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CHARACTERIZATION OF CD9 AND CD44 SURFACE MARKERS ON STRESS ERYTHROPOIESIS PROGENITOR CELLS

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ABSTRACT

Stress erythropoiesis is a distinct pathway of red blood cell development when the body is under hypoxic stress. Our understanding of this process is dependent on the knowledge we have of the stages of stress erythroid progenitor development. To determine if CD9 and CD44 surface markers could be used to identify $Kit^{+}/SCA1^{+}$ and $Kit^{+}/SCA1^{-}$ progenitor cell populations, we ran flow cytometry on stress erythroid progenitors generated by culturing bone marrow cells isolated from C57BL/6J mice in expansion and differentiation media. We then analyzed how the expression of CD9 and CD44 changes on the $Kit^{+}/SCA1^{+}$ and $Kit^{+}/SCA1^{-}$ cell populations. We found that the expression of both CD9 and CD44 changes as the progenitor cells develop. Both surface markers were expressed more frequently in the $Kit^{+}/SCA1^{+}$ cell population when compared to the $Kit^{+}/SCA1^{-}$ cell population. These results suggest that CD9 and CD44 may be used as a distinguishing cell surface marker when isolating these progenitor cell populations. An increased understanding of how to isolate precise populations of the stress progenitor cells will expand our understanding of the developmental process of stress erythropoiesis and can lead to treatment advancements for patients with anemia.

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Chapter 1

Introduction

Steady State Erythropoiesis

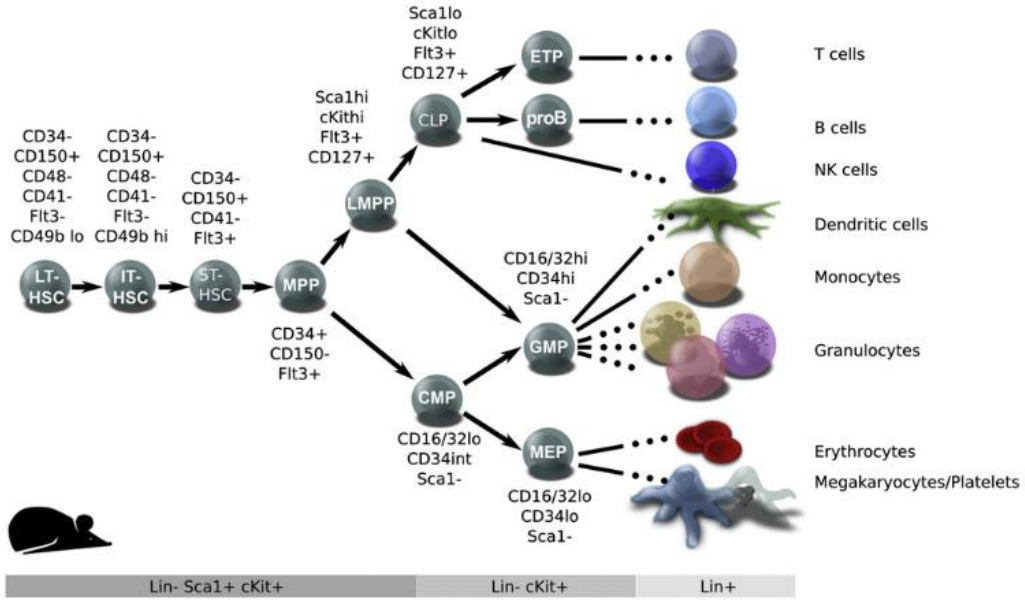
Hematopoiesis is responsible for creating and regenerating all blood cells within the body. Proper hematopoiesis is crucial due to the inability of blood cell populations to self-renew and the massive daily regeneration requirements (Doulatov et al., 2012). Cells resulting from this process originate from multipotent hematopoietic stem cells (HSC) residing in the bone marrow (BM) (Dzierzak & de Pater, 2016). The HSC is necessary to ensure proper renewal of HSCs as others differentiate (Pronk et al., 2007). From the HSC, through a step-wise process of differentiation and lineage restriction, mature erythrocytes form through erythropoiesis (Akashi et al., 2000; Zivot A et al., 2018).

Erythropoiesis occurs in a series of three highly regulated phases: engagement, precursor differentiation, and erythroid differentiation. Within the engagement phase of differentiation, HSC begin to commit to the myeloid cell lineage beginning with multipotent progenitors (MPPs) and then common myeloid progenitor (CMP) cell (Doulatov et al., 2012; Zivot A et al., 2018). Based on continued growth factor signaling the CMP cells further differentiate into the megakaryocyte-erythroid progenitors (MEPs) after which differentiation into burst-forming unit-erythroid (BFU-E) cells marks the end of the first phase. BFU-E represent the first cell committed exclusively to the erythroid lineage (Zivot A et al., 2018). BFU-Es further differentiate into colony forming-unit erythroid and the second phase begins, which is marked by continued differentiation of the progenitor cells into proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, and orthochromatic erythroblasts. Throughout this phase, the cells decrease in size, gain hemoglobin and eventually lose the nucleus. This development occurs on erythroblastic

islands, which culminates in the release of reticulocytes or immature erythrocytes that mature and are capable of transporting oxygen throughout the body (Zivot A et al., 2018).

A current model for the hematopoietic pathways can be seen in **figure 1** for humans and mice. The understanding of these pathways relies heavily on the characterization of the surface markers that distinguish the distinct populations of cells. An understanding of the surface markers that characterize each progenitor cell is useful when treating disease of the blood that result from one of these pathways being dysregulated. Much of what we know about the regulation and cell populations of these pathways, especially erythropoiesis, comes from studies using a murine model.

A.



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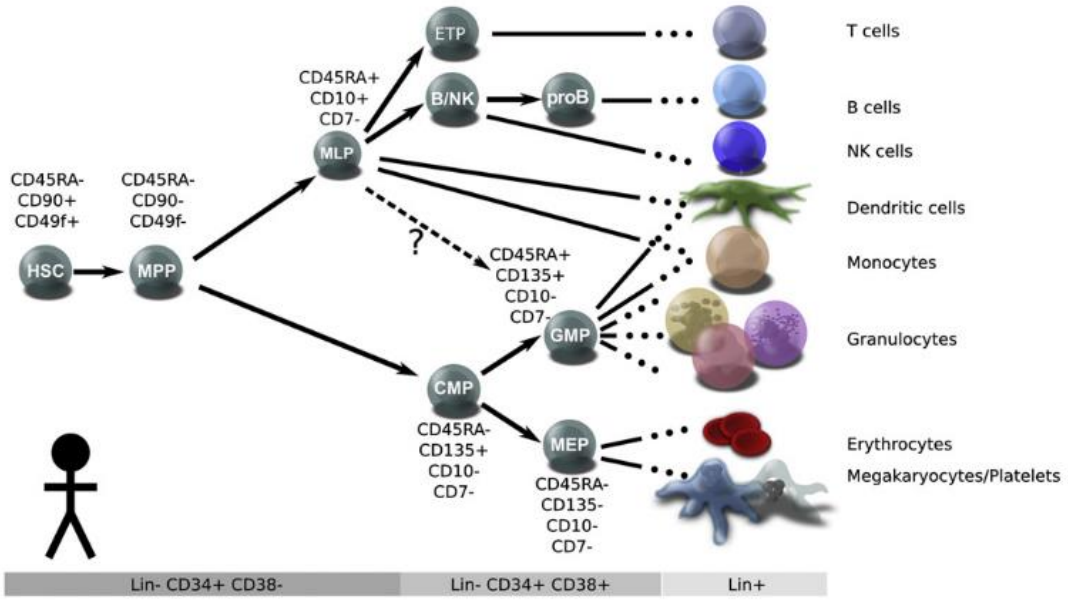


Figure 1. Current Models of Lineage Determination in the Adult Mouse and Human Hematopoietic

The major classes of stem and progenitor cells described in the text are defined by cell surface phenotypes, which are listed next to each population and in the gray bars below each schematic. Terminally differentiated cells are shown on the right, and inferred lineage relationships are depicted with arrows. In mice (A), HSCs can be separated into long-term (LT), intermediate-term (IT), and short-term (ST) classes based on the duration of repopulation. In humans (B), HSCs are defined by the expression of CD49f and other markers, but their heterogeneity has not been investigated. In mice, differentiation of HSCs gives rise to transiently engrafting multipotent progenitors (MPPs), and a series of immature lymphoid-biased progenitors (such as LMPPs) that undergo gradual lymphoid specification. In humans, MPPs can be identified by the loss of CD49f expression; however, only one population of immature lymphoid progenitors (MLPs) has been described. Both mice and humans have well-defined populations of myelo-erythroid progenitors: CMPs, GMPs, and MEPs. Lin: cocktail containing cell surface markers for all terminally differentiated populations (B cell; T cell; NK; dendritic cell, monocyte, granulocyte, megakaryocyte, and erythrocyte). *Figure produced by Doulatov et al. (2012).

The process of erythropoiesis is highly regulated by a variety of signaling factors (cytokines, growth factors, accessory proteins)—most notably, erythropoietin (EPO). EPO is responsible for acting on hematopoietic progenitor cells to increase the level of erythropoiesis and erythroid progenitor cells (Grover et al., 2014). Upon binding of EPO to its receptor, erythropoietin receptor (EPOR), signaling pathways are activated that result in the downstream production of erythrocytes (Bunn, 2013; Tsiftsoglou et al., 2009). EPO expression is regulated by hypoxia inducible factor-1 (HIF-1) which is made up of an oxygen sensitive α subunit and an oxygen insensitive β subunit (Israels & Israels, 2003). In times of normoxia, the alpha subunit of hypoxia inducible factor-1 (HIF-1) is marked for degradation by an E3

ubiquitin ligase, von Hippel-Lindau protein (pVHL), and is then degraded through the proteasomal pathway. Since HIF-1α is degraded, the HIF-1β subunit remains inactive and EPO expression is not activated. However, in response to hypoxic conditions, EPO expression increases due to HIF-1 αβ subunits dimerizing (Berlian et al., 2019; Liu et al., 2006). Due to the decreased presence of oxygen, pVHL does not interact with HIF-1α allowing it to stabilize and heterodimerizes with HIF-1β. (Israels & Israels, 2003). The increased levels of active HIF-1 result in increased EPO mRNA production leading to increased levels of EPO (Bunn, 2013).

In addition to EPO, stem cell factor (SCF) has been shown to play a pivotal role in erythrocyte development (Wang et al., 2008). SCF interacts with its receptor, Kit, leading to the dimerization and autophosphorylation of the tyrosine kinase. This phosphorylation provides the phosphate necessary for other signaling molecules to bind resulting in downstream alterations in gene expression, survival signals and the early proliferation of primitive cells. (Munugalavadla & Kapur, 2005; Wang et al., 2008). It has also been shown that SCF and EPO may act sequentially during erythropoiesis to support the proliferation and survival of the erythroid progenitor cells, respectively (Wang et al., 2008).

In addition to the individual cytokines that regulate erythropoiesis, cell types, like macrophages, also make up the regulatory network. Macrophages secrete signaling factors, such as insulin-like growth factor, which can stimulate erythroid cell production (Zivot A et al., 2018). However, more notably, central macrophages play a pivotal role in erythrocyte development within erythroblastic islands. These macrophages are needed to phagocytize nuclei from orthochromatic normablasts leading to the formation of a reticulocyte and eventually a mature erythrocyte. Lastly, these central macrophages also provide the iron needed for erythropoiesis (Tsiftsoglou et al., 2009). The balance between these signaling factors and the central macrophages are crucial for efficient red blood cell development.

Anemia

Acute anemia results in the rapid production of erythrocytes through stress erythropoiesis to mitigate the tissue hypoxia (Xiang et al., 2015). Anemia describes a variety of disorders (anemia of inflammation, β-Thalassemia, anemia of chronic disorders for example) affecting red blood cells (RBCs) resulting in ineffective steady state erythropoiesis (Cherukuri et al., 2004; Crielaard & Rivella, 2014; Fraenkel, 2017). Generally, anemia results in hypoxia due to a reduced ability of RBCs to transport oxygen throughout the body because of a reduction in RBC count or hemoglobin levels. Anemia can manifest acutely or chronically depending on the mechanism that leads to decreased erythropoiesis or blood loss (Janz et al., 2013).

The classification of anemia can be based on either the size of RBCs—microcytic, macrocytic, or normocytic—or the mechanism (e.g. decreased RBC production) that causes the disorder. Thus, in order to classify and diagnose the specific anemia, tests must focus on identifying the cause of the anemia. In order to do this, a variety of tests may be used, among which include a determining the person's hematocrit, conducting a complete blood count, a reticulocyte count, and/or blood smears (Cascio & DeLoughery, 2017). Symptoms of anemia include fatigue, weakness, and shortness of breath and result in a significantly decreased quality of life for patients ("Anemia", 2018). The proper diagnosis is critical for effective treatment.

Forms of treatment can range from nutritional supplements to blood transfusions and/or hormone treatment with recombinant human erythropoietin (rhEPO) and depend on the diagnosis and severity of anemia (Jacob et al., 2012; "Anemia", 2018). While these treatments can be effective, they do not come without their inherent risks. Blood transfusions carry the risk of transmitting pathogens, transfusion-associated circulatory overload, and adverse immune reactions (Goodnough & Panigrahi, 2017). Treatment with rhEPO can be effective at increasing EPO stimulation of erythropoiesis; however, it can cause flu-like symptoms, anaphylactic reactions, and potentially plays a role in stimulating cancer progression (Jacob et al., 2012). A better characterization of early erythroid progenitor cells can help

provide safer treatment options provided to anemia patients by allowing for specific cell populations to be isolated or enhanced in the transfusions. This requires further research on the expression levels of specific surface markers on progenitor cells in hematopoiesis.

Stress Erythropoiesis

In times of stress or fetal development, when the body must produce more erythrocytes than manageable by steady state erythropoiesis, the body relies on the stress erythropoiesis pathway to rapidly produce RBCs. The stress erythropoiesis pathway is best understood in mice where it occurs in the fetal liver and adult spleen (Paulson et al., 2011). This pathway, like steady state erythropoiesis, is heavily regulated. However, unlike steady state erythropoiesis, stress erythropoiesis utilizes a distinct population of stress progenitor cells and additional signaling molecules to regulate the rapid expansion of RBCs (Bennett et al., 2019).

In stress erythropoiesis, stress erythroid progenitor cells are derived from the short-term HSCs, In the bone marrow these cells are multipotential, but they become are restricted to the erythroid fate once they migrate from the BM to the spleen (Liao et al., 2018). These short-term HSCs are distinct because they express CD34+, Kit+, Lin- surface markers, a combination of stem cell markers (i.e. CD34) and hematopoietic markers (Kit) (Bresnick et al., 2018; Xiang et al., 2015). Once in the spleen, the short-term HSCs cells proliferate and eventually differentiate into stress BFU-Es which terminally differentiate into erythrocytes. Stress erythropoiesis progenitor cells can be characterized by their simultaneous expression of immature (e.g. Kit and Sca1) and late erythroid markers (e.g. CD71 and TER119) (Xiang et al., 2015). Additionally, the stress progenitor BFU-Es exhibit properties that are distinct from steady state BFU-E. They were shown to require only EPO to form colonies *in vitro* while steady state erythropoiesis BFU-Es require EPO and a burst promoting-signal (Paulson et al., 2011). This alteration in signaling regulation is consistent with the need to induce rapid proliferation and differentiation of the cells. In addition to this

property, the stress BFU-Es are also morphologically distinct as they generate BFU-E colonies that are larger and have more satellite colonies in comparison to the steady state BFU-Es (Lenox et al., 2005).

In addition to the differences in surface markers and growth characteristics, stress erythropoiesis progenitor cells are also regulated by distinct signaling molecules that are required in addition to the steady state erythropoiesis signals discussed in the 'Steady State Erythropoiesis' section (Millot et al., 2010). The regulation of the stress progenitors is depended on hypoxia, SCF, bone morphogenetic protein 4 (BMP4), and hedgehog (Perry et al., 2009; Xiang et al., 2015). Hypoxia potentiates the activation of signaling pathways that trigger the stress progenitor cells, Lin-Kit+CD34-, to become responsive to BMP4. Progenitor cells in the BM migrate to the spleen and once in the spleen they encounter Hedgehog ligands. Indian hedgehog is the most likely family member involved. Hedgehog signaling leads to increased expression of BMP4 and the two signals act together to specify the stress erythroid fate (Perry et al., 2009). During normoxia, SCF only increases the size of BFU-Es. In hypoxic conditions, however, SCF result in the increased burst size and proliferation rate of the BFU-Es, and BMP4 signaling in the spleen results in stress BFU-E cell population expansion (Paulson et al., 2011). Hypoxia also regulates the production of BMP4. In hypoxic conditions, HIF2α binds to hypoxia response elements on the *Bmp4* gene resulting in expression of BMP4 (Wu & Paulson, 2010).

Continued research will provide a better understanding of the signals and progenitor cells involved in the stress erythropoiesis pathway. These data will be advantageous in developing enhanced therapeutics for patients suffering with anemia, patients undergoing chemotherapy, and patients recovering from massive blood loss (Bresnick et al., 2018). A key to improving our understanding of human stress erythropoiesis is the analysis of the signaling factors required for stress progenitor cell proliferation and differentiation, which will improve our understanding of how to culture the progenitor cells *in vitro.* Moreover, by characterizing the cell surface markers of the specific stress progenitor cell populations, a better understanding of how to isolate specific cell populations can be gained. Both avenues can provide a necessary understanding of the stress pathway to improve the current

understanding of diseases, like anemia, and how to treat patients with improper RBC development (Mori et al., 2015).

Thesis Rational

Previous work characterized a series of markers—Kit, SCA1, CD34 and CD133—on RBC progenitor cells. Using the patterns of marker expression, a developmental series of progenitors in erythropoiesis was identified. These markers were later combined with CD71 and Ter119 to characterize later progenitors in the process (Arndt et al., 2013; Dulmovits et al., 2017; Xiang et al., 2015). This set of markers enabled the Paulson lab group to characterize the development of stress erythroid progenitors *in vivo* and *in vitro*, however, the identification of new surface markers would enable further characterize these populations progenitor cells.

Work from a graduate student in the Paulson Lab analyzed the differential gene expression in rapidly proliferating ($PKH26^{Hi}$) and slowly proliferating ($PKH26^{low}$) immature stress erythroid progenitor cells. In this analysis, markers including CD44 and CD9 were identified as surface markers that are highly expressed in $PKH26^{Hi}$ cells. CD44 is a cell adhesion protein that binds to hyaluronan which has been shown previously to play a role in a number of cell signaling pathways—including hematopoiesis. CD44 functions in ligand binding, co-receptor activity, and as a link between the cell membrane and the cytoskeleton (Ponta et al., 2003). Notably, CD44 has been used previously to characterize erythroid progenitor cells. CD44 decreases as the RBC differentiate from a proerythroblast to the orthochromatic erythroblast indicating that CD44 decreases as these cells differentiate (Chen et al., 2009). CD9, on the other hand, has been identified as a marker associated with megakaryocyte differentiation and has not previously been analyzed in erythroid progenitors (Mori et al., 2015). CD9 is a tetraspanin molecule that functions as an adhesion molecule. It has been shown to play a role in leucocyte adhesions, exosome

biogenesis, and interacts with the c-Kit indicating it plays a prominent role in hematopoietic differentiation (Reyes et al., 2018; Termini & Gillette, 2017).

Characterization of CD44 and CD9 during differentiation *in vitro* could identify new populations of progenitors that would further delineate the developmental steps in the expansion and differentiation of stress erythroid progenitors. The goal of my thesis is to characterize how the expression patterns of CD9 and CD44 surface markers change during the differentiation of early ($Kit^{+}/SCA1^{+}$) and late ($Kit^{+}/SCA1^{-}$) progenitor cells in C57BL/6J wild type mice. We tested the expression of these proteins in stress erythroid progenitors from *in vitro* cultures using flow cytometry to analyze how the progenitor cells alter the expression of the CD9 and CD44 during expansion and differentiation.

Chapter 2

Materials and Methods

Bone Marrow Isolation

To isolate red blood cell (RBC) progenitor cells, two C57BL/6J wild type mice were euthanized by CO2 overdose. The hind limb femur dissection and preparation for long bone dissection followed the protocol outlined in Amend, Valkenburg, and Pienta (Amend et al., 2016). An 18-gauge needle was used to isolate the bone marrow cells from each mouse with 3 mL of phosphate-buffer saline (PBS). The needle was filled with the PBS, inserted into the medial condyle of the femur, and the PBS was expelled to flush the progenitor cells into a 15 mL conical tube. Cells from each mouse were kept in separate tubes and centrifuged at 10,000 rpm for 5 minutes following extraction. The supernatant was discarded and the cells were resuspended in 1 mL of Red Blood Cell Lysis solution and incubated on ice for 10 minutes. 1 ml of PBS was added to the cells and they were then centrifuged (10,000 rpm, 5 minutes). This supernatant was discarded and each set of cells were cultured as described in 'Cell Culture Conditions.'

Cell Culture Conditions

Isolated murine bone marrow cells were cultured in stress erythropoiesis expansion media (SEEM) for 5 days and switched to stress erythropoiesis differentiation media (SEDM) for 3 days. SEEM contains Gibco IMDM (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products), 1% penicillin-streptomycin, 10 mg/ml insulin, 200 mg/ml transferrin, 2mM L-glutamine, 0.01g/ml bovine serum albumin, 7 ml/L 2-mercaptoethanol, 30 ng/ml GDF15 (Novoprotein), 15 ng/ml BMP4 (R&D systems), 50 ng/ml SCF (Goldbio) and 25 ng/ml SHH (GoldBio). SEDM contains Gibco IMDM, all supplements in SEEM and 3U/ml EPO. Cells were incubated at 2% O2, 5% CO2 when cultured in SEDM.

Cell Collection

To collect cells for antibody staining and subsequent flow analysis, 6 mL of media from each flask was collected and placed into separate 15 mL conical tubes. The tubes were then centrifuged (10,000 rpm, 5 minutes). 6 mL of new SEEM was added to each flasks to maintain a sufficient volume of nutrients and signaling factors for cell growth. After discarding the supernatant, the cells in each conical tube were resuspended in 1 mL of PBS and 50 µL from each conical was placed in individual 1.7 mL tubes for cell counting. 50µL of trypan blue dye was added to each of the tubes to label dead cells and the cells were counted as explained in the Cell Viability Testing with Trypan Blue Exclusion Method published by the National Institute of Environmental Health Sciences ("Cell Viability Testing").

Antibody Staining

To visualize the surface markers during flow cytometry, antibodies with specific fluorochromes attached were used to label the cells. Dead cells were determined by propidium iodine (PI). A list of antibodies was provided in Appendix A: Supplemental Information table 1. 200 µL aliquots of cells were portioned for one unstained control for each mouse, and single stain (SS) controls, fluorochrome minus one (FMO) controls, and a viability control (PI) that would be shared between each mouse. Then 250 µL was aliquoted out for CD9 and CD44 samples from each mouse into 1.7 mL microcentrifuge tubes. Following this, the tubes were centrifuged (3,500 rpm, 4 minutes). The supernatant was discarded and all tubes were resuspended in 200 µL of PBS.

Flow Cytometry

To determine the expression pattern of the surface markers, a BD Accuri C6 Flow Cytometer was used for Flow Cytometry to excite the fluorochromes. The cytometer was run using the Accuri C6

protocol (BD Accuri C6, 2012). For control samples, the event limit was set to 5,000 and the event limit was set to 200,000 for the CD9 and CD44 samples. FlowJO version 10.6.1 was used to analyze the expression results produced from the cytometer.

Chapter 3

Results

Previous research in the Paulson lab identified that CD44 and CD9 were expressed in PKH26^{Hi} cells. I sought to determine whether these markers could be used to identify this population, which would allow the lab to specifically analyze these cells. I isolated BM cells from the femur of two C57BL/6J mice and ran flow cytometry on these cells to further explore the expression of CD44 and CD9 in the early stage (Kit^{+}/SCA1^{+}) progenitor cells and the late stage (Kit^{+}/SCA1^{-}) progenitor cells.

Kit^{+}/SCA1^{+} progenitor cells express CD44 more frequently than Kit^{-}/SCA1^{+} progenitor cells

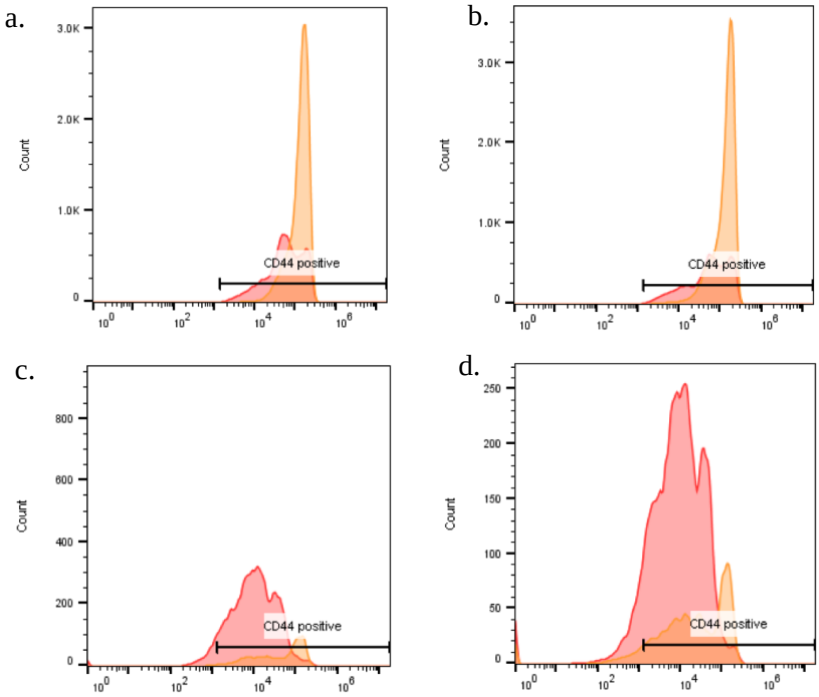
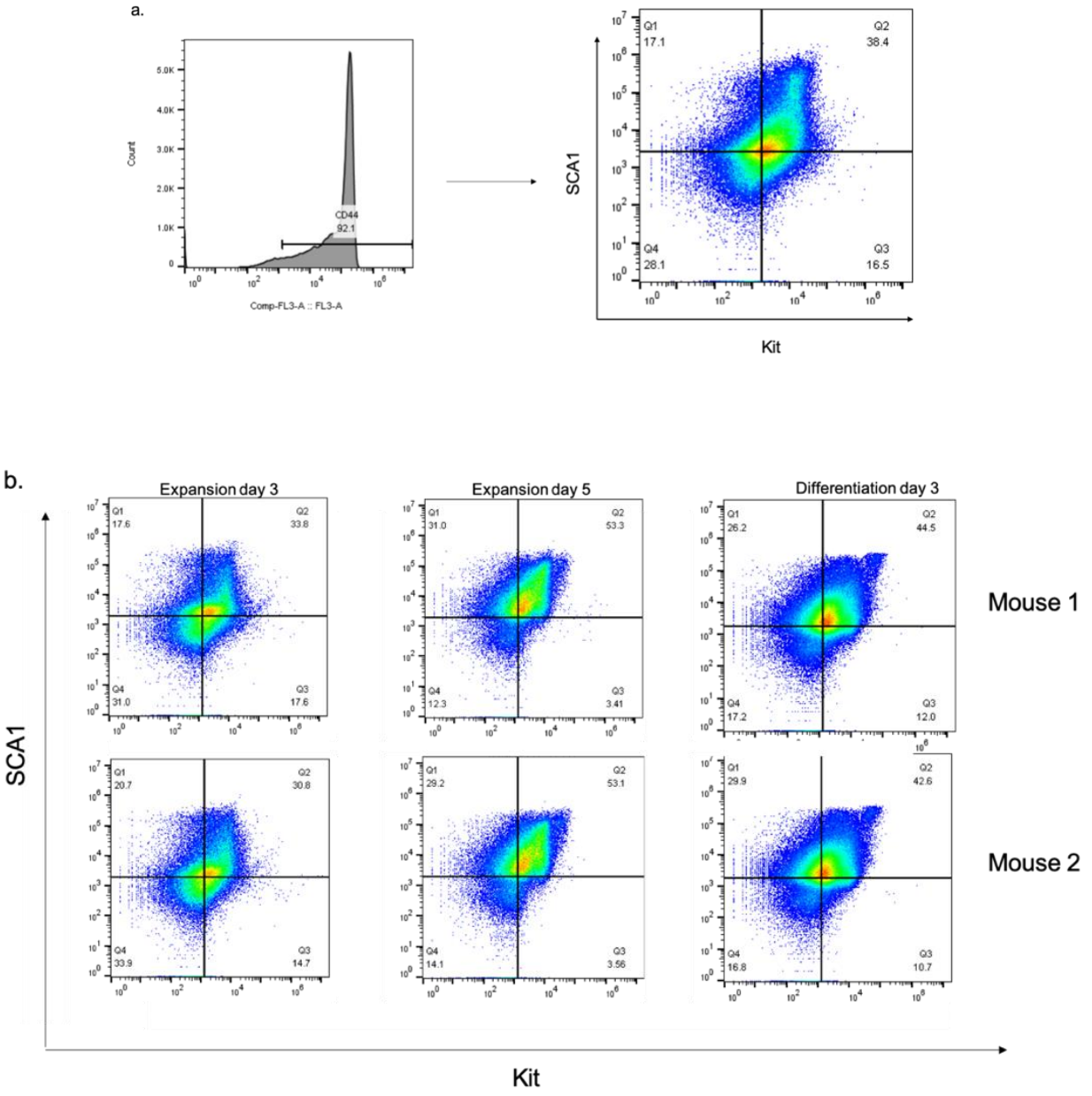


Figure 2. Change in CD44 Kit+/SCA1+ and Kit+/SCA1- surface markers

(A and B) Count of progenitor cells with Kit+/SCA1+ expression for mouse 1 (A) and mouse 2 (B). Expansion day 3 is graphed in red. Expansion day 5 is graphed in orange. Differentiation day 3 is graphed in blue. Expression was quantified using an Accuri C6 Flow Cytometer.
(C and D) Count of progenitor cells with Kit+/SCA1- expression for mouse 1 (C) and mouse 2 (D). Expansion day 3 is graphed in red. Expansion day 5 is graphed in orange. Differentiation day 3 is graphed in blue. Expression was quantified using an Accuri C6 Flow Cytometer. Flow cytometry analysis was done using FlowJo 10.6.1.

For CD44 surface marker expressing cells, **Figure 2A and 2B** shows that CD44^{+} expressing Kit^{+}/SCA1^{+} progenitor cells increase in count from expansion day 3 (E3) to expansion day 5 (E5) by

nearly four-fold for both replications. While **Figure 2C and 2D** showcase that the CD44+ Kit+/SCA1- population decrease by almost 3-fold from E3 to E5 in each replication. Additionally, there were more Kit+/SCA1+ cells expressing CD44+ (about 3,000) than there are Kit+/SCA1- cells expressing CD44+ (about 100) counted in E5 suggesting that as the progenitor cells mature they lose expression of CD44.



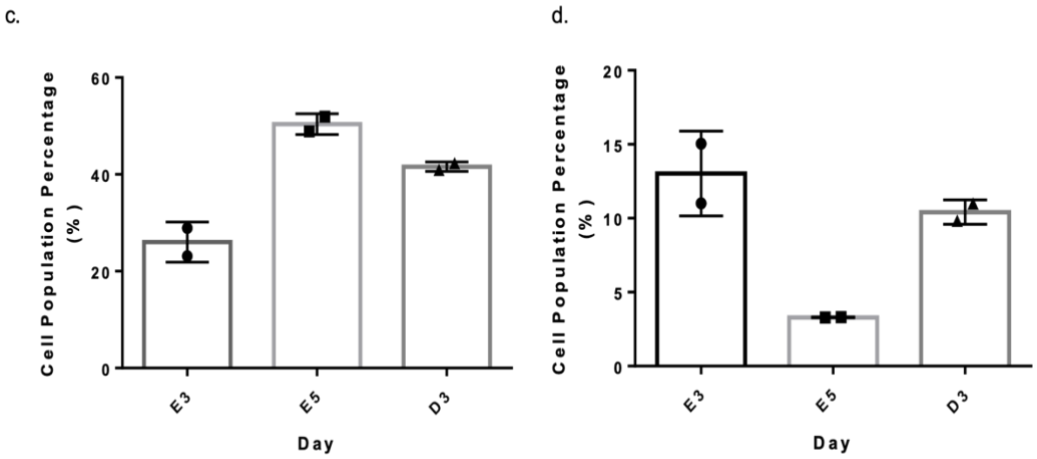


Figure 3. Representative CD44 Flow Cytometry Expression Data

(A) Experimental overview of CD44 gating and flow cytometry analysis.
(B) The representative flow cytometry results of CD44+ cells in mice at different time points during culture.
(C and D) Percentage of CD44+ progenitor cell population that are also Kit+/SCA1+ (C) or Kit+/SCA1-
(D) expression at different days during culture (n=2). E3 is expansion day 3. E5 is expansion day 5. D3 is differentiation day 3. Graph represents the average expression level and the error bars represent the variation between each mouse. Flow cytometry analysis was done using FlowJo 10.6.1.

Quantification of the CD44 expression level in Kit+/SCA1+ and Kit+/SCA1- characterized the expression of this surface marker as the progenitor cells mature. Data in **Figure 3A** gives an overview of how the expression levels were obtained. FlowJo was first used to isolate my analysis to only the living CD44 expressing cells. Then from that population, I gated the for the expression of Kit and SCA1 on days E3, E5, and D3 (**Figure 3B)**. It can be seen that for each mouse, the percentage of CD44 Kit+/SCA1+ progenitor cells are greater than the percentage of CD44 Kit+/SCA1- progenitor cells. This observation, again, suggests that the expression of CD44 decreases as the progenitor cells mature from the early to late stages.

I then took the percentage of the Kit+/SCA1+ and Kit+/SCA1- cell populations from Figure 3B and graphed the average from each replication in **Figure 3C and 3D**, respectively. On E3, we observed an average of 32.2% of CD44 expressing cells were Kit+/SCA1+ and 16.15% were Kit+/SCA1-. On E5, Kit+/SCA1+ cells expressing CD44 increased to 53.2% (a 20.9% increase from E3) while the Kit+/SCA1- cell population decreased to 3.49% (a 9.65% decrease from E3) was seen. After being exposed to EPO, at the D3 timepoint, the Kit+/SCA1+ population expressing CD44 had a slight decrease to 43.55% (about a

10% decrease from E5) and the Kit+/SCA1- population had an increase to 11.35% (an increase of about 8%). These results indicate that the expression of CD44 is variable for both cell population but most frequently expressed on the Kit+/SCA1+, early progenitor, population.

Kit+/SCA1+ progenitor cells express CD9 more frequently than Kit-/SCA1+ progenitor cells

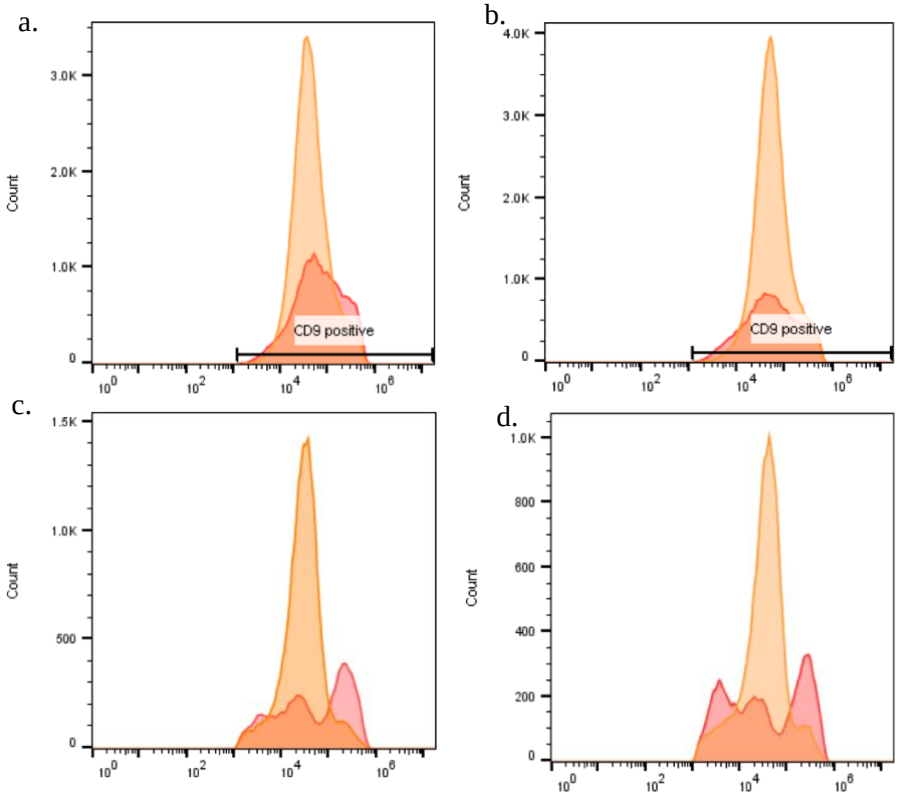
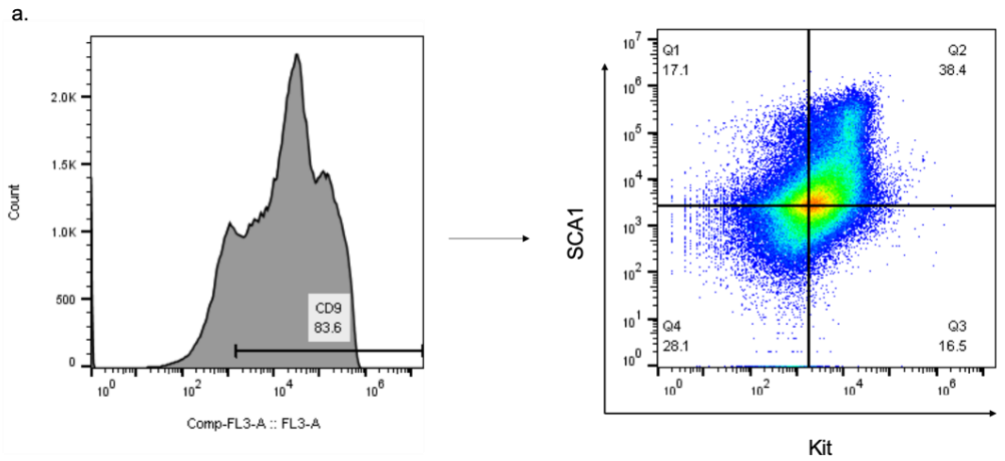


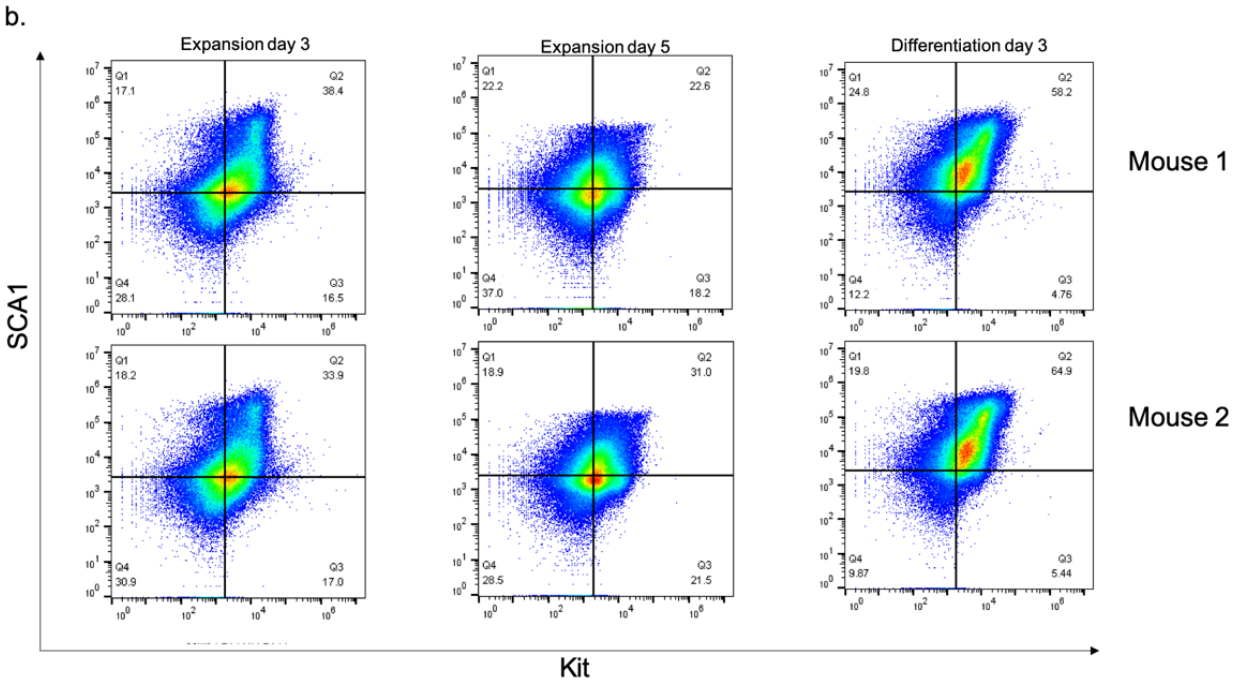
Figure 4. Change in CD9 kit+/SCA1+ and Kit+/SCA1- surface markers

(A and B) Count of progenitor cells with Kit+/SCA1+ expression for mouse 1 (A) and mouse 2 (B). Expansion day 3 is graphed in red. Expansion day 5 is graphed in orange. Differentiation day 3 is graphed in blue. Expression was quantified using an Accuri C6 Flow Cytometer.
(C and D) Count of progenitor cells with Kit+/SCA1- expression for mouse 1 (C) and mouse 2 (D). Expansion day 3 is graphed in red. Expansion day 5 is graphed in orange. Differentiation day 3 is graphed in blue. Expression was quantified using an Accuri C6 Flow Cytometer. Flow cytometry analysis was done using FlowJo 10.6.1.

I next analyzed the expression of CD9 on the Kit+/SCA1+ and Kit+/SCA1- cell populations. Data in **Figure 4A-4D** show the count of Kit+/SCA1+ (Fig. 4A-4B) and Kit+/SCA1- (Fig. 4C-4D) I found on E3 and E5. Both cell populations studied had an increase by about 3-fold in CD9 expressing cell count from

E3 and E5 with a greater count found in the Kit+/SCA1+ population compared to the Kit+/SCA1- population (~3,500 vs. ~1,000).





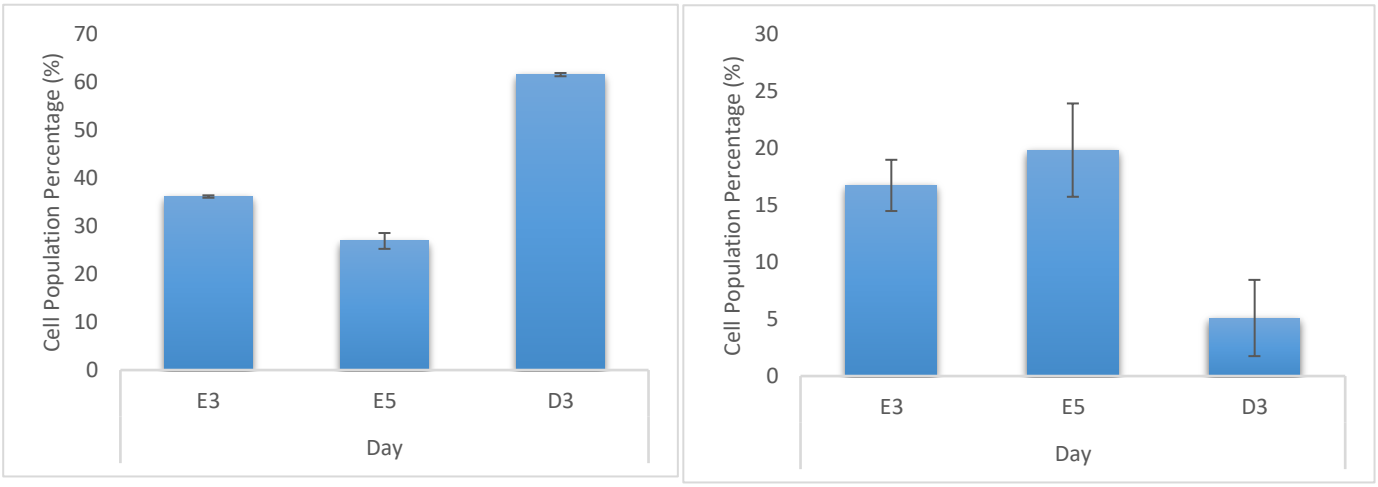


Figure 5. Representative CD9 Flow Cytometry Expression Data

(A) Experimental overview of CD9 gating and flow cytometry analysis.
(B) The representative flow cytometry results of CD9+ cells in mice at different time points during culture.
(C and D) Percentage of CD9+ progenitor cell population with Kit+/SCA1+ (C) and Kit+/SCA1- (D) expression at different days during culture (n=2). Shapes on each of the bars represent the individual mice. E3 is expansion day 3. E5 is expansion day 5. D3 is differentiation day 3. Graph represents the average expression level and the error bars represent the standard error during each step. Flow cytometry analysis was done using FlowJo 10.6.1.

Similar to my study for CD44 expression, I characterized the expression of CD9 using flow cytometry on E3, E5, and D3 (**Figure 5A)**. I included only living CD9 expressing cells and looked for the expression of Kit and SCA1. The data in **Figure 5B** represents the flow cytometry data for each time point. Similar to expression of CD44, I found that more $Kit^{+}/SCA1^{+}$ progenitor cells expressed CD9 than $Kit^{+}/SCA1^{+}$ on E3, E5 and D3. This suggests that the CD9 surface marker is present in early progenitor cells but is progressively lost as the cells mature.

I then graphed the flow cytometry data which is represented in **Figure 5C-5D**. On E3, an average of 36.15% of the CD9 expressing cells were $Kit^{+}/SCA1^{+}$ while only 16.75% were $Kit^{+}/SCA1^{-}$. The percentage of $Kit^{+}/SCA1^{+}$ cells decreased to 26.8% (a decrease of 9.35%) on E5. On the other hand, the $Kit^{+}/SCA1^{-}$ cells increased slightly to 19.85% (an increase of 3.1%) on E5. Lastly, data on D3 showcased that the $Kit^{+}/SCA1^{+}$ progenitor population had a notable increase in CD9 expression with an average of 61.55% indicating this early population expresses CD9 as it differentiates. The $Kit^{+}/SCA1^{-}$ cell population, however, decreased to 5.1% indicating that as these cells mature they decrease in CD9 expression.

Chapter 4
Discussion

Previous research characterized the expression pattern of RBC surface markers including: Kit, SCA1, CD34, CD133, CD71, and Ter119. These markers have since been used to explain the developmental series of RBC progenitors (Arndt et al., 2013; Dulmovits et al., 2017; Xiang et al., 2015). CD44 and CD9 are differentially expressed surface markers on PKH26^{Hi} cells. Here, I further characterized CD44 and CD9 in early (Kit^{+}/SCA1^{+}) and late (Kit^{+}/SCA1^{-}) stress erythroid progenitors. My results indicate that both CD44 and CD9 are expressed on the surface of the Kit^{+}/SCA1^{+} and Kit^{+}/SCA1^{-} cell populations.

Focusing on CD44, almost all expression was seen in the Kit^{+}/SCA1^{+} progenitor cell populations when compared to the Kit^{+}/SCA1^{-} population (Fig. 2). Additionally, there was a significant increase in mean fluorescence intensity (MFI) from E3 to E5 in the Kit^{+}/SCA1^{+} cell population. However, the Kit^{+}/SCA1^{-} population, on the other hand, had a decreases in MFI during this time. Scatterplots of these stress erythroid progenitors during E3, E5, and D3 gated for SCA1 and Kit expression also showed this distinction in the progenitor populations (Fig. 3B). The fact that CD44 is expressed more frequently in the Kit^{+}/SCA1^{+} when compared to the Kit^{+}/SCA1^{-} progenitor cells indicates that CD44 is a reliable surface marker to use for distinguishing these cell populations.

Moving to CD9 expression, a more interesting expression pattern was seen in the Kit^{+}/SCA1^{+} stress progenitor cells on E3. There is a noticeable fluctuation in MFI with three peaks (Fig. 4A and 4B). This suggests that there may be a high, medium, and low subset of these cells which become predominantly medium by E5. The Kit^{+}/SCA1^{-} cells do not have this different expression pattern seen on E3. They have a single expression patter E3 which increases

on E5 (Fig. 4C and 4D). Further experiments should work to determine if the high, medium, and low population of Kit+/SCA1+ seen on E3 represent unique stages of development.

Further comparison of CD9 expression revealed another difference between the Kit+/SCA1+ and Kit+/SCA1- progenitor populations. Differentiation media resulted in a shift in CD9 expression for both populations. Kit+/SCA1+ cells had a notable increase in CD9 expression. I saw an increase of about 30% in the CD9 Kit+/SCA1+ expressing cells in the flow cytometry data whereby the majority of CD9 expressing cells were the Kit+/SCA1+ progenitors (Fig. 5B and 5C). Contrastingly, I found that differentiation media had the opposite effect on the Kit+/SCA1- progenitors. The media caused these cells to decrease their expression of CD9 by nearly 15% (Figure 5B and 5D). The pattern of expression is likely due to the role of CD9 as a cell adhesion molecule; CD9 has previously been shown to play a role in immune cell extravasation (Reyes et al., 2018). However, for the case of mature RBCs, which do not want to move between the blood vessels naturally and would, thus, likely not need CD9 expressed in a mature state. Whatever the case for this shift in CD9 expression, the differences in expression between the Kit+/SCA1+ and Kit+/SCA1- stress progenitor populations make it a potential marker to be used for identifying these populations of cells.

This characterization is a start; however, this thesis should be expanded on. Further research must be conducted before definitely using these markers to distinguish the Kit+/SCA1+ and Kit+/SCA1- stress erythroid cell populations from one another. The use of the Accuri C6 limited the number of fluorescent markers that could be used to four fluorochromes. This limited the number of surface markers I was able to characterize at one time. This work was being done but was not able to be completed due to the COVID-19 pandemic. Therefore, a more comprehensive study still must be completed where other surface markers on these stress

erythroid progenitor cell populations are labeled and characterized with a more expansive flow cytometer. This characterization would give a broader understanding of the expression pattern of CD44 and CD9 in relation to previously identified surface markers.

In conclusion, my results have further characterized the expression of CD44 and CD9 in $Kit^{+}/SCA1^{+}$ and $Kit^{+}/SCA1^{-}$ stress progenitor cells. Both CD44 and CD9 expression was markedly higher in the $Kit^{+}/SCA1^{+}$ population in comparison to the $Kit^{+}/SCA1^{-}$ population. This provides support for the potential of using these markers in identifying and further characterizing the developmental pattern of stress erythroid progenitor cell populations. This enhanced understanding of stress erythropoiesis will help to advance our ability to isolate specific progenitor cells which may be helpful in the treatment of blood disorders including anemia.

Appendix A: Supplemental Material

Table S1. Antibody Streak Overview

Antibody	**Fluorochrome**	**Accuri C6 Channel**
CD9	PE-Cy7	FL3
CD44	PE-Cy7	FL3
CD117 (c-Kit)	Alexa Fluor 647 (APC)	FL4
Sca1 (Ly-6A/E)	FITC	FL1
Viability Stain	Propidium Iodine (PI)	FL2

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EDUCATION

- Pennsylvania State University Schreyer's Honors College Graduation: May 2020
- Major: Immunology and Infectious Disease
  - Minor: Bioethics and Medical Humanities

WORK EXPERIENCE

Janssen Immunology Translational Science & Medicine Intern June 2019-August 2019

- Compiled clinical trial portfolios comparing competitors' trial designs for disease areas of interest
- Collaborated with physicians and scientists to design a patient engagement presentation to gain feedback from patients on clinical trials
- Participated in literature searches for TSM team projects and presented findings to the TSM team

Thesis Research: Red Blood Cell Progenitor Characterization August 2018-May 2020

- Conducted thesis research using flow cytometry to characterize CD9 and CD44 expression on red blood cell progenitor cells

Pediatric Oncology Student Training (POST) Fellow June 2018-August 2018

- Investigated role of Delta-like protein 3 (DLL3) in neuroblastoma cells as a potential immunotherapeutic target under Dr. John Maris's (2017, NCI Investigator of the Year) supervision
- Optimized Gateway Cloning to overexpress DLL3 in low expressing cell lines
- Developed skills in cell culture, ELISA, and gateway cloning

Zombie Ants Research January 2017-May 2018

- Mapped behavioral patterns of infected ants
- Collaborated with graduate student to study the altruistic behaviors in ant colonies
- Compiled data for graduate students in Excel
- Maintained ant colonies

LEADERSHIP EXPERIENCE

Penn State Crew President January 2018-May 2020

- Lead executive board, coaching staff, and team of 70 members
- Managed budget of over $120,000
- Collaborated with board to plan team events and annual Nittany Lion Chase Regatta

Team Captain 2018-2020; Novice Representative 2016-2017;

Molecular Biology Lab Teaching Assistant August 2017-May 2018; August 2019-May 2020

- Instructed 24 undergraduate students
- Assessed student proficiency in scientific communication, plasmid DNA isolation, microscopy, PCR, gel electrophoresis, and use of bioinformatic tools

Molecular Biology Learning Assistant August 2017-December 2017; January 2019-May 2019

- Acted as an extension of the instructor to facilitate learning during lecture and scheduled office hours

- Answered students' questions regarding course content
- Advised students in study techniques

Penn State Club Sports Council Secretary August 2019-May 2020

VOLUNTEER EXPERIENCE

PSU LifeLink January 2018-May 2020

- Mentor a student with disabilities from the State High Area School District
- Aid in the acquisition of social and lifestyle skills
- Adapt to fit student's needs

Discovery Space January 2017-January 2018

- Facilitated the use of museum exhibits that teach children fundamental scientific concepts to help foster a love for learning at an early age
- Worked with museum staff to maintain a clean and safe environment

Back Mountain Memorial Library Summer Reader Summer 2015-Summer 2017

- Planned a story hour to a group of adolescent children age 2 and ages 3-5
- Taught children fine motor skills through finger plays and interactive dances, social interactions through group activities, and basic knowledge such as the ABCs, counting, and what noises animals make

Geisinger ER Department Volunteer July 2017-August 2017

- Worked with other Emergency Room staff in order to ensure patients have a comfortable time waiting by offering amenities such as warm blankets, water, and updates on wait time

ACADEMIC ACCOMPLISHMENTS

- College of Agricultural Science Honor Roll (2016-2017)
- Galen Dreibelbis Endowment for Excellence in Agriculture
- Rosemarie C. and Howard R. Peiffer Scholarship in the College of Agriculture Sciences
- Gamma Sigma Delta