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**Creating an in-vitro co-culture model of Human Megakaryocytic-Erythroid Progenitors
and Endothelial Cells**

Clerin John, Vanessa Scanlon, PhD

ABSTRACT

Previous studies have suggested that endothelial cells promote red blood cell (RBC) production in human bone marrow, which has implications for improved treatments for anemia, cancer, and reproduction. However, investigation of this phenomenon is hindered by a dearth of studies that have successfully isolated primary endothelial cells from human bone marrow. Using flow cytometry, magnetic cell separation, and primary cell culture techniques we tested the hypothesis that adult bone marrow-derived endothelial cells can be isolated from previously frozen bone marrow samples by selecting for CD144+ expressing cells and culturing them *in vitro*. Magnetic cell separation utilizing the marker CD144 to isolate adult bone marrow-derived endothelial cells was not effective from human bone marrow samples that had already been depleted of CD34+ cells. In future experiments we will test the ability to magnetically separate CD144+ adult bone marrow derived endothelial cells from freshly collected bone marrow prior to CD34+ selection. Results of these studies will generate a model that we can use in the future to test the molecular mechanisms by which human bone marrow-derived endothelial cells promote erythroid differentiation.

INTRODUCTION

More than 10% of inpatient hospital procedures require blood transfusions, and our ability to generate transfusable platelets and red blood cells is still insufficient ¹. Therefore, patients heavily rely on peripheral blood donors. Unfortunately, there is a chronic shortage of blood donations since blood can only stay fresh outside of the body for 7 days, and there is a risk of disease transmission or immune reaction to foreign cells¹. These drawbacks provide the rationale to improve the scale of *in vitro* production protocols to make clinically relevant amounts of platelets (PLTs) and red blood cells (RBC) for transfusion medicine. The hematopoietic system has the capability of cell proliferation and differentiation to meet the demand of blood and immune cell production. The Megakaryocytic-Erythroid Progenitor (MEP) is an upstream hematopoietic progenitor that gives rise to the lineage-committed erythroid progenitor (ErP), which differentiates into RBC, and megakaryocytic progenitor (MkP), which differentiates into megakaryocytes producing PLTs ². By understanding the mechanisms by which we can expand the MEP population and control their lineage decision, we can increase the production of RBC and/or platelets *in vitro*.

To test progenitor cell fate, we perform colony-forming unit (CFU) assays, which enable researchers to identify the expansion ability and lineage commitment of progenitor cells *in vitro*, more specifically they are single-cell assays that have improved our understanding of hematopoiesis and provide us with a vigorous method for assessing the dynamics of lineage commitment.³

Preliminary evidence has shown that coculturing MEP with human umbilical vein endothelial cells (HUVECs) in CFU assays confers a strong bias in the MEP lineage decision to increase

erythroid production.⁴ It remains to be determined if primary human bone marrow-derived endothelial cells recapitulate an erythroid bias in cocultured MEP.

CD144⁺ endothelial cells are found in the vascular wall and maintain integrity through tight junctions⁵. These cells are a major component of the bone marrow microenvironment that regulates the trafficking and homing of hematopoietic stem and progenitor cells⁴. The goal of this study was to isolate primary human bone marrow endothelial cells and establish a coculture system with MEP as a more physiologically relevant *in vitro* model with which we can test the mechanisms that underlie the erythropoietic effect of endothelial cells on MEP. Our expectation was that the marker CD144⁺ would separate the endothelial cells from the multitude of additional cell types present in human bone marrow samples to facilitate isolation and subsequent coculture with MEP.

MATERIALS/METHODS

Flow Cytometry and Cell Sorting

Human granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood was CD34⁺ enriched (CliniMACS; Miltenyi) and stained with Lineage cocktail-BV510 (BD Biosciences [BD]), CD34-BV421 (Biolegend), CD38- PECE594 (BD), CD45Ra-BV711 (Biolegend), CD135-PE (Biolegend), CD36-PerCPCy5.5 (BD), CD110-APC (BD), and CD41a-APCH7 (BD) antibodies. Human MEPs (Lin⁻CD34⁺CD45Ra⁻CD135⁻CD38^{mid}CD110⁺CD36⁻CD41a⁻) were sorted on a FACSAria, as previously described.⁶

Dual Mk/E Colony Formation Assays

MEPs were cultured in MegaCult C Medium Plus Lipids (Stem Cell Technologies) mixed with Collagen solution (Stem Cell Technologies) to a final concentration of 1.2 mg/mL with varying

combinations of cytokines as defined in the text including 3.0 U/mL rhEPO, 10 ng/mL rhIL-3, 10 ng/mL rhIL-6, 25 ng/mL rhSCF, and 50 ng/mL rhTPO. All cytokines were purchased from ConnStem (Cheshire, CT) except rhEPO (Amgen).

Dual Mk/E Colony Forming Unit Assays in Coculture with Endothelial Cells

EC cytokines were added to the CFU media formulation for the HUVEC cells. The HUVEC cell media was composed of 10 uL hydrocortisone, 200 uL rhFGF-B, 50 uL IGF, 50 uL heparin, 50 uL ascorbic acid, 50 uL rhEGF, 50 uL VEGF, 50 uL GA, and 1000 uL FBS.

Magnetic cell separation

We attempted to isolate CD144+ cells from aliquots of human bone marrow that were previously depleted of CD34+ cells and refrozen utilizing two different anti-human CD144+ antibodies conjugated to magnetic beads from Miltenyi Biotec (MACS Microbeads) to allow for magnetic cell separation and isolation of bone marrow endothelial cells.

Endothelial Cell Culture

Following the isolation protocol, we cultured the HUVEC cells in 10 uL hydrocortisone, 200 uL rhFGF-B, 50 uL IGF, 50 uL heparin, 50 uL ascorbic acid, 50 uL rhEGF, 50 uL VEGF, 50 uL GA, and 1000 uL FBS. The primary endothelial cells were in MSC media which is composed of 10 mL FBS, 0.5 mL AA, and 39.5 mL alpha MEM.⁷

Detection of Mk/E Colonies with Immunofluorescence

Colonies were stained in situ 10–14 days post-plating with an antibody staining cocktail comprised of Iscove's Modified Dulbecco's Media, anti-CD41-PE or CD41-AlexaFluor 488

(Biolegend), anti-CD235a conjugated to allophycocyanin (APC) (BD), and CD71 conjugated to APC (Biolegend). Colony types were assessed by fluorescence microscopy on a Molecular Devices ImageXpress Micro HT.ai high content imager.

Human subjects

All work was conducted according to the Declaration of Helsinki principles. Primary human cells were purchased from the Yale Cooperative Center of Excellence in Hematology. Collection and use of human cells were approved by the Yale University Institutional Review Board. Healthy donors who were already donating cells for allogeneic transplantation provided written informed consent prior to the use of surplus G-CSF mobilized cells or bone marrow collections for research.

Statistical Analysis

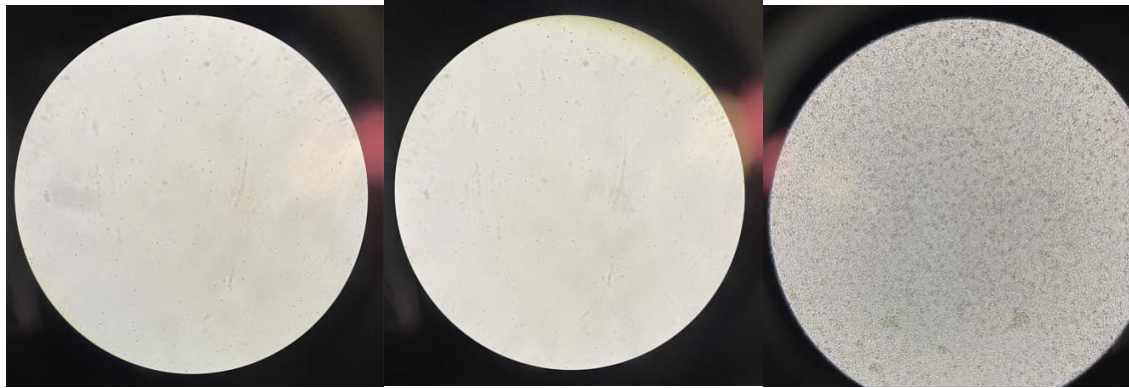
Data are plotted as mean plus standard deviation. Statistical analyses were performed using an analysis of variance or Student's t-test with a p-value ≤ 0.05 considered statistically significant.

RESULTS

Isolation of CD144+ cells from human bone marrow

Two trials were conducted in order to determine if CD144+ isolation was successful. The cells were previously depleted of CD34+ cells and were refrozen. In both trials we can see that neither of the CD144 antibodies used to select worked on the human bone marrow samples. Figure 1 demonstrates how the cells look after being in the endothelial cell media overnight. These cells

have not adhered to the bottom of the plate and are floating, which means that the cells are dead.



Pre-isolation

Pre-isolation

Post-isolation

Figure 1. Brightfield images of cells pre- and post-isolation with anti-CD144.

There was no enrichment for CD31+ cells in the CD144+ fraction compared to CD144- or preselected sample, which indicates that the cell selection failed. In both trials, we expected for the flow cytometry results to shift towards population 3 which means that there wasn't any added signal since the CD144+ didn't shift. Ultimately the CD144+ cells were not selected.

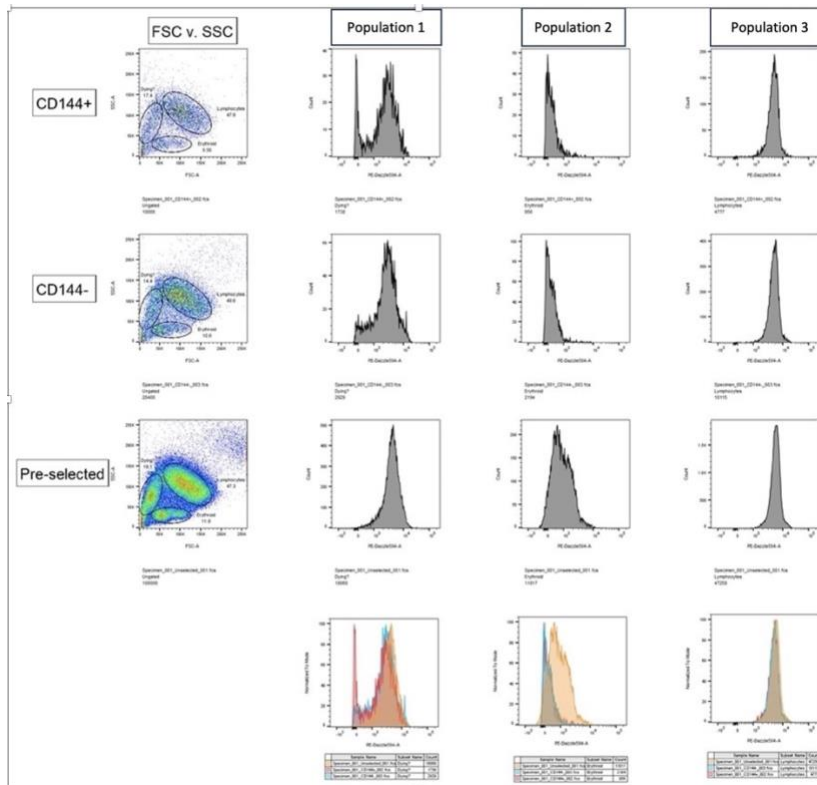


Figure 2. Measurement of bone marrow endothelial isolation purity with flow cytometry.

Post-CD144+ magnetic cell selection, the positive fraction and the negative fraction were stained with anti-CD31 (another marker of endothelial cells) to confirm the degree of purity. Flow plots shown are for samples selected with anti-CD144+.

Confirmation that coculture of MEP with HUVEC cells confers an erythroid bias.

To confirm if coculture of MEP with HUVEC cells confers a skewing toward erythroid production, we cocultured MEP with varying concentrations of HUVEC cells, *in situ* stained with antibodies against lineage markers, imaged the plate with fluorescence to detect the lineage commitment of daughter cells within the colonies (Figures 4, 5, 6, and 7), and quantified the number and types of colonies grown (Figure 7). HUVEC alone wells did not grow colonies, confirming that HUVEC cells don't spontaneously make megakaryocytic or erythroid colonies.

In order to keep the HUVEC cells alive in coculture, we modified the CFU assay media formulation to include endothelial cell-supportive cytokines. To confirm that endothelial cell-supportive cytokines don't skew MEP fate, we compared MEP grown alone in standard MEP CFU assay culture conditions, and MEP grown alone in CFU assay conditions containing both MEP and endothelial-supportive cytokines. We found no significant difference in the number and colony types grown between the two conditions. When we compared the number and types of colonies grown by MEP cocultured with increasing concentrations of HUVEC cells to MEP grown alone, we saw a significant reduction in mixed Mk/E colonies, confirming preliminary data that suggested that endothelial cells in coculture with MEP lose bipotency and acquire an erythroid bias.

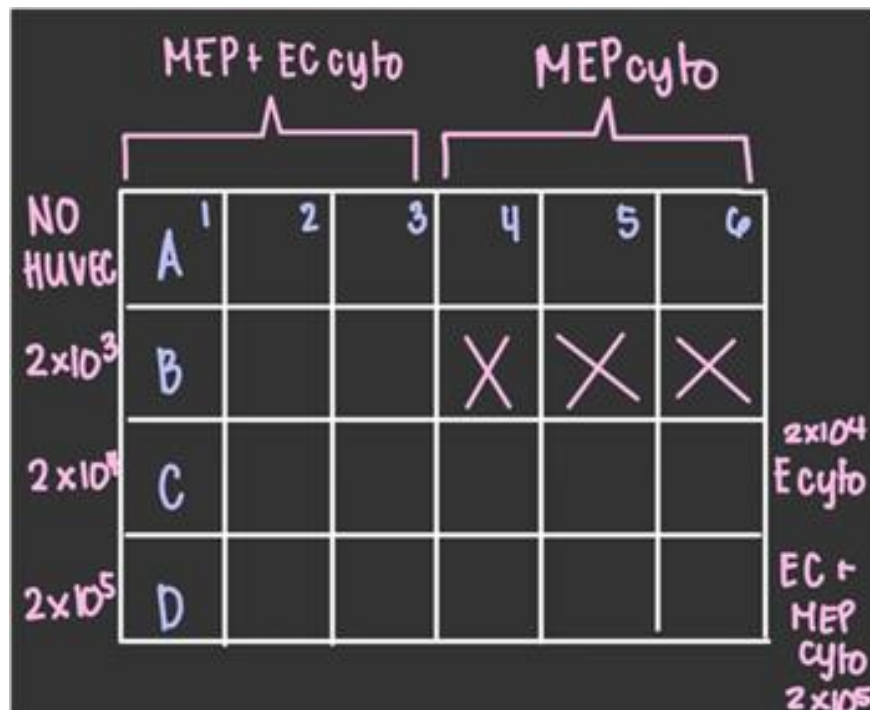


Figure 3: HUVEC cell plate organization

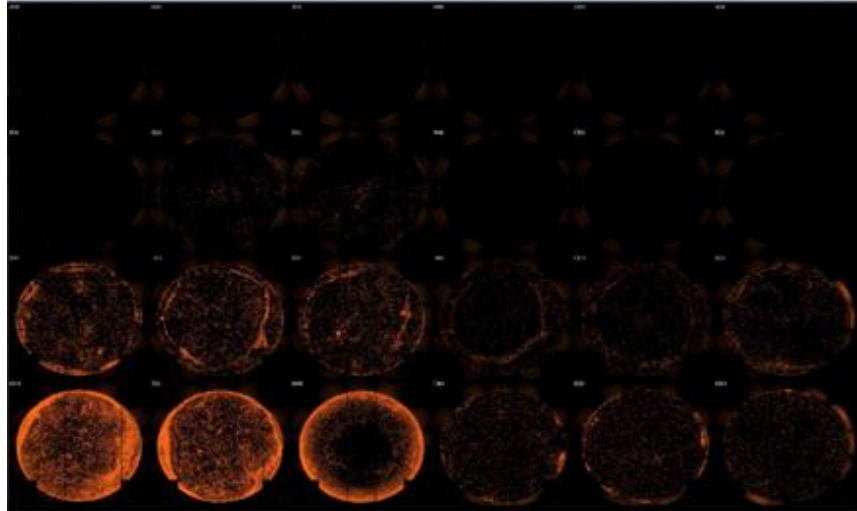


Figure 4: HUVEC Cells.

HUVEC cells stably express the fluorescent reporter mCherry, allowing for visual confirmation of the varying concentrations of HUVEC cells plated in each well.

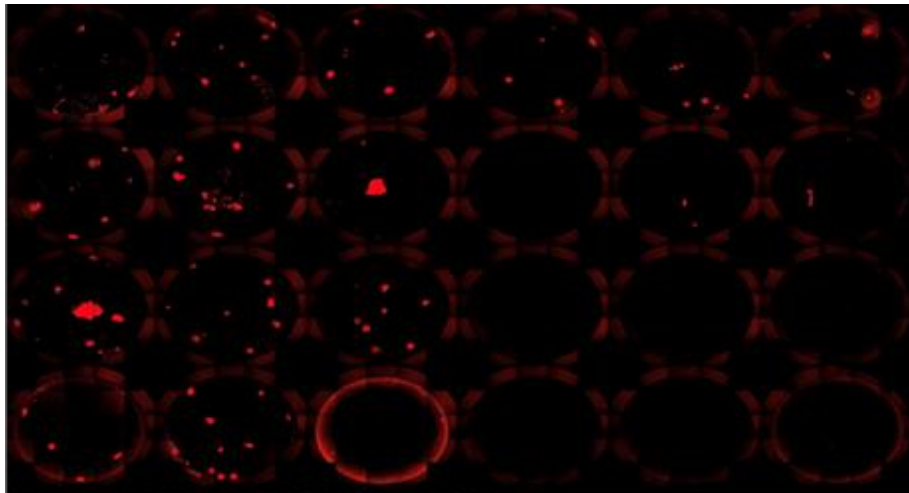


Figure 5: Erythroid-containing colonies.

Anti-CD71 and anti-CD235a label erythroid-committed daughter cells in the colonies with a far red fluorescence.

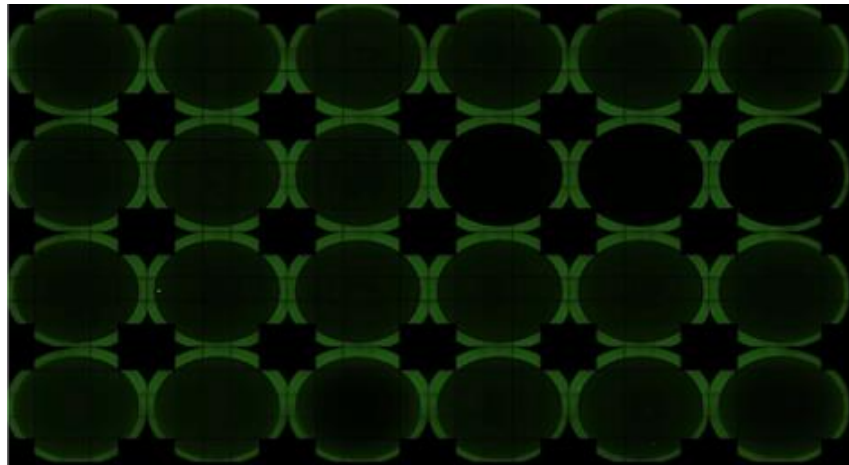


Figure 6: Megakaryocyte colonies.

Anti-CD41 labels megakaryocytic-committed daughter cells in the colonies with a green fluorescence.

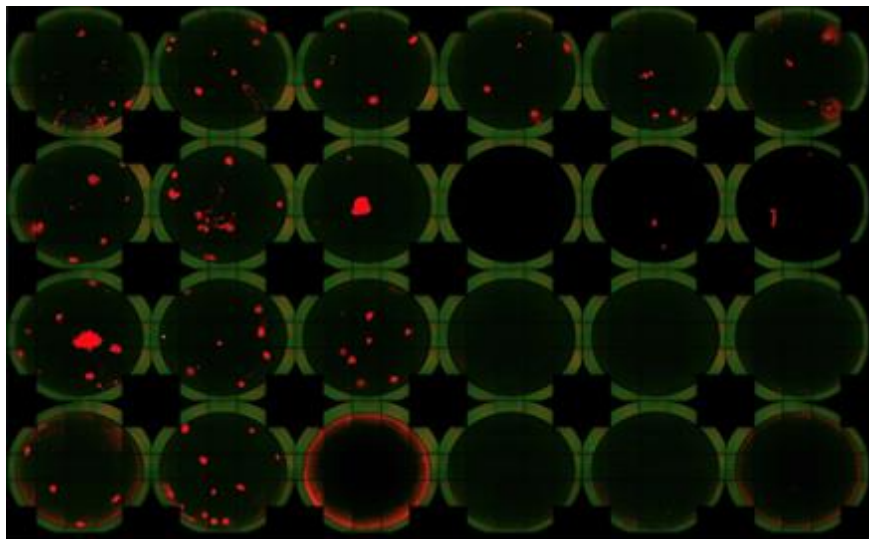


Figure 7: Overlay image.

Colonies that contain both green and red labeled cells contain both Mk-committed and erythroid-committed daughter cells and is counted as a mixed Mk/E colony.

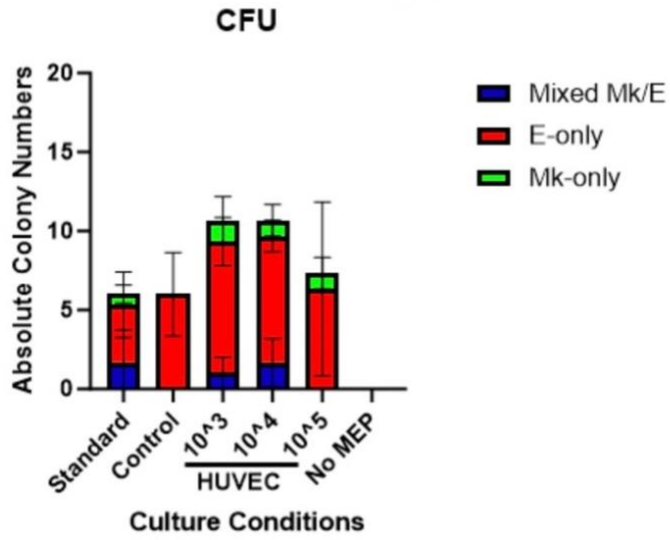


Figure 8: Colony counts grown from MEP in various conditions ($n=3$, $p<0.05$).

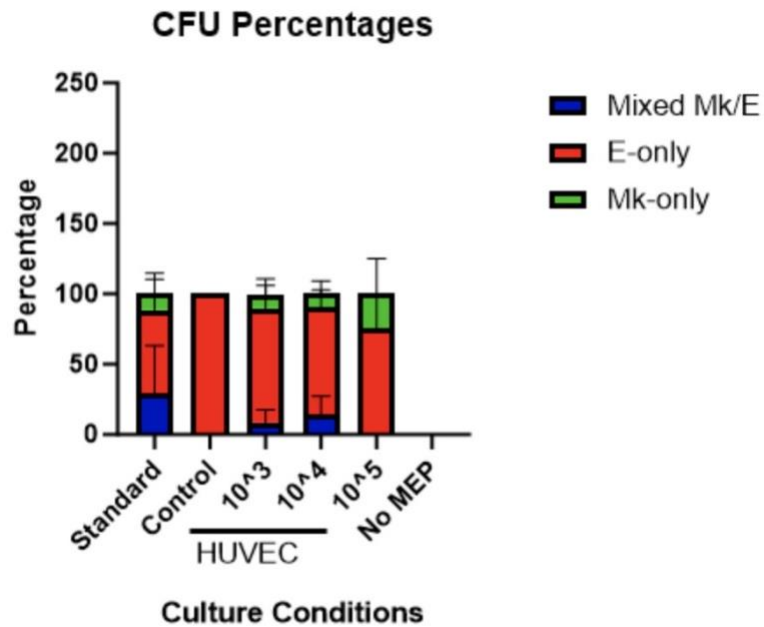


Figure 9: Percentage of colony counts grown from MEP in various conditions.

DISCUSSION

Our results demonstrate that magnetically separating CD144+ cells from human bone marrow samples that were previously depleted of CD34+ cells and refrozen is unsuccessful. This may be due to inefficient magnetic cell separation and require further optimization of the cell isolation protocol. Alternatively, it may indicate that bone marrow endothelial cells do not persist after a freeze/ thaw cycle and may need to be isolated from freshly collected bone marrow. An additional possibility is that the CD144+ endothelial cells also express some level of CD34 and were already depleted from the sample before they were refrozen. Further experiments using freshly isolated human bone marrow and measuring the frequency of CD144+ cells in the CD34+ isolated fraction will enable us to differentiate these possibilities and move forward with establishing a protocol for isolating primary human bone marrow-derived endothelial cells.

We were able to confirm preliminary data that MEP can form colonies in the presence of endothelial-supportive cytokines, and that coculture with HUVECs confers a lineage bias towards erythroid. This coculture system can be used to initiate mechanistic studies to understand the molecular signaling crosstalk between endothelial cells and MEP that promote erythroid commitment and differentiation.

The CD144+ cells needed to be selected in order to conduct the ideal experiment – plating with MEP in CFU assays. Since the CD144+ cells did not select successfully, we were unable to proceed with the experiment by plating with the MEP. However, we were able to successfully coculture HUVEC with the MEP and show that there is a strong lineage decision to the E colony. In the future, we will look into current research techniques on BMEC cell separation as we

suspect that endothelial cells may be sensitive to the freeze thaw process, so we need to develop an approach where we never previously thaw the cells. Given the data found with the HUVEC cells, this is a promising start.

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