothelial cells derived from human embryonic stem cells form durable blood vessels in vivo.* Nat Biotechnol, 2007. **25**(3): p. 317-8.
62. James, D., et al., *Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFbeta inhibition is Id1 dependent.* Nat Biotechnol, 2010. **28**(2): p. 161-6.
63. Djordjevic, S. and P.C. Driscoll, *Targeting VEGF signalling via the neuropilin co-receptor.* Drug Discov Today, 2012.
64. Liu, Y., et al., *Tip110 maintains expression of pluripotent factors in and pluripotency of human embryonic stem cells.* Stem Cells Dev, 2012. **21**(6): p. 829-33.
65. Bailey, J.L., et al., *Collagen oligomers modulate physical and biological properties of three-dimensional self-assembled matrices.* Biopolymers, 2011. **95**(2): p. 77-93.
66. Prasain, N., J.L. Meador, and M.C. Yoder, *Phenotypic and functional characterization of endothelial colony forming cells derived from human umbilical cord blood.* J Vis Exp, 2012(62).
67. Nourse, M.B., et al., *VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering.* Arterioscler Thromb Vasc Biol. **30**(1): p. 80-9.

68. Lu, S.J., et al., *Generation of functional hemangioblasts from human embryonic stem cells.* Nat Methods, 2007. **4**(6): p. 501-9.
69. Carmeliet, P., et al., *Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele.* Nature, 1996. **380**(6573): p. 435-9.
70. Ferrara, N. and T. Davis-Smyth, *The biology of vascular endothelial growth factor.* Endocr Rev, 1997. **18**(1): p. 4-25.
71. Gerber, H.P., et al., *VEGF is required for growth and survival in neonatal mice.* Development, 1999. **126**(6): p. 1149-59.
72. Staton, C.A., et al., *Neuropilins in physiological and pathological angiogenesis.* J Pathol, 2007. **212**(3): p. 237-48.
73. Takashima, S., et al., *Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3657-62.
74. Shalaby, F., et al., *Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice.* Nature, 1995. **376**(6535): p. 62-6.
75. Kitsukawa, T., et al., *Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs.* Development, 1995. **121**(12): p. 4309-18.
76. Evseenko, D., et al., *Mapping the first stages of mesoderm commitment during differentiation of human embryonic stem cells.* Proc Natl Acad Sci U S A. **107**(31): p. 13742-7.
77. Kuilman, T., et al., *The essence of senescence.* Genes Dev. **24**(22): p. 2463-79.
78. Lippmann, E.S., et al., *Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells.* Nat Biotechnol. **30**(8): p. 783-91.

Curriculum Vitae

J. Luke Meador

Education

Indiana University Southeast, New Albany, IN

- Bachelor of Science in Chemistry ACS certified; Degree Awarded 2010
  Specialization: Biochemistry
  Grade Point Average: 3.59 on 4.0 scale

- Bachelor of Arts in Chemistry and Business Management; Degree Awarded 2009
  Specialization: Analytical Chemistry and Business Management
  Grade Point Average: 3.56 on 4.0 scale

Awards

Indiana University School of Medicine, Indianapolis, IN

- T32 Hematopoiesis training fellowship; 2011-2012

Indiana University Southeast, New Albany, IN

- IUS Paul Ogle Scholarship; 2005-2010
  $5000/yr; Awarded for Outstanding High School G.P.A. and SAT scores

- IUS Chemistry Malcholm Kochert Scholarship; 2005-2009
  $1000/yr increasing; Awarded for outstanding High School achievement for the pursuit of a degree in Chemistry

- Outstanding Chemistry Student; 2009
  1 of 2 students selected for excellence in academics

- Dean's List; 2005-2010
  Awarded for obtaining a G.P.A. of 3.5or higher

- Who's Who Among Students in American Universities and Colleges
  1 of 2 Nominated by faculty for excellence in Academics in Chemistry

Teaching Experience

Indiana University Southeast, New Albany, IN

- Teaching Assistant; Summer 2008
  Teaching assistant for Introduction to Chemistry

- Supplemental Instructor; 2007-2010
  Conducted outside tutor sessions at the request of faculty
  Taught: Principles of Economics, Principles of Chemistry, College Algebra

- Math Lab Tutor; 2009-2010
  Provided a friendly and welcoming atmosphere to students in need of help with mathematics.

Activities and Community Service

Floyd Central High School, Floyds Knobs, IN
- Head Diving Coach and Assistant Swimming Coach; 2005-2010

Indiana University Southeast, New Albany, IN
- Chemistry Club Member; 2006-2010

Avon High School, Avon, IN
- Diving Coach; 2011-2012

Indiana University School of Medicine
- Tour the Life Blog: Go Big or Go Home

Publications

Prasain N., **Meador J. L.**, Yoder M. C., Phenotypic and Functional Characterization of Endothelial Colony Forming Cells Derived from Human Umbilical Cord Blood, *Journal of Visualized Experiments*, 2011.

Nutan Prasain*, Man Ryul Lee*, Sasidhar Vemula, **Jonathan Luke Meador**, Momoko Yoshimoto, Michael J. Ferkowicz, Alexa Fett, Manav Gupta, Michael Ginsberg, Brian M. Rapp, Reza Saadatzadeh, Younghee Lee, Michael P. Murphy, Sherry L. Voytik-Harbin, Shahin Rafii, Hal E. Broxmeyer, and Mervin C. Yoder**.** Novel protocol to derive Endothelial Colony Forming Cells from human pluripotent stem cells that display high clonal proliferative potential, extensive replicative capacity, and in vivo vessel forming potential. *In preparation for submission to Nature Medicine,* 2013.