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Derivation of Late Outgrowth Endothelial Progenitor Cells from Adult Human Peripheral Blood Mononuclear Cells

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Short Abstract:

Late outgrowth EPCs have the capacity for clonal growth, and tolerate passaging in culture. These features facilitate the use of late outgrowth EPCs to study the biology of the endothelium in various disease states. The following article describes the methods for their derivation and characterization from adult human subjects.

Long Abstract:

Coronary artery disease is the leading cause of death and heart failure in the United States. Endothelial cells play several critical roles in the pathogenesis of coronary disease: 1) Surface molecules of endothelial cells prevent thrombus formation within the vessel lumen, 2) Endothelial cells regulate local blood flow within the vessel by secretion of substances that regulate vascular tone. Understanding the mechanisms that control the development of endothelial dysfunction and the pathways involved in stimulation of new vessel formation in humans is essential to development of new therapies for coronary artery disease and reversal of heart failure in ischemic heart disease. Endothelial progenitor cells (EPCs) provide a model system to understand the biology of human endothelium under normal conditions and in disease.

EPCs are defined as blood borne cells that form endothelial cells or

contribute to new blood vessel formation *in vivo*. Late outgrowth EPCs are distinguished from early outgrowth EPCs by their growth in culture as colonies over ten to thirty days on collagen coated surfaces. Late outgrowth EPCs will proliferate as colonies when plated in limiting dilution, indicating they have the capacity for clonogenic growth, and also form new blood vessels *de novo* when transplanted *in vivo*. Late outgrowth EPCs also have a distinct transcriptional profile when compared with early outgrowth EPCs and blood monocytes, and have a high degree of overlap with authentic endothelial cells.

Given that late outgrowth EPCs have features of bonafide endothelial cells, they provide an important model system to understand the biology of endothelial cells from humans, can be conveniently obtained through a peripheral venous blood draw, and facilitate studies of how different disease states modify their biology in humans. These features provide an important model system in humans to develop new approaches to normalize dysfunction and growth in disease.

Introduction

Coronary artery disease is the leading cause of heart failure and death in the United States. Endothelial cells play several critical roles in the pathogenesis of coronary disease: 1) surface molecules of endothelial cells prevent thrombus formation within the vessel lumen, 2) endothelial cells regulate local blood flow within the vessel by secretion of substances that regulate vascular tone. Endothelium becomes damaged, dysfunctional, and senescent when exposed to toxins including substances in tobacco smoke, elevated or oxidized lipoproteins, and exposure to elevated shear stress in hypertension (1). These factors impair the normal functions of endothelium resulting in formation of atheromatous plaque and in some cases occlusion of the vessel lumen due to thrombus formation. Once a vessel is occluded by thrombus or atheromatous plaque, the cardiac tissue surrounding the blocked blood vessel will lose oxygen supply, and contractile function, resulting in heart failure symptoms. Understanding the mechanisms that control the development of endothelial dysfunction and the pathways involved in stimulation of new vessel formation in humans is essential to development of new therapies for coronary artery disease and reversal of heart failure in ischemic heart disease. Endothelial progenitor cells (EPCs) provide a model system to understand the biology of human endothelium under normal conditions and in disease.

EPCs are rare (0.0001% of blood mononuclear cells), blood borne cells that form endothelial cells or contribute to new blood vessel formation *in vivo* (2). Ashahara and co-workers (3) initially described EPCs as a population of cells present in the mononuclear cell fraction of blood that bears features of endothelium when cultured under permissive conditions, and show perivascular incorporation *in vivo*. Subsequent work showed that there are at least two distinct types of endothelial progenitor cells, defined by their growth properties in culture (4). Early outgrowth EPCs are defined by formation of colonies in culture after less than seven days in culture on fibronectin coated surfaces (5), and cell surface expression of the antigens CD45, CD133 and VEGFR2. Recent evidence

showed that early outgrowth EPCs contribute to the formation of new vessels by facilitating angiogenesis but cannot form *de novo* blood vessels *in vivo* (6). Additionally, early outgrowth EPCs have marker expression and biologic activities consistent with a monocyte lineage hematopoietic stem cell (7) including the ability to phagocytose bacteria (6).

Late outgrowth EPCs are distinguished from early outgrowth EPCs by their growth in culture as colonies over ten to thirty days (depending on the age of the donor's mononuclear cells) (6) on collagen coated surfaces. Late outgrowth EPCs will proliferate as colonies when plated in limiting dilution, indicating they have the capacity for clonogenic growth, and will form new blood vessels de novo when transplanted *in vivo* (6). Very few specific molecular markers distinguish early and late outgrowth EPCs from each other, and there is overlapping expression of endothelial cell surface molecules in both cell types. Late outgrowth EPCs can be purified from blood mononuclear cells by sorting for positive expression of CD34 and CD146, and the absence of CD45 (8). However, late outgrowth EPCs have a distinct transcriptional profile when compared with early outgrowth EPCs and blood monocytes, indicating their biologic features are clearly different and have a high degree of overlap with authentic endothelial cells (9).

Due to their relatively recent discovery and characterization, the biology of late outgrowth EPCs, and their responses to normal and abnormal physiologic stimuli remain to be defined. While the effects of cardiovascular disease risk factors on the quantity of early outgrowth EPCs (10, 11) have been established, similar experiments have not been performed to determine how CVD risk factors affect the quantity and angiogenic activity of both early and late outgrowth EPCs. The interrelationship of the early and late outgrowth EPCs in disease remains to be determined in detail, and how the interplay of these two cell types may be altered to either regulate vessel formation, or be impaired in disease. For example, the effects of moderate to high concentrations of high-density lipoprotein on cell viability and senescence has been explored using both early and late outgrowth EPCs in culture (12). In another example, the quantity of both CD34+ CD133+ VEGFR2+ EPCs and late outgrowth EPCs were quantified in the blood of patients with rheumatoid arthritis, and the quantity of both cell types correlated with disease activity (13). The culture of late outgrowth EPCs may also hold promise to identify patients at increased risk for thrombosis in myeloproliferative neoplasms. In a retrospective study, increased late outgrowth EPCs correlated with patients that had a history of blood clotting disorders (14). While a prospective test of this concept is required, the finding suggests that the biology of the late outgrowth EPC may provide clinically useful information that may be used to alter therapy for patients to prevent disease. While late outgrowth EPCs are rare in adult human blood and challenging to isolate in culture, a particular advantage of the late outgrowth EPC is that the cells can be passaged while the early outgrowth EPCs cannot, facilitating characterization of the effects of disease on endothelial cells in disease. Evaluation of the biology of both early and late outgrowth EPCs will be important to understand the biology of human endothelium and regeneration in disease states.

Given that late outgrowth EPCs have features of bonafide endothelial cells, they provide an important model system to understand the biology of endothelial cells from humans, can be conveniently obtained through a peripheral venous blood draw, and facilitate studies of how different disease states modify their biology in humans. These features provide an important model system in humans to develop new approaches to normalize dysfunctional endothelial cells in disease, and regulate their growth in ischemic and malignant disease states. The following article describes the methods for isolation and characterization of late outgrowth endothelial progenitor cells from adult humans and in a large animal model of cardiovascular disease using pigs (15). Importantly, our goal is to standardize the approach to derivation of late outgrowth EPCs, and place our approach with adult human blood in the context of other leaders in the field of late outgrowth EPC biology to provide insights into our approach that may be compared and contrasted with the approach of others in the field (16, 17).

Protocol Text:

1.) Pre Study Considerations

1.1) Obtain approval for human subjects studies from the local institutional review board, and obtain informed consent from study subjects. The results shown were obtained under an approved protocol with the University at Buffalo Health Sciences IRB (MED6690710E). For studies of EPCs involving animals, obtain approval to obtain blood from animals from your institutional IACUC panel.

2) Preparation of Culture Medium and Coating of Cultureware

2.1) Prepare the EGM-2 culture medium with all growth factor supplements provided for growth of late outgrowth EPCs. Remove 50 mL of the culture medium from the bottle and replace with 40 mL of fetal bovine serum for a final concentration of 10% FBS. Batch testing of fetal bovine serum may be required as not all lots of FBS support growth of late outgrowth EPCs. Add the growth factor supplements to the vial of FBS provided, then add directly to the medium. The EGM-2 medium should be prepared at least monthly for optimal results.

2.2) Prepare rat tail collagen solution. Add glacial acetic acid (0.575 mL) to sterile, endotoxin free water (495 mL) and sterile filter. Rat tail collagen solution is added to the diluted acetic acid solution to obtain a final concentration of 50 μg/mL. Consult the spec sheet for your specific lot of rat tail collagen to determine its concentration. The collagen solution is then added to culture dishes (2mL per well of a six well dish). Incubate dishes with collagen solution for 24 hours prior to plating of mononuclear cells in a humidified incubator.

3.) Preparation of Blood Mononuclear Cells

3.1) Blood mononuclear cells are obtained using standard venipuncture methods.

3.2) Collect 10 tubes of venous blood in Vacutainer CPT tubes. Gently mix the blood with the citrate fluid by inversion. DO NOT CHILL the collected blood in the CPT tubes. This will impair separation of red blood cells in the Ficoll density gradient.

4.) Centrifugation of Blood Mononuclear Cells

4.1) Spin the blood collection tubes at 1500 x g for 30 minutes at room temperature with no brake to prevent disturbing the separated layers of blood.

4.2) Blood collection tubes should be opened in a biosafety cabinet. Pipette the plasma layer from the collection tubes. Avoid disturbing the plasma monocyte/lymphocyte band. Use a 1000 μL pipettor removing 500μL with each aspiration. Dispense the plasma into a waste container using sterile technique.

5.) Washing the Blood Mononuclear Cell Fraction

5.1) Carefully collect the lymphocyte/monocyte fraction from the tube, avoiding any debris or particulates that may not have spun through the Ficoll cushion, and dispense the cells into a separate 50mL conical tube.

5.2) From ten tubes of blood, the volume of lymphocytes/monocytes is usually 10 mL. Slowly add EGM-2 medium with 10% FBS (pre-warmed to 37C) to the lymphocyte/monocyte fraction and mix the medium with the cells by gentle swirling of the tube. Bring the volume of medium up to 40 mL using additional EGM-2 medium. Pellet the lymphocytes/monocytes by centrifugation at 300 x g for 15 minutes. Gently resuspend the pellet again in 10 mL of EGM-2. Wash the cells three times.

5.3) Remove a small aliquot of the cell preparation for cell counting after the third wash of the cells. 10-50 x 10^6 mononuclear cells are obtained from ten tubes of venous blood.

6) Plating of Mononuclear Cells for Culture of Late Outgrowth EPCs

6.1) Remove the collagen solution from pre-coated culture dishes and wash the wells once with EGM-2 medium with 10% FBS to remove residual acetic acid.

6.2) Gently transfer 10 x 10^6 cells of the lymphocyte/monocyte fraction in a 2.5mL volume to one well of a six well plate. Distribute the cells throughout the culture well and return the dish to the culture incubator.

7) Culture of Mononuclear Cells for Derivation of Late Outgrowth EPC Colonies

7.1) After one day in culture, remove 80-90% of the culture medium by

aspiration. Wash the well once with fresh EGM-2 + 10% FBS containing medium (2.5 mL), then resume culture of the cells at 37°C/5% CO2.

7.2) Change the medium daily for the first seven days of culture. Then medium changes can be switched to every other day until colonies are visualized.

7.3) Review the culture wells with a phase contrast microscope using the 2X or 4x objective. After three to four weeks in culture, colonies should appear.

8) Subculture of Late Outgrowth Endothelial Progenitor Cells

8.1) Wash the culture well with pre-warmed PBS three times. Cloning cylinders can be placed around the identified LOEPC colonies to assure only these cells are passaged. Add trypsin (0.2mL per cloning cylinder) for 3 minutes at 37C. Observe the cells to assure they have rounded up after trypsin treatment.

8.2) Add 0.2 mL of EGM-2 medium containing 10% FBS to the cloning cylinder, or 0.5 mL medium to the culture dish if cloning cylinders are not used. LOPEC colonies are aspirated directly off the culture dish using a pipettor. Collect the dissociated cells into a 15 mL conical tube. Pellet the cells by centrifugation at 300 x g for 7 minutes.

8.3) Carefully remove the supernatant and resuspend the cells in 4mL EGM-2 medium. Plate the cells into two collagen coated culture wells.

9) Characterization of Late Outgrowth Endothelial Progenitor Cells by Microscopy

9.1) Grow late outgrowth EPCs to near confluence on a 24 well dish as above.

9.2) Wash cells three times with PBS (pre-warmed to 37°C), then fix cells by incubation with undiluted methanol (500 μL per well, pre-chilled to -20°C) for 15 minutes. Incubate cells at 4°C during fixation period. Then carefully remove methanol and rehydrate cells in pre-warmed PBS.

9.3) Block non-specific antibody binding by incubation in 5% donkey serum in PBS (blocking solution) for 1 hour at 4°C.

9.4) Remove blocking solution and replace with PBS. Add antibodies (listed in Materials Table,1:100 dilution). Incubate at 4°C for at least two hours.

9.5) Remove primary antibody solution. Wash cells three times in pre-warmed PBS incubating 5 minutes per wash.

9.6) Add secondary antibodies (listed in Materials Table, 1:500 dilution). Incubate cells at 4°C for one hour. Then aspirate secondary antibody solution. Wash cells

twice in pre-warmed PBS incubating 5 minutes per wash.

9.7) Add bis-benzamide to counterstain nuclei (1:500 dilution); incubate at room temperature for 10 minutes. Remove bis-benzamide solution and replace with warm PBS.

9.8) Visualize with an immunofluorescence microscope.

Representative Results:

The number of late outgrowth EPC colonies derived from each subject varies between individuals, and between blood draws due to the fact that the cells are a rare event in the blood stream (0.0001% of mononuclear cells). Most subjects will derive one to two colonies from 80 mLs of blood. In our experience, we derive 1.9 ± 0.4 (mean $\pm$ SEM, n=10) colonies per subject from 80 mL of blood, with an efficiency of 36%. Late outgrowth EPC colonies are easily distinguished from the surrounding background of mononuclear cells as darker, flatter, and spindle shaped than the surrounding round, phase bright, mononuclear cells (Figure 1A and B). Figure 1A provides an example of the earliest stages of colony outgrowth of late outgrowth EPCs, and Figure 1B shows a more fully developed colony. Late outgrowth EPC colonies will appear in cultures after three to five weeks in culture. When late outgrowth EPC colonies are not obtained in culture only the background of phase bright mononuclear cells are obtained. A lack of late outgrowth EPC colony formation, or lack of detection of endothelial specific antigens may suggest a problem with reagents (growth medium, serum, or collagen solution). Troubleshooting steps for the technique are outlined in the Discussion section.

After late outgrowth EPC colonies are derived and expanded at least one passage they must be characterized to assure their endothelial cell type, and lack of leukocyte markers. Figure 2 shows representative immunofluorescence microscopy images of late outgrowth EPCs obtained from normal human subjects after one passage in culture. The cells should express the cell surface antigens PECAM, CD34, CD146 and VE-Cadherin as illustrated in Figure 2. Additionally, intracellular von Willebrand Factor can be visualized as intracellular needle like structures that are Weibel-Palade bodies containing the protein, and are a hallmark of endothelial cells (18). Cells that do not express these antigens may represent contamination of dermal fibroblasts, mesenchymal cells or monocytes. In our hands other cell types are not obtained using the culture conditions and colony focused passaging techniques described.

Flow cytometry should be used to assess the purity and quality of the late outgrowth EPCs derived after passaging of initial outgrowth colonies. Live cells are stained in suspension using previously described methods (19). Both unstained cells and cells incubated with mouse IgG controls (APC, FITC or PE) are used to determine background fluorescence of the cells (Figure 3, panel A). Figure 3, panel B shows representative results of flow cytometry analysis of late outgrowth EPC cultures stained for the endothelial cell antigens PECAM,

VEGFR2, CD146, and CD34. The derived cells homogeneously express these endothelial cells antigens, while lacking expression of markers found in leukocytes or monocytes (CD45, CD133 or CD14) shown in Figure 3 panel C. Additional markers of mesenchymal cell types (CD90, Stro-1, and NG2-proteoglycan) were also negative (not shown).

Table and Figure legends:

Figure 1-Representative Late Outgrowth Endothelial Progenitor Colonies Derived from Adult Human Peripheral Blood Mononuclear Cells. Representative phase contrast images of late outgrowth EPC colonies after three (Panel A) and four (Panel B) weeks in culture from the same individual. Late outgrowth EPC colonies appear phase dark, flat and spindle shaped, and grow in clusters, while the surrounding monocytes are round, phase bright, and randomly scattered throughout the culture dish. Results are representative of findings from >5 adult human subjects. Scale bars indicate 200 μm.

Figure 2-Representative Immunofluorescence Photomicrographs of Adult Human Late Outgrowth Endothelial Progenitor Cells. Late outgrowth EPCs from an adult human subject stained with antibodies to the endothelial specific antigens PECAM, CD34, CD146, VE-Cadherin, and von Willebrand Factor (vWF)**.** The arrows denote vWF staining in a rod-like configuration consistent with Weibel-Palade bodies. Nuclei are stained with bis-benzamide. Results are representative of findings from >5 adult human subjects. Scale bars indicate 100 μm.

Figure 3-Representative Flow Cytometry Analysis of Adult Human Late Outgrowth Endothelial Progenitor Cells. Late outgrowth EPCs were harvested for flow cytometry analysis. Live cells were analyzed unstained, or with Mouse IgG-FITC, APC or PE conjugated antibodies (Panel A). Quadrants were set to background fluorescence levels. Panel B illustrates representative flow cytometry findings of LOEPCs stained with conjugated antibodies to typical endothelial cell antigens (PECAM-FITC, VEGFR2-APC, CD146-FITC, CD34-APC). Panel C importantly shows that LOEPCs lack the pan leukocyte antigen CD45, CD133, or the monocyte marker CD14. Results shown are representative of findings in >5 human subjects.

Discussion:

The method for derivation, sub culturing, and characterization of adult human late outgrowth EPCs detailed provides a means to study the biology of human endothelial cells obtained by a simple venous blood draw. The important advantage of the late outgrowth EPC model versus early outgrowth EPCs includes the ability to passage the cells beyond initial derivation. Importantly, late outgrowth EPCs provide the only source of endothelial cells from blood that will

form functional vessels when transplanted in vivo, and can be serially passaged, while early outgrowth EPCs do not possess these capacities (6).

In terms of the derivation of late outgrowth EPCs, there are several critical points to emphasize that may improve the reproducibility and reliability of the experimental model: 1) The age of the reagents used (less than one month) and screened lots of fetal bovine serum are critical to the success of this method in our hands. 2) The duration of culture needed to obtain late outgrowth EPC colonies generally depends on the age of the donor blood mononuclear cells. For umbilical cord blood mononuclear cells, colonies may be initially visualized after seven to ten days. Mononuclear cells from adult human donors require three to four weeks of culture before a clearly distinguishable colony can be identified. 3) Generally one small to medium size colony containing several hundred cells can be replated to one well of a six well culture dish without inducing cell cycle arrest. Smaller colonies may be replated to smaller culture vessels if needed. Generally late outgrowth EPCs obtained from adult humans will tolerate replating to passage 3 or 4 without becoming senescent in fetal bovine serum containing medium. We have had success passaging cells to passage 9 using EBM2 medium supplemented with human platelet lysate with no appreciable changes in the quality or character of the cells (20).

If no late outgrowth EPCs are obtained from a subject there are several troubleshooting steps to proceed through. 1) Assure that the EGM-2 medium is less than one month old. The medium contains recombinant growth factors that will degrade over time when diluted in medium. 2) Determine if the rat-tail collagen solution used to coat the culture dishes is less than 1 month old as this may decay in solution as well. 3) Some subjects have fewer numbers of late outgrowth EPCs per 80 mL of blood. It may require a larger volume of blood sampling to obtain late outgrowth EPC colonies. 4) Some lots of fetal bovine serum to not support the growth of late outgrowth EPCs. Two control experiments to perform lot testing on include deriving late outgrowth EPCs from a donor that has produced colonies previously, or derivation of the cells from umbilical cord blood mononuclear cells. Late outgrowth EPC colonies are more frequent and readily derived from this cell preparation. 5) Another possible outcome is derivation of large appearing endothelial cells containing vacuoles. This generally is observed when there are issues with the EGM-2 medium, either the medium is not fresh or the serum batch is not supportive of cell growth.

There are several potential limitations of the late outgrowth EPC model. While late outgrowth EPCs are a convenient model to study the biology of these circulating endothelial progenitors in culture, at this point it is unclear what their origin is, and if they contribute significantly to new vessel formation after injury in vivo.

How the biology of late outgrowth EPCs relates to endothelial cells in specific tissues of including coronary, aortic, pulmonary, or other organ arterial or venous endothelial cells remains to be determined. Tissue specific features of endothelium varies between organs and it is unknown if these features are acquired by the environment or intrinsic to the tissue type they are derived from. Future work to determine the origin of late outgrowth EPCs and their contribution

to endothelial regeneration in adults will require cell type specific labeling methods that have not been developed to date. The model may prove to be clinically relevant to predict future cardiovascular disease risk based on the quantity of cells obtained, number of colonies obtained from initial outgrowth, or replating efficiency. Future applications of the cells may include a model to perform drug screening assays to identify novel pathways that may prevent or reverse the onset of senescence in human endothelium; a feature of the development of atherosclerosis in vivo. Additionally important clues regarding the stimuli that may augment or suppress their growth, and how age impacts the proliferative capacity of neonatal cord blood derived late outgrowth EPCs versus adult late outgrowth EPCs remain important questions to be answer about these cells, and provide important insights into the biology of human endothelium in aging and disease.

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Disclosures (Conflict of Interest Statement):
None

Table of Equipment and Reagents

Name of Reagent/Material	Company	Catalog Number
Collagen I, rat tail	BD Biosciences	354236
EGM-2 Bullet Kit	Lonza	CC-3162
BD Vacutainer CPT tube with sodium citrate	BD Biosciences	362761
6 well culture plate	BD Biosciences	353934
Defined FBS	Hyclone	SH30070.01
0.05% Trypsin-EDTA (1X), Phenol Red	Invitrogen	25300054
Sterile Cloning Cylinders	Fisher Scientific	07-907-10
Phosphate Buffered Saline	Invitrogen	14190250
Trypan Blue Stain	Invitrogen	15250-061
Glacial Acetic Acid	Fisher Scientific	A38-500
Sample Needles & Collection sets	BD Bioscienes	367344
Vacutainer Holder	BD Biosciences	364815
Methanol	JT Baker	9070-01
Normal Donkey Serum	Millipore	S30-100mL
Bis-benzamide	Invitrogen	H3569
Mouse anti-human PECAM (Clone P2B1)	Santa Cruz Biotechnology	sc-20071
Mouse anti-human CD146 (Clone P1H12)	Santa Cruz Biotechnology	sc-18837
Mouse anti-human CD34 (Clone QBEnd/10)	Santa Cruz Biotechnology	sc-52312
Goat anti-human VE-Cadherin	Santa Cruz Biotechnology	sc-6458
Alexa Fluor 488 Donkey anti-Mouse IgG	Invitrogen	A-21202
Alexa Fluor 555 Donkey anti-Goat IgG	Invitrogen	A-21432
Mouse IgG1-APC	BD Biosciences	550854
Mouse IgG1-FITC	BD Biosciences	349041
Mouse IgG1-PE	BD Biosciences	555787
Mouse anti human CD34-APC	BD Biosciences	340441
Mosue anti-human CD133-PE	Miltenyi Biotec	130-080-801
Mouse anti-human CD31 FITC	eBioscience	11-0319-42
Mouse anti-human CD146 FITC	Santa Cruz Biotechnology	sc-18837 FITC
Mouse anti-human VEGFR2 APC	R&D Systems	FAB357A
Mouse anti-human CD45 FITC	BD Biosciences	347463
Mouse anti-human CD14 FITC	eBioscience	8011-0149-120

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*Figure 1
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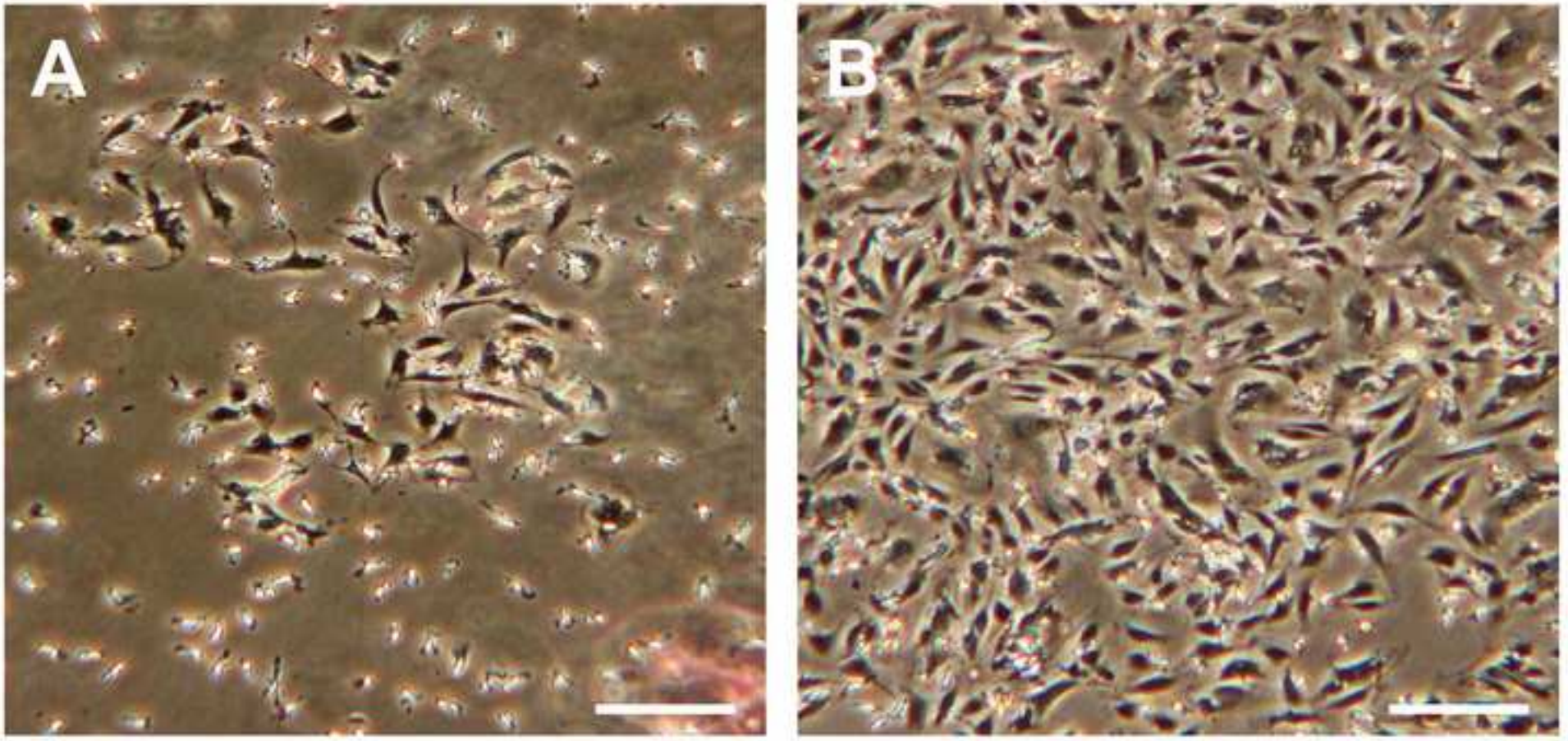

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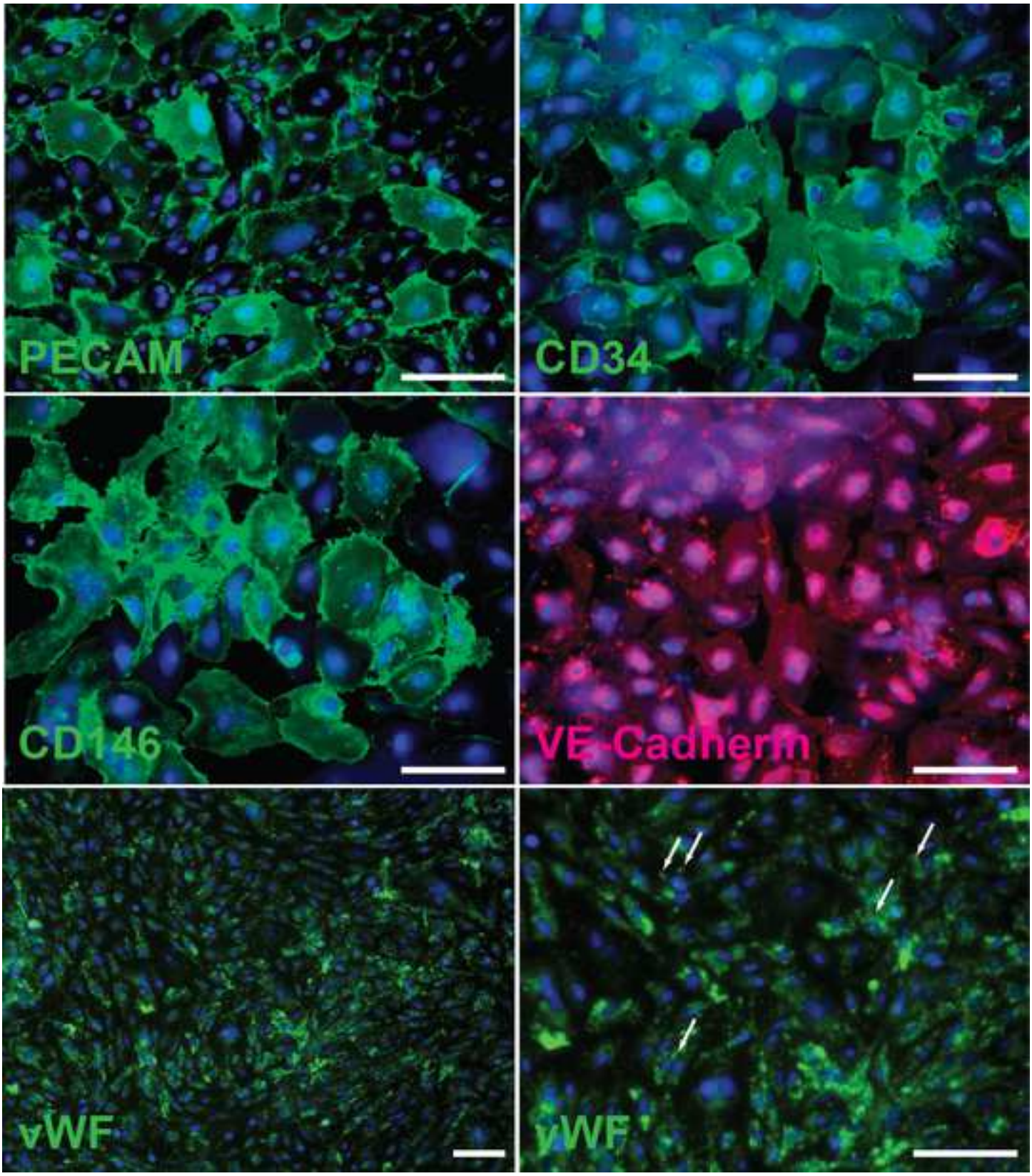

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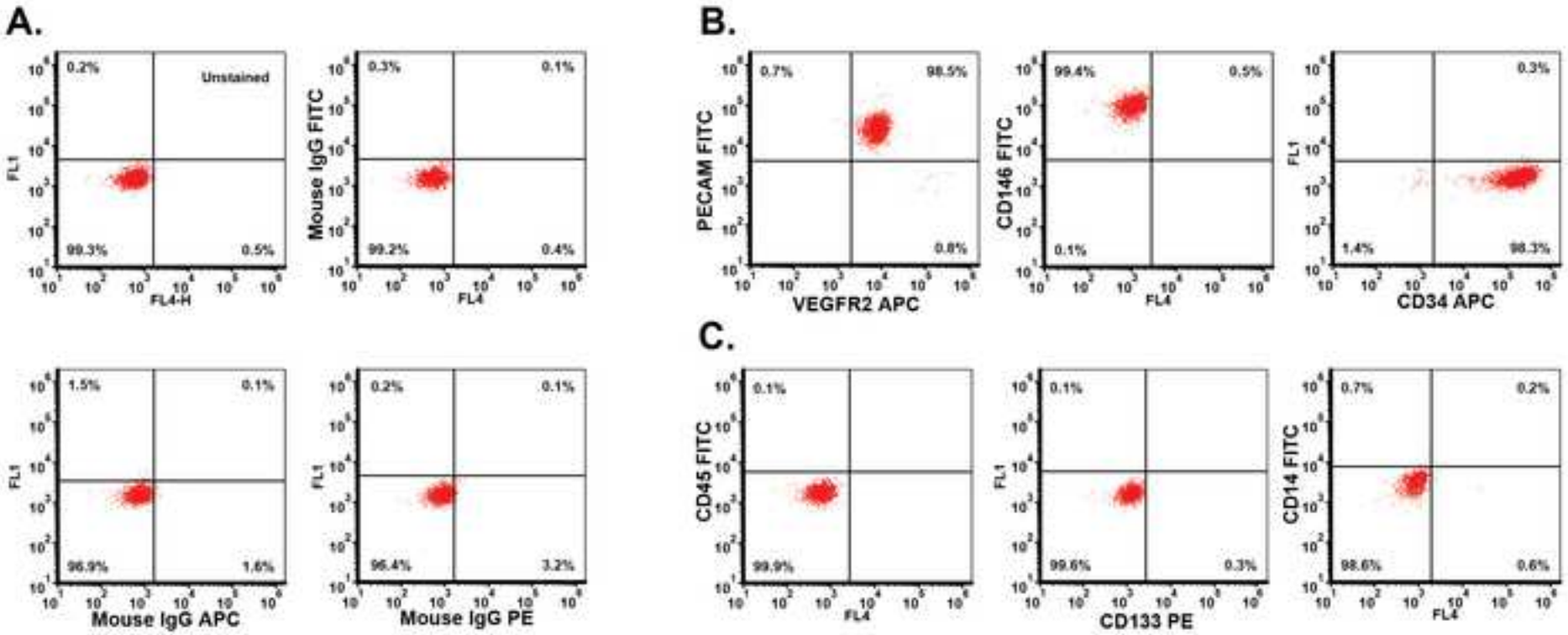

*Table of Reagents/ Materials Used
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Table of Equipment and Reagents

Name of Reagent/Material	Company
Collagen I, rat tail	BD Biosciences
EGM-2 Bullet Kit	Lonza
BD Vacutainer CPT tube with sodium citrate	BD Biosciences
6 well culture plate	BD Biosciences
Defined FBS	Hyclone
0.05% Trypsin-EDTA (1X), Phenol Red	Invitrogen
Steriile Cloning Cylinders	Fisher Scientific
Phosphate Buffered Saline	Invitrogen
Trypan Blue Stain	Invitrogen
Glacial Acetic Acid	Fisher Scientific
Sample Needles & Collection sets	BD Bioscienes
Vacutainer Holder	BD Biosciences
Methanol	JT Baker
Normal Donkey Serum	Millipore
Bis-benzamide	Invitrogen
Mouse anti-human PECAM (Clone P2B1)	Santa Cruz Biotechnology
Mouse anti-human CD146 (Clone P1H12)	Santa Cruz Biotechnology
Mouse anti-human CD34 (Clone QBEnd/10)	Santa Cruz Biotechnology
Goat anti-human VE-Cadherin	Santa Cruz Biotechnology
Mouse anti-human Von Willebrand Factor	Dako
Alexa Fluor 488 Donkey anti-Mouse IgG	Invitrogen
Alexa Fluor 555 Donkey anti-Goat IgG	Invitrogen
Mouse IgG1-APC	BD Biosciences
Mouse IgG1-FITC	BD Biosciences
Mouse IgG1-PE	BD Biosciences
Mouse anti human CD34-APC	BD Biosciences
Mosue anti-human CD133-PE	Miltenyi Biotec
Mouse anti-human CD31 FITC	eBioscience
Mouse anti-human CD146 FITC	Santa Cruz Biotechnology
Mouse anti-human VEGFR2 APC	R&D Systems
Mouse anti-human CD45 FITC	BD Biosciences
Mouse anti-human CD14 FITC	eBioscience

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Author(s): Jennifer K. Lang and Thomas R. Cimato

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Article Title: Derivation and Characterization of Late Outgrowth Endothelial Progenitor Cells from Adult Human Peripheral Blood Mononuclear Cells

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February 20, 2013

Angela Messmer-Blust PhD
Science Editor, Clinical and Translational Medicine
Journal of Visualized Experiments
17 Sellers St.
Cambridge, MA 02139

Dear Angela,

I would like to re-submit our manuscript entitled “Derivation of Late Outgrowth Endothelial Progenitor Cells from Adult Human Peripheral Blood Mononuclear Cells” for review and publication after peer review. I have carefully considered the reviewers comments and have made adjustments to the manuscript text, as well as provided additional data that strengthen the manuscript. In the text below I will address the comments provided by the four reviewers.

Editorial comment; “Figures: missing scale bars on figures 1 and 2”

I have added scale bars to figures 1 and 2 as requested. The size of the scale bars is provided in the figure legends.

Reviewer’s comments (I indicated the reviewers comments in bold and follow the comments with my response):

Reviewer 1: “Unfortunately, the Methods section is sloppy and lacks sufficient detail as to the antibodies used. Here FACS analysis to validate the imaging results and to clearly distinguish the two populations is interesting…In Fig. 2 especially (isotype/or a negative control-unstained control would be useful), esp. in a Methods paper. Also which antibodies are used?”

We appreciate the frank nature of the reviewer’s comments and agree with the conclusion that the protocol is not clear regarding details to characterize late outgrowth endothelial progenitor cells. Proper assessment of the late outgrowth EPCs is a critical quality control step to assure the cells obtained are truly an endothelial cell, match the results of others in the literature, and do not express markers found in lymphocytes or monocytes.

I have addressed the reviewer’s criticism in two ways: **1)** I have added an additional pair of images to Figure 2 illustrating expression of von Willebrand Factor protein in late outgrowth EPC cultures at low and high magnification to illustrate two points; A) the expression of intracellular von Willebrand Factor (vWF) is noted throughout the culture which supports that the derived cells are homogenously endothelial in nature. B) The higher magnification image of cells stained with vWF show a hallmark property of endothelial cells; concentration of the protein in punctate needle like structures known as Weibel Palade bodies, a feature specific to endothelial cells. **2)** I have included representative flow cytometry analysis of late outgrowth EPCs from one individual (Figure 3). The figure includes unstained cells and cells stained with Mouse IgG conjugated with APC, FITC and PE to assess the background staining of the cells. Data are also provided to indicate the cells express hallmark antigens of endothelium, and importantly

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lack expression of lymphocyte and monocyte antigens. We feel the combination of the added microscopy data showing intracellular vWF and homogenous nature of the cultures by flow cytometry provide evidence of the robust nature of the protocol. We do not observe two populations of cell types after initial derivation of the cells as documented by the homogenous staining of the cells and the lack of staining for lymphocyte and monocyte markers as suggested by the reviewer. The antibodies used for the microscopy and flow cytometry experiments are indicated in the table of reagents.

"Fig. 1 is a key figure. However, there is no effort to assess the angiogenic ability of the cells identified. Eg. Through isolation and wound healing or tube formation."

There are multiple additional assays that may be employed to document the angiogenic ability of late outgrowth EPCs including migration assays, tube formation in Matrigel. Additional proof that the isolated cells are late outgrowth EPCs includes the lack of absorption of fluorescent-labeled bacteria, and colony formation under clonogenic plating conditions. While these experiments are important, the space provided to describe the methods involved in these assays does not allow for us to provide these data. We have previously published the methods and data for Matrigel tube formation and clonogenic colony formation of porcine late outgrowth EPCs in our paper entitled "Statins enhance clonal growth of late outgrowth endothelial progenitors and increase myocardial capillary density in the chronically ischemic heart" in PLoS One (reference 15 in the current manuscript). We can provide evidence to document these properties in adult human late outgrowth endothelial progenitor cells but description of the assays in detail will require further space in the protocol section of the manuscript.

"The methods are not well written and not clear"

While this comment is not specific regarding what areas are unclear I have extensively refined the protocol section of the manuscript to improve the clarity. I have reworded statements that lack clarity and precision, and I have made clear reference to the table of reagents to guide the reader more clearly. I have also included a section in the methods detailing how I perform immunofluorescent staining of the cells to characterize the cells properly by microscopy.

"In short. Apart from providing a nice review of EPC isolation from human samples and the current thinking in the field, the methods do not provide sufficient detail that would. i) allow a researcher to determine the efficacy of the method described over another; and ii) allow a researcher to reproduce the data in a manner that might derive similar results."

As stated above, we have refined the protocol section to provide the essential details required to obtain late outgrowth EPCs from adult human subjects. We have provided examples of pitfalls that are essential for troubleshooting the technique and have arisen in the protocol in our experience. We did not endeavor to provide a comparison of available methods versus our approach. We feel the protocol has been revised with adequate detail for a researcher to reproduce the data that will derive similar results.

Reviewer 2-Major concerns;

"Why the range of cell density to be plated in a well is so large (10-50 million/well)?"

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We routinely obtain 10-50 million mononuclear cells per blood draw of 80 mLs per subject. We have no found a significant difference in the number of late outgrowth EPCs derived based on the plating density within this range.

"Do the authors routinely plate 10 or 50 millions?"

We routinely plate 10 million mononuclear cells per well on a 6 well plate to derive late outgrowth EPCs. I have updated the protocol text to clarify this point **(point 6.2 on page 6 of the manuscript).**

"While the results can be expressed as number of colonies per mL of blood, cell density might be critical for a colony to appear and therefore might not be a linear relationship between amount of blood, cell density, and number of colonies. Have the authors tried different cell densities?"

The reviewer raises an interesting point. We have tested the plating density and efficiency of colony derivation at lower plating densities, but not higher than 100 million cells per well of a 6 well dish. We find the lowest plating density that supports the appearance of late outgrowth EPC colonies at 10 million cells per well. We did not observe a significant increase in the number of colonies derived when plating at higher densities up to 100 million. We have not gone beyond this plating density as this would require drawing larger quantities of blood from each donor.

"In general, discussion of troubleshooting lacks important information: i) is there any demonstration that non-fasted samples are less efficient for deriving late EPC?"

We have not directly tested if fasted versus non-fasted samples result in higher or lower numbers of EPC colonies derived. We do note (and it is generally known) that blood drawn in the post-prandial state will result in significant turbidity in the serum layer from lipoproteins that make separation of the plasma layer and buffy coat after centrifugation challenging to discern properly. **I have removed this point from the discussion section on page 12** since we have not formally tested this concept specific to EPC colony derivation, however it is our general practice to obtain blood from human subjects only in the fasted state for this reason.

"ii) 80 mL of blood is very much and it is unclear what the authors mean for "it may require a larger volume of blood sampling to obtain late outgrowth EPC colonies"

Late outgrowth EPCs are a rare cell contained in the blood stream in adults (see Arterioscler Thromb Vasc Biol (2012) 32: 1045-53). In our hands we derive 1.9 ± 0.4 (mean $\pm$ SEM, n=10) colonies per subject from 80 mL of blood. Given that late outgrowth EPCs are a rare cell type in peripheral blood we surmise (but have not formally tested) that a larger number of cells would result in a greater number of colonies derived. **I have deleted this statement from the discussion section for clarity since we have not tested this concept formally.**

"iii) cell density"

We addressed the considerations of cell density above; within the range of 10 million to 100 million cells per well of a six well dish we have noted no discernable difference in the derivation of colonies when blood from the same donor was used.

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"iv) factors that can be used to stimulate formation of colonies and be used as positive controls"

Specific factors in human subjects that stimulate formation of late outgrowth EPC colonies have not been identified to my knowledge. We previously showed treatment of pigs with the HMG-CoA reductase inhibitor pravastatin increases the number of late outgrowth EPC colonies obtained in vivo, and increases the number of colonies derived when pravastatin is added to blood mononuclear cells at the time of plating in vitro. We have not further tested the effects of pravastatin on the derivation of human late outgrowth EPC colonies.

The best positive control available for the derivation of late outgrowth EPCs is umbilical cord blood mononuclear cells as the number of colonies derived per mL of blood is >8 fold higher in cord blood than in adult human blood. Alternatively, we use blood derived from a donor known to reliably produce late outgrowth EPC colonies as a positive control.

"The 1st sentence of the long abstract and intro should read "Coronary artery disease is the leading cause of heart failure and death in the United States"

We made the suggested change to the first sentence of the long abstract.

"Which is the meaning of the sentence "A lack of late outgrowth EPC colony formation, or lack of detection of endothelial specific antigens is uninterpretable for the experiment"

We clarified this sentence in the discussion to read "A lack of late outgrowth EPC colony formation, or lack of detection of endothelial specific antigens suggests a problem with reagents (growth medium, serum, or collagen solution).

Reviewer #3:

"This paper describes the methods for the isolation and characterization of late outgrowth EPCs from humans and large animal models. Briefly, they isolate mononuclear cells from human blood and through a specific culture of the cells they obtain what they call "late outgrowth EPCs". The results demonstrate the authors isolate and culture cells which express surface antigens PECAM, CD34, CD146, and VE-Cadherin. In general it is worrying that the authors do not discuss the fact that the term EPC is up for discussion since no one so far has convincing shown the in vivo differentiation of EPCs to endothelial cells. Moreover, these so called EPCs are now being thought to represent population of monocytes sharing many characteristics of endothelial cells that affect existing cells through paracrine mechanisms. Also the authors should go through the references again since I do not find good correlation with the text and references in general. Overall, as a reviewer I do not find the approach for conducting this study relevant."

We thank reviewer #3 for reminding us that the field of EPC biology is controversial and several important issues remain to be determined to understand the contribution of EPCs to new blood vessel formation. Unfortunately, the late outgrowth EPCs that are the subject of the current study do not have specific distinguishable antigens that would facilitate a lineage mapping study that would allow one to understand the function of late outgrowth EPCs in angiogenesis models in vivo. This does not mean that the cells and the model are irrelevant. As discussed in the introduction, the growth properties of late outgrowth EPCs have shown good correlation with

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subjects who have myelodysplastic disorders who have a propensity for thrombosis (page 4). Additionally, the cells themselves may be used for cell therapy in animal models and eventually human subjects studies in diseases of vascular insufficiency like coronary disease or peripheral vascular disease. Additionally, the reviewer states that EPCs are a resident population of monocytes. These features refer to the early outgrowth EPCs and are a key feature that distinguishes the late outgrowth EPC. The late outgrowth EPC does NOT have monocyte like features. These points are discussed in the introduction of the article (see page 3 of the introduction). I have reviewed the references and do not find any significant discrepancies between the manuscript text and the reference list as noted by the reviewer.

Reviewer #4:

"The manuscript 'Derivation of Late Outgrowth Endothelial Progenitor cells from Adult Human Peripheral Blood Mononuclear Cells' describes research methodology to isolate and culture a specific subtype of endothelial progenitor. Although the methodology is not novel, it is of great importance to standardize research practice and protocols, therefore I believe this manuscript will be extremely useful for researchers in the Vascular Biology field. Nevertheless, there are some issues that will certainly need to be clarified before this manuscript is accepted for publication"

1) As mentioned before, this is not a novel protocol; therefore previous similar protocols isolating these late outgrowth endothelial progenitor cells should be highlighted. As a minimum, these two papers appearing in JoVE should be cited…."

We appreciate the reviewer recognizing the importance of standardization of the methodology to derive late outgrowth endothelial progenitor cells, and its potential usefulness to vascular biologists. We have added the following sentences to the end of the introduction (bottom of page 4) to acknowledge how this manuscript fits in the context of the two JoVE articles previously published:

> "Importantly, our goal is to standardize the approach to derivation of late outgrowth EPCs, and place our approach with adult human blood in the context of other leaders in the field of late outgrowth EPC biology to provide insights into our approach that may be compared and contrasted with the approach of others in the field (16, 17)."

The two relevant JoVE articles are now referenced in the current manuscript.

2) What is the efficiency/success rate for the isolation of these late EPCs? Authors indicate vaguely that "generally most subjects will derive one or two colonies from 80 mL of blood, and that some subjects will generate no colonies at all". Based on the five human subjects used for this study, can authors state the efficiency of isolation in percentage? It is commonly accepted in the EPC research community that isolation efficiency from human peripheral blood is very low (~5-50%). Authors should clearly state this, as readers can be mislead into believing that isolation of these EPCs is very easy.

We agree with the reviewer that 1) our findings were stated vaguely, and 2) that it may be misleading to researchers that isolation of these cells is "easy" when it is not.

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We modified the first two sentences of the representative results section (top of page 8) in the following manner to address the comments:

> "The number of late outgrowth EPC colonies derived from each subject varies between individuals, and between blood draws due to the fact that the cells are a rare event in the blood stream (0.0001% of mononuclear cells). Most subjects will derive one to two colonies from 80 mLs of blood. In our experience, we derive 1.9 ± 0.4 (mean ± SEM, n=10) colonies per subject from 80 mL of blood, with an efficiency of 36%."

3) "In the first and third paragraph for the discussion section, it is stated that late outgrowth EPCs cannot be cultured beyond 3-4 passages before becoming senescent. This is probably not always the case as other labs have successfully grown cells for over 10 passages."

The reviewer astutely points out that late outgrowth EPCs do not always become senescent in culture and refers to two previously published papers. The referenced paper by Ingram et al studied the derivation and characterization of late outgrowth EPCs from adult human peripheral blood and umbilical cord blood. Only the umbilical cord blood derived cells, but not the adult human EPCs, could be passaged sequentially in medium containing fetal bovine serum. More recently we have adopted a method using human platelet lysate in place of fetal bovine serum that facilitates expanded passaging and growth of adult human late outgrowth EPCs to passage 9 in our hands with no appreciable change in the quality or character of the cells, and no apparent senescence. I have modified the text of the discussion (middle of page 12) to reflect these findings as shown below:

> "Generally late outgrowth EPCs obtained from adult humans will tolerate replating to passage 3 or 4 without becoming senescent in fetal bovine serum containing medium. We have had success passaging cells to passage 9 using EBM2 medium supplemented with human platelet lysate with no appreciable changes in the quality or character of the cells (20)."

4) "It is indicated that after isolation, late outgrowth EPCs should be characterized to assure their endothelial phenotype. This is of critical importance and while authors suggest the use of immunofluorescence for CD31, CD34 and CD146, it is well accepted that flow cytometry-based immunophenotyping is required not only to identify endothelial and progenitor markers, but also to exclude the possibility of hematopoietic/mesenchymal cell contamination in the mass culture. This point should be highlighted in the results section."

Again we agree with the viewpoint of the reviewer. We have included flow cytometry characterization of the cells in the representative results (Figure 3), and provided the antibodies used and catalog numbers in the table of reagents.

Minor concerns:

1) "page 4, protocol step 4.1. Details concerning the breaks in the centrifuge for density gradient centrifugation should be added."

We agree with the reviewer that this oversight should be corrected; We amended this line of the protocol with the following statement:

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"4.1) Spin the blood collection tubes at 1500 x g for 30 minutes at room temperature with no brake to prevent disturbing the separated layers of blood."

2) "Page 4, protocol step 6.2. Details on the volume used in a single well from a six well plate should be added as research groups use 2-4 mLs."

We modified the protocol to read:

"6.2) Gently transfer 10 x 10^6 cells of the lymphocyte/monocyte fraction in a 2.5mL volume to one well of a six well plate. Distribute the cells throughout the culture well and return the dish to the culture incubator."

3) "Page 7, first paragraph is not very clear and should be re-written. "when late EPC colonies are not obtained in culture, only the background of phase bright mononuclear cells are obtained. A lack of late outgrowth EPC colony formation, or lack of detection of endothelial specific antigens is uninterpretable for the experiment". What do the authors exactly mean by this comment?"

We agree with the reviewer that the statement above is unclear. We modified this sentence (top of page 8, end of first paragraph) to clarify this statement in the following manner:

"When late outgrowth EPC colonies are not obtained in culture only the background of lack of detection of endothelial specific antigens suggests a problem with reagents (growth medium, serum, or collagen solution). Troubleshooting steps for the technique are outlined in the Discussion section."

4) "Page 7, second paragraph. It is stated that "other cell types generally will not expand under the culture conditions used". This is not completely accurate as it has been reported that some mesenchymal cell types can be easily amplified under this very same culture conditions."

We agree with the reviewer that others have reported derivation of mesenchymal cell types from umbilical cord blood mononuclear cells, and it is plausible one may observe this result in adult human mononuclear cells, although we have not thus far. Additionally, we have refined the methods section to passage the initial outgrowth colonies using cloning cylinders or by directly picking the colonies from the plate with a micropipettor to avoid potential contamination issues suggested above. To address this issue we have modified this sentence (end of second paragraph, page 8) in the following manner:

"Cells that do not express these antigens may be found from contamination of dermal fibroblasts, mesenchymal cells, or monocytes. In our hands other cell types are not obtained using the culture conditions and colony focused passaging techniques described."

We worked diligently to take into account all of the reviewer's comments and feel the manuscript is significantly improved and a valuable addition to the vascular biology literature at this point.

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We hope you are in agreement. Please contact me if there are further questions regarding the manuscript.

Sincerely,

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