THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF IMMUNOLOGY AND INFECTIOUS DISEASE

Role of RNA Binding Protein Smaug in Metabolic Regulation of Erythropoiesis

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ABSTRACT

Erythropoiesis is a critical process in which erythroid progenitor cells proliferate and differentiate into mature red blood cells. Current findings have determined two pathways of erythropoiesis, the standard steady state pathway of erythropoiesis and the BMP4-dependent stress erythropoiesis pathway, which is activated during situations of infection and inflammation. Stress erythropoiesis is a complex process involving the function of many proteins and regulatory pathways. Though, the purpose and impact of these proteins and pathways are not fully understood by the scientific community. A candidate RNA-binding protein, Smaug, is presumed to function in the efficacy of erythroid differentiation processes and mitochondrial respiration events. In turn, this research focuses on the preliminary phases of Smaug protein analysis, ultimately questioning whether the lack of Smaug expression results in the inhibition of stress erythroid differentiation. To investigate Smaug mRNA expression levels, an expansion and differentiation (SEEM/SEDM) cell culture system developed by the Paulson Lab was utilized, along with an in-vivo model of inflammatory anemia, heat-killed brucella abortus (HKBA). Both cell sample models were tested using RT qPCR methods, in which the expression of Smaug was compared to 18S rRNA. Ultimately, results suggested that Smaug expression rises during expansion, and decreases during differentiation, which is consistent with our hypothesis that Smaug regulates the presence of respiratory complex RNAs and mitochondrial respiration levels.

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Chapter 1

Introduction

Hematopoiesis

The processes involved in the production of cells and plasma within an organism's blood supply can be defined as hematopoiesis. Several lineages of blood cells are produced consistently in the body of organisms throughout their lifespan, including the development of erythrocytes, leukocytes, platelets, and a variety of other innate and adaptive immune cells such as T and B lymphocytes, macrophages, and mast cells (Orkin, 1995). The progression of hematopoiesis in organisms, such as humans, and the resulting blood components produced, originate from hematopoietic stem cells. These specific stem cells comprise the beginning stages of hematopoiesis and continue through multiple, complex proliferation and differentiation events to become the specialized cells and components apparent in the blood (Tavian et. al., 2010). Given the complexity of hematopoietic processes, it is evident that strategic and comprehensive regulatory systems are involved. The pivotal role of hematopoiesis in organism homeostasis and immune function, along with the intricate involvement of hematopoietic stem cells, makes it a prominent topic within stem cell biology. Therefore, it is essential to determine the origins and mechanisms involved in hematopoiesis to further understand how this elaborate process is regulated in different organisms.

In humans, hematopoiesis can be described and divided into two lineage branches: myeloid and lymphoid (Liggett et. al., 2020). Both lineages are involved in the function of different processes, involving participation in nutrient transport systems, immune functions, hemostasis, and more (Liggett et. al., 2020). Furthermore, the myeloid and lymphoid lineages encompass the differentiation and production of several cell types, which function as fundamental components in the maintenance of an organisms

homeostatic and immunologic responses. The myeloid lineage is primarily involved in the function and development of innate immune components. Erythrocytes, megakaryocytes, leukocytes, and dendritic cells are included in the cell types produced through the myeloid lineage. In turn, myeloid progenitors display several key functions aside from innate immune activities, such as oxygen transport and hemostasis (Liggett et. al., 2020). Conversely, the lymphoid lineage is involved predominantly in adaptive immune cell production and function. Specifically, B lymphocytes, T lymphocytes, and natural killer cells include some of the adaptive components produced within the lymphoid lineage of hematopoiesis (Liggett et. al., 2020). Despite key differences between the myeloid and lymphoid lineages, the corresponding components produced by each lineage work collaboratively to ensure an optimal, protective response within the organism.

Moreover, as continual studies are conducted determining novel discoveries of hematopoietic mechanisms and genomics, evolving models of hematopoiesis arise. Currently, there is a classic, continuum, and punctuated continuum model of hematopoiesis, each involving differences in the distinguishment of cell fates, lineage contribution, and differentiation hierarchies. The classic model defines hematopoiesis as a compilation of different states, with each state depicting the fate of future differentiated cells produced (Liggett et. al., 2020). In turn, the continuum model of hematopoiesis focuses on a continual differentiation process, instead of stepwise, with an emphasis on lineage commitment properties (Liggett et. al., 2020). The punctuated continuum model displays similarities to the classic model, however, the various states of cells involved within the process are associated with heterogenous pools of cells, in which differing lineage commitments and differentiation events arise (Liggett et. al., 2020).

The complexity involved with hematopoietic processes and the vast genomic nature associated throughout differentiation and proliferation events offers expansive opportunities for future studies. Subsequently, erythropoiesis, a specific process associated with hematopoiesis, focuses precisely on the generation of erythrocytes from stem cell and progenitor cell differentiation (Nandakumar et. al., 2016).

Genetic mechanisms and mutation events are distinctly important when discussing erythropoiesis, as several erythroid disorders have been investigated in humans. Continual expansion in the understanding of hematopoietic events, such as erythropoiesis, with an emphasis on the genomic properties involved within these processes, will allow for the further understanding of the mechanisms that regulate erythroid progenitor cell proliferation and differentiation.

Erythropoiesis

The process of erythropoiesis culminates in the generation of mature red blood cells (erythrocytes). Specifically, the mature red blood cells are produced by the differentiation of erythroid progenitor cells in the bone marrow (Nandakumar et. al., 2016). Once red blood cells reach maturation, their primary function is to transport oxygen to the tissues comprising the organism's body. Given the complexity of this process, the maturation of red blood cells is known to be strictly regulated by several factors, including hormones and immune signaling components such as cytokines (Nandakumar et. al., 2016). Studies have discovered significant differences in erythropoiesis between embryos and adults. As an embryo develops, the location of erythropoiesis transitions from the yolk sac to the liver and spleen, then ultimately to the bone marrow (Nandakumar et. al., 2016).

In mammals, embryonic erythropoiesis can be defined by several developmental waves. First, in the yolk sac of an embryo, primitive erythroid progenitor cells found in blood islands differentiate into erythroblasts, which produce embryonic hemoglobin (Tang and Wang, 2023). A shift in erythropoiesis location from the yolk sac to the liver and spleen occurs between week 6-8 of embryo gestation (Tang and Wang, 2023). Both the liver and spleen begin to produce erythropoietin hormone (Epo), which stimulates the process of erythropoiesis (Tang and Wang, 2023). Furthermore, erythro-myeloid progenitor cells and hematopoietic stem cells migrate toward these organs and colonize (Nandakumar et. al., 2016). After the

second trimester, the process transitions from the liver and spleen areas to the bone marrow, which then becomes the hematopoietic center and site of erythropoiesis activity at birth (Tang and Wang, 2023).

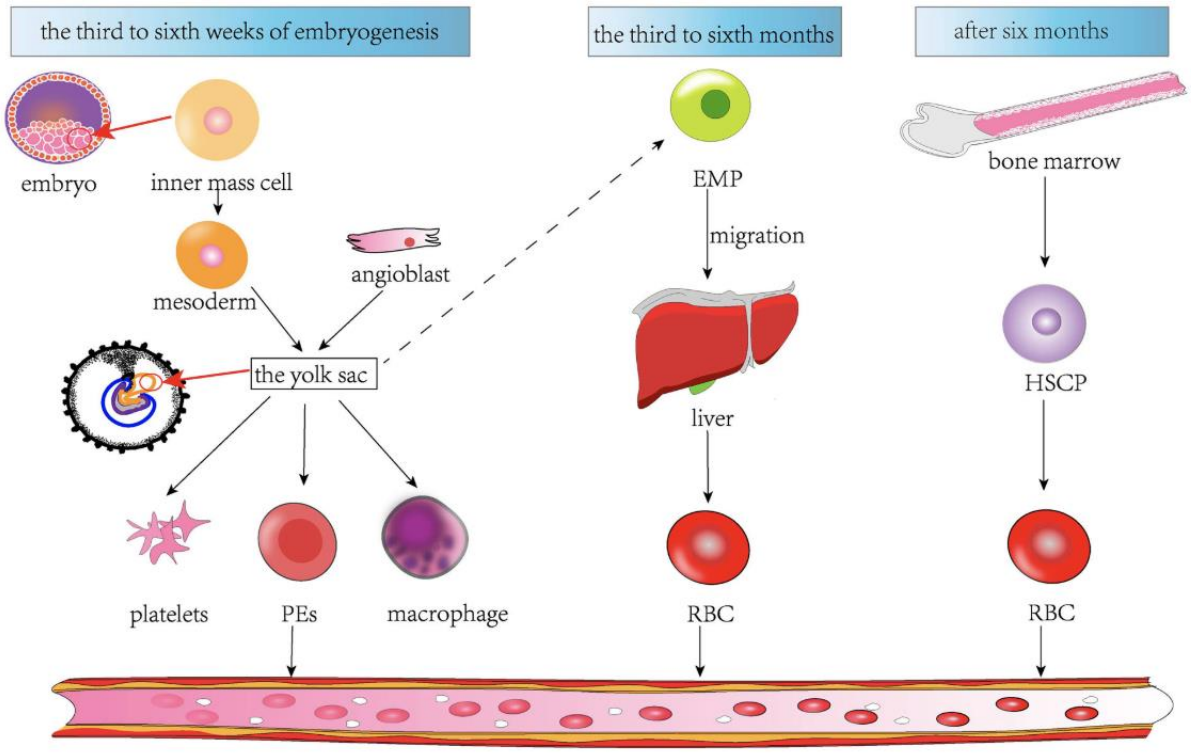

Figure 1. Overview of embryonic erythropoiesis, including transition locations

Erythropoiesis process begins in the embryo yolk sac between the first three to six weeks of embryogenesis. Within the yolk sac, platelets and macrophages are produced, along with primitive erythroblasts (PE) which then enter the embryo blood stream. Transition to the embryo liver and spleen occur around month three of embryogenesis. Erythro-myeloid progenitor cells (EMP) migrate to the liver and differentiate into red blood cells, which enter the embryo blood stream. The final transition to the bone marrow occurs after six months of embryogenesis. Hematopoietic stem cell progenitors (HSCP) differentiate into red blood cells and enter the embryo blood stream. From "Regulation of erythropoiesis: emerging concepts and therapeutic implications" by P. Tang and H. Wang, 2023, Hematology, 28:1, Figure 1 (https://doi.org/10.1080/16078454.2023.2250645).

Following birth and into adulthood, erythropoiesis activity persists in the bone marrow. Hematopoietic stem cells start by producing megakaryocytes, along with erythroid bursting-forming units (BFU-E), and erythroid colony-forming units (CFU-E) which are a continuous stage of erythroid progenitor cells (Tang and Wang, 2023). Furthermore, proerythroblasts (Pro-E) are created, followed by basophils (Bas-E), polychromic phage (Poly-E), and positively stained erythrocytes (Ortho-E).

Ultimately, the cells become reticulocytes as the cell size decreases with enucleation and hemoglobin levels increase (Tang and Wang, 2023). The changes associated with the progression from hematopoietic stem cells to reticulocytes are the precursors necessary for the complete maturation of an erythrocyte (Tang and Wang, 2023).

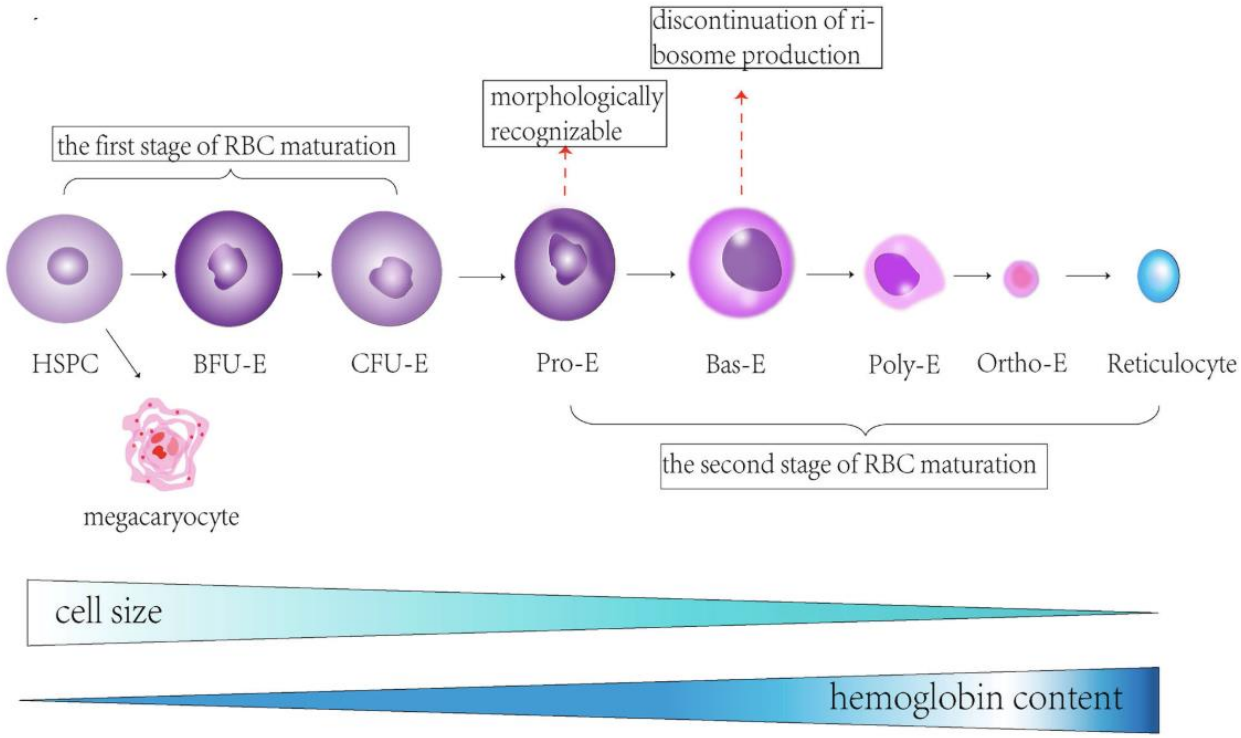

Figure 2. Overview of red blood cell maturation and differentiation in the bone marrow

Displays the stages of red blood cell (RBC) maturation from hematopoietic stem cells (HSPC) to reticulocytes. As cell production and differentiation to reticulocyte continues, cell size decreases and hemoglobin content increases. From "Regulation of erythropoiesis: emerging concepts and therapeutic implications" by P. Tang and H. Wang, 2023, Hematology, 28:1, Figure 2 (https://doi.org/10.1080/16078454.2023.2250645).

Influence and Impact of Stress Erythropoiesis

Erythropoiesis is an intricate process involving elaborate mechanisms, differentiation events, and regulatory components. Erythrocyte production is typically maintained at a steady state by homeostatic mechanisms, known as steady state erythropoiesis (Paulson et. al., 2020). This process ensures a

consistent production of new erythrocytes for efficient delivery of oxygen to body tissues, while also controlling the removal of aging erythrocytes (Paulson et. al., 2020). Although, there are several situations in which steady state erythropoiesis is impacted and unable to maintain standard homeostatic levels. Infections resulting in tissue damage and inflammation are known to negatively impact steady state erythropoiesis, resulting in the use of an alternative pathway for erythrocyte production called the BMP4-dependent stress erythropoiesis pathway (Paulson et. al., 2020).

Studies analyzing the BMP4-dependent stress erythropoiesis pathway demonstrate a different pathway of erythrocyte production in comparison to steady state erythropoiesis (Paulson et. al., 2020). Specifically, BMP4-dependent stress erythropoiesis has been shown to develop a large wave of new erythrocyte production at once, instead of a consistent rate of erythrocytes over time (Ruan and Paulson, 2023). The increased production of erythrocytes at one time point is necessary to maintain homeostasis until steady state erythropoiesis resumes (Ruan and Paulson, 2023). Given the influence of inflammatory signaling on BMP4-dependent stress erythropoiesis pathway activation, along with the apparent differences between steady state and BMP4-dependent stress erythropoiesis, metabolic regulatory components and proteins impacting the function of both pathways must be analyzed and distinguished.

The Smaug Protein

Erythropoiesis and other events involved in the maturation of red blood cells in mammals is tightly regulated and stimulated by the function of multiple genes and proteins. SAMD5, which will further be referred to as Smaug, is a known RNA-binding protein found in organisms such as flies and mammals (Bruzzone et. al., 2020). This protein has multiple presumed functions ranging from mammalian neurogenesis to mitochondrial regulation (Baez and Boccaccio, 2005). Specifically, Smaug protein orthologs often bind to mRNA transcripts that contain Smaug recognition elements (SREs) within stem-loops in their secondary structure (Fernández-Alvarez et. al., 2022). Smaug then functions as a

translational regulator, in which it binds to a variety of mRNAs resulting in the destabilization or repression of translation (Amadei et. al., 2015).

The repressed mRNAs resulting from Smaug protein binding have been found in specific membrane-less organelles, termed Smaug foci or bodies (Fernández-Alvarez et. al., 2022). Smaug bodies have been analyzed and apparent in components of mammalian nervous tissues, and in the muscles of fly embryos and adults (Fernández-Alvarez et. al., 2022). Despite several studies depicting the nature of Smaug protein function and effect on cellular translation components and events, limited information regarding the regulatory mechanisms of Smaug expression is known throughout the scientific community. Therefore, additional analysis regarding pathways of regulation for the Smaug protein in both mammals and flies in needed to further understand the overall impact of Smaug in cellular processes occurring within these organisms (Fernández-Alvarez et. al., 2022).

Smaug and Mitochondrial Respiration

During both stress erythropoiesis and steady state erythropoiesis, mitochondrial dynamics provide key regulatory functions. As red blood cell maturation occurs, mitochondrial activities affect differentiation events, heme biosynthesis, iron metabolism processes, and the progression of glycolytic to oxidative cellular metabolism (Gonzalez-Ibanez, 2020). Recent studies examining the significance of mitochondrial function and erythropoiesis have found that mitochondrial dynamics, involving the fission and fusion events associated with mitochondrial turnover and other signaling events, assume a pivotal role in the commitment of erythroid progenitors to differentiation during early erythropoiesis (Gonzalez-Ibanez, 2020). Significantly, data has shown that the progression of erythroid differentiation, keeping cells in the proerythroblast stage of development, occurs when mitochondrial fusion events are diminished or damaged (Gonzalez-Ibanez, 2020). Considering the apparent correlation between

mitochondrial dynamics and erythropoiesis, analysis of proteins associated with mitochondrial functions and assessment of protein expression changes on erythroid differentiation processes, must be investigated.

The RNA-binding protein Smaug is suspected to have a prominent function in the regulation of mitochondrial respiration events (Fernández-Alvarez et. al., 2022). Furthermore, mitochondrial respiratory complex gene expression increases during times of stress erythropoiesis (Fernández-Alvarez et. al., 2022). Despite possible functional correlations between Smaug protein expression and mitochondrial respiration, the methods by which they are related to erythropoiesis progression and efficacy is unknown. The goal of this research is to test the role of Smaug in the increase of mitochondrial respiration response and determine whether loss of Smaug expression results in the inhibition of stress erythroid differentiation.

Chapter 2

Methods

Animals

Wild type and C57BL/6 mice were maintained and tested in accordance with guidelines and regulations previously approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Both male and female wild type and C57BL/6 mice were used for experimentation. Wild type mice were used in the heat-killed brucella abortus (HKBA) model for in vivo testing. Mice were injected with 10^8 particles of HKBA per 20g mouse intraperitoneally. All C57BL/6 mice were between 8-10 weeks of age.

Stress Erythropoiesis Expansion/Differentiation (SEEM/SEDM) Cultures

Stress erythropoiesis expansion and differentiation was modeled in C57BL/6 mice cells using the protocol developed by the Paulson Lab. On day 0, C57BL/6 mice were euthanized, and bone marrow cells were harvested. Bone marrow cells were obtained by flushing of the central bone marrow canal with phosphate buffered saline (PBS) solution. The PBS and bone marrow cell suspension was filtered and centrifuged for 5 minutes at 400-700g. The cells were then resuspended in 3-5mL of erythrocyte lysis buffer and chilled on ice. After 5 minutes on ice, an additional 5mL of PBS was added to stop cell lysis and the cells were filtered, centrifuged, and resuspended with 5mL of PBS once again. The cells were then diluted, dyed, and counted. Following the counting process, approximately 2.5 million cells were added into a flask with 5mL of SEEM media and incubated at 37C in 5% CO_2. Once proper incubation was completed, cultures were moved to SEDM media. Flasks with approximately 2 million cells were mixed with 5mL of SEDM media, which is SEEM media containing 3 U/mL of erythropoietin (Epo). The SEDM culture flasks were placed into a 2% O_2 incubator and flow staining occurred. For the purposes of

this experiment model, graduate students of the Paulson Lab provided unfractionated bone marrow as a control, SEEM day 5 cultures, and SEDM day 3 cultures for testing.

In Vivo HKBA Cell Cultures

Wild type mice were injected with 10^8 particles of HKBA per 20g mouse intraperitoneally. Following injection, spleen cells were harvested from the mice at different time points. The spleen red blood cells from the mice were lysed and the remaining cells were used for RNA samples. For the purposes of this experiment model, graduate students of the Paulson Lab provided HKBA cells from days 0, 4, 6, and 8 after injection. The day 0 HKBA cells were used as a control.

Quantitative Reverse Transcription Polymerase Chain Reaction

Stress erythropoiesis SEEM/SEDM and HKBA cell culture cDNA was detected and quantified using quantitative reverse transcription polymerase chain reaction (RT-qPCR). After the samples were acquired, SEEM/SEDM and HKBA samples were prepared. Three samples from day 0 unfractionated bone marrow, SEEM day 5, and SEDM day 3 cell cultures were provided by the Paulson Lab graduate students. Similarly, three samples from HKBA day 0, 4, and 6, and two samples from HKBA day 8 cell cultures were provided by the Paulson Lab graduate students. Calculated quantities of RNA from the SEDM/SEEM samples were mixed with nuclease-free water and master mix solution obtained from the qScript cDNA Synthesis Kit. Furthermore, RNA from the HKBA cell cultures were diluted with nuclease-free water, and the diluted RNA was mixed with nuclease-free water and master mix solution obtained from the qScript cDNA Synthesis Kit. After preparation, each sample from the SEEM/SEDM and HKBA cell cultures were 10 μL. Following the manufacturers protocol, cDNA from the SEEM/SEDM and HKBA samples were obtained using reverse transcription polymerase chain reaction

(RT-PCR). After cDNA for each sample was acquired, the samples were prepared for qPCR. A combined qPCR mix was made by proper calculation and addition of nuclease-free water, Perfecta qPCR SuperMix, and either Smaug or 18s probes. Both probes were bought and provided by graduate students from the Paulson Lab. After both probe qPCR mixtures were prepared, 2 μL of cDNA from the SEEM/SEDM and HKBA samples were added to 8 μL of combined Smaug probe mix. Similarly, 2 μL of cDNA from the SEEM/SEDM and HKBA samples were added to 8 μL of combined 18s probe mix. Following the manufacturers protocol, qPCR for all samples was ran and results were derived using Applied Biosystems\StepOne Software.

Chapter 3

Results

To analyze mRNA expression of Smaug in cell culture samples, relative expression of Smaug was compared to the relative expression of the 18S rRNA housekeeping control. First, qPCR results for SEEM/SEDM samples were quantified and calculated to compare the relative expression of Smaug to 18S rRNA endogenous control expression. Individual ΔCt values, using the Ct values from Smaug and 18S qPCR results, were calculated for the three samples from the unfractionated bone marrow, SEEM day 5, and SEDM day 3 cultures. The ΔCt values represent the difference in the Ct values from the Smaug gene and the 18S housekeeping gene (Livak and Schmittgen, 2001). Following this, the ΔCt values were subtracted from the calculated unfractionated bone marrow control average, and $2^{(-\Delta\Delta C(t))}$ values for the samples were calculated and averaged. The individual and averaged $2^{(-\Delta\Delta C(t))}$ values allowed for convenient analysis of relative changes in Smaug gene expression. These calculations were repeated separately for the HKBA day 0, 4, 6, and 8 samples (Livak and Schmittgen, 2001).

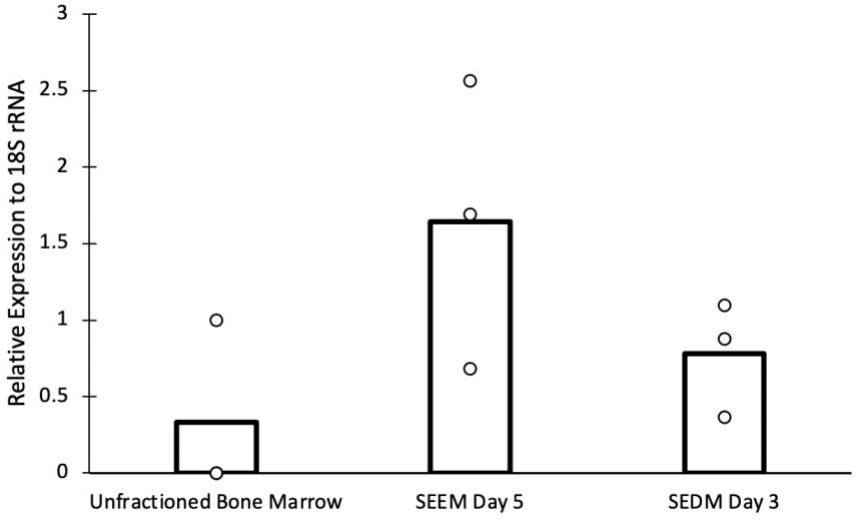

Figure 3. Relative expression of Smaug compared to 18S rRNA from unfractionated bone marrow, SEEM, and SEDM samples

Individual and averaged $2^{(-\Delta\Delta C(t))}$ values for samples tested in the unfractionated bone marrow, SEEM day 5, and SEDM day 3 cultures. N=3 for unfractionated bone marrow, SEEM day 5, and SEDM day 3. Averages are shown in bar form, and individual $2^{(-\Delta\Delta C(t))}$ values as points.

Unfractionated bone marrow showed minimal expression of Smaug. In contrast, during expansion, Smaug expression increased. This observation is consistent with our hypothesis that as mitochondrial respiration is low, respiration complex RNAs are being held by Smaug. After the addition of Epo, differentiation occurs, and Smaug expression decreases in comparison to the expansion results. This observation fits the idea that during differentiation, increased mitochondrial respiration results from the subsequent translation of the respiration complex RNAs that were held by Smaug, and that Smaug is no longer needed.

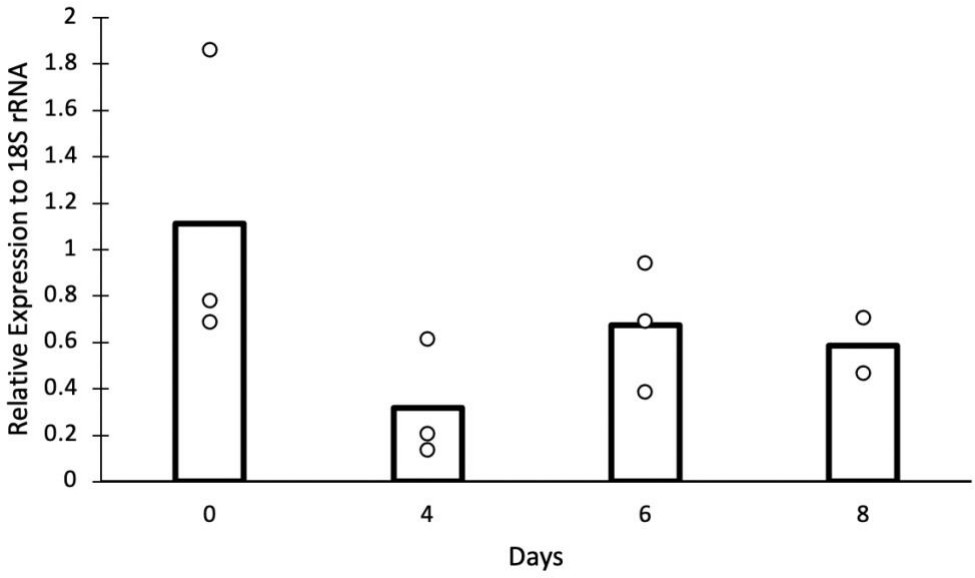

Figure 4. Relative expression of Smaug compared to 18S rRNA from HKBA cell samples

Individual and averaged $2^{(-\Delta\Delta C(t))}$ values for samples tested in the HKBA cell cultures from days 0, 4, 6, and 8. N=3 for HKBA cell cultures from days 0, 4 and 6. N=2 for HKBA cell cultures from day 8. The $2^{(-\Delta\Delta C(t))}$ averages are shown in bar form, and individual $2^{(-\Delta\Delta C(t))}$ values as points.

We next examined Smaug expression in an in vivo model of inflammatory anemia, HKBA treatment. The day 0 spleen samples expressed high levels of Smaug, which was different than unfractionated bone marrow in Figure 3. We observed the lowest expression on day 4. While expression of day 6 and 8 increase, they are still less than day 0. Although days 6 and 8 do correspond to differentiation, day 4 is not clearly corresponding to expansion stage. More time points would be needed

to make a better comparison between the in vitro culture and the in vivo model. In addition, the HKBA treated samples were whole spleen cells, while the in vitro culture samples were enriched for stress erythroid progenitors (SEPs). The extra cells in the spleen samples confound the analysis.

Chapter 4

Discussion

Stress erythropoiesis is a complex process involving the maturation of red blood cells and is known to occur in a variety of mammalian species (Paulson et. al., 2020). Given the intricate nature of stress erythropoiesis and the mechanisms involved with this process, continual analysis of the regulatory proteins associated with the differentiation of erythroid progenitors is necessary. Specifically, this project analyzed the Smaug RNA binding protein and focused on determining the role of this gene in association with mitochondrial responses and erythroid differentiation ability. Samples of spleen cells from HKBA treated mice and in vitro cultured SEEM/SEDM cells from several time points were used to test the relative expression of Smaug in comparison to 18S rRNA. Relative gene expression comparison was completed through data and results provided by RT-qPCR. Overall, the data calculated via the qPCR showed that the Smaug protein gene expression increased during expansion cultures and begins to decrease in expression during differentiation events. These results are consistent with our hypothesis that Smaug plays a role in regulating mitochondrial respiration during erythroid differentiation. The data from Figure 3 showed that Smaug gene expression is up during expansion and experiences a decrease during differentiation. During expansion cultures shown in Figure 3, glycolysis is occurring while mitochondrial respiration is low. These low levels of respiration allow the Smaug protein to bind and hold RNAs for respiratory complexes. Therefore, since the Smaug protein is thought to actively bind to RNA when respiration levels are low, a rise in expression level would be expected. In contrast, differentiation cultures shown in Figure 3 experience metabolism changes and continual glycolysis, but mitochondrial respiration is high. These high respiration levels result in the translation of respiratory complex RNAs, resulting in a decreased Smaug expression, since there are no remaining RNAs to bind. Similar conclusions can be made after analysis of the in vivo data in Figure 4. However, the interpretation is more complex, day 4 HKBA samples may be starting to differentiate, while day 6 and day 8 HKBA samples are model differentiation. Although the HKBA day 4 sample appears to have a low Smaug expression in

comparison to the other tested days, Smaug expression is clearly seen to decrease in expression between the HKBA day 6 and day 8 samples. It is not clear whether these differences are significant. These data provide further reasoning to continue the study and analysis of the Smaug protein as a regulatory mechanistic component involved in the process of stress erythropoiesis.

Despite the qPCR data and the speculated role of the Smaug protein, additional studies are necessary to confirm Smaug function and impact on mitochondrial response and corresponding erythroid differentiation processes. Future experiments designed to investigate the function of Smaug will involve knockdown of the protein using CRISPR gene editing technology. Once Smaug knockdown is complete, studies testing the ability and efficacy of stress erythroid progenitor differentiation without Smaug expression will be completed. Furthermore, based on the qPCR data from the tested SEEM/SEDM and HKBA cell samples, knockdown of the Smaug protein would support our hypothesis that Smaug loss causes inhibition of stress erythroid differentiation.

Following the information provided by the results obtained from the analysis of Smaug mRNA expression in samples from SEEM/SEDM cultures and spleen cells isolated from HKBA treated mice and qPCR results, we would predict that the mutant model derived from CRISPR gene editing technology would undergo stress erythropoiesis inefficiently, since proper differentiation of stress erythroid progenitor cells would not occur. With Smaug protein expression absent, differentiation events would not function optimally due to variances in mitochondrial respiration levels and decreased presence of respiratory complex proteins. Further analysis of the mutant model via methods such as flow cytometry, colony assays, and analysis of respiratory protein expression will provide further data regarding the function of the Smaug protein on stress erythroid differentiation abilities.

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- Assist with laboratory experiments and tasks focusing on the study of stress erythropoiesis and gene analysis

Research Scholar Program, Lehigh Valley Hospital, Allentown, PA **June 2021 - August 2021**
- Worked with Dr. James K. Wu, Chief of Cardiothoracic Surgery
- Conducted research on the Impella Device and evaluated the effect on the mortality rate of patients with Cardiogenic Shock
- Published: Mechanical Circulatory Support using Impella Devices for Cardiogenic Shock