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A History of Stress Erythropoiesis: Past, Present, and Future

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ABSTRACT

Steady state erythropoiesis maintains erythroid homeostasis and occurs in the bone marrow. However, there are times when the bone marrow cannot keep up with the demand for red blood cells. Stress erythropoiesis makes up for this deficit through rapid red blood cell production in the spleen. Although the existence of the stress erythropoiesis pathway was known for fifty some odd years, characterization of the real mechanism did not begin until twenty years ago. This work consolidates the research on the pathway through a discussion of what is known and what outstanding questions need to be answered. In the process, the inner workings of stress erythropoiesis are explained, its connection to a vital physiological pathway examined, and future studies proposed based on this information.

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Chapter 1

Introduction to Stress Erythropoiesis

The importance of the red blood cell cannot be understated, as it carries out the absolutely vital role of transporting oxygen to the various tissues to the body. More than 2 million red blood cells are made every second in a process called erythropoiesis.[1] The creation of red blood cells during homeostatic conditions is known as steady state erythropoiesis and is the primary method for maintaining the optimal level of erythrocytes in the blood. During steady state erythropoiesis, the bone marrow is tasked with supplying the necessary erythroid precursors to generate new red blood cells. There is a hierarchy of these erythroid precursors, beginning with the hematopoietic stem cell (HSC), which develops into the common myeloid progenitor (CMP), the megakaryocyte-erythroid progenitor (MEP), the burst forming unit-erythroid (BFU-E), the colony forming unit-erythroid (CFU-E), and erythroblast, in that order.[1] In addition to supplying these progenitor cells, the bone marrow also has a particular niche that supports erythropoiesis, known as the erythroblastic island (EBI). An EBI is composed of a macrophage that is surrounded by erythroblasts, or developing erythroid progenitors. The macrophage supports the development of these erythroblasts by both directing and contributing to their growth through delivery of various signals, secreted factors, and iron for heme biosynthesis. In addition, the macrophage is involved in the final differentiation step to a red blood cell, as it phagocytoses the nuclei of an enucleating erythroblast, leading to the formation of a reticulocyte.[1] The reticulocyte then enters the blood circulation, where it matures into an erythrocyte. Erythropoietin (Epo) is constantly secreted at low levels to compensate for the clearance and removal of senescent or worn out red blood cells, which is carried out by macrophages in the spleen.

When steady state erythropoiesis is not working properly or cannot produce enough red blood cells to carry oxygen to all of the tissues of the body, anemia results. Anemia, by definition, is the insufficiency of red blood cells, and can lead to significant pathology. Debilitating symptoms such as extreme fatigue, weakness, and difficulty breathing have severe impacts on quality of life, and can even lead to death if not treated in a timely or proper fashion. There are various types of anemias, with some being more common than others. Anemia can be the result of an intrinsic defect to the erythropoietic process or an extrinsic problem. For example, iron-deficiency anemia is the most common anemia[2] and is an extrinsic defect that results when a lack of iron prevents sufficient synthesis of hemoglobin, the oxygen-binding and carrying component of the red blood cell, and in turn results in a shortage of viable red blood cells. In contrast, sickle-cell anemia is an intrinsic defect that is particularly common in Africa, where it confers resistance to *Plasmodium* infection, the causative agent of malaria. Sickle cell anemia is so aptly named because under hypoxic conditions, some red blood cells adopt a sickle shape due to the polymerization of mutant beta globin. One simple alteration in the amino acid of the beta globin protein, from glutamic acid to valine, causes this debilitating anemia. Production of sickle-cell hemoglobin (HbS) not only reduces the amount of correctly functioning and available red blood cells, but sickle-cells, due to their unwieldy shape, can block capillaries, compromising blood circulation and leading to cardiac events. Furthermore, sickle-cell erythrocytes are quite fragile, often leading to lysis, which releases free hemoglobin and heme into the blood stream. These factors are potent pro-inflammatory mediators that induce inflammation and contribute to the pulmonary and cardiovascular problems observed in sickle-cell disease patients. Finally, anemia can also occur due to the natural immune response of

inflammation, especially in cases of chronic inflammation, and is quite common. An entire chapter will be dedicated to discussing anemia of inflammation, as it is of great importance.

The prospect of steady state erythropoiesis becoming incapable of maintaining an adequate red blood cell count and the ensuing consequences of anemia is ameliorated by a pathway that can rapidly make up the deficit of red blood cells, referred to as “stress erythropoiesis.” Compared to steady state erythropoiesis, stress erythropoiesis utilizes a different strategy. Instead of constant production as is the case in steady state erythropoiesis, stress erythropoiesis generates a bolus of new red blood cells to compensate the anemia. These erythrocytes are thought to maintain homeostasis until bone marrow steady state erythropoiesis can resume. Stress erythropoiesis occurs in the spleen as opposed to the bone marrow. Furthermore, signals for stress erythropoiesis are distinct from those of steady state erythropoiesis.

Mouse genetics were instrumental in uncovering the workings of the stress erythropoiesis pathway. The genesis of these studies began with one intriguing example of anemia uncovered in the 1930s involving the flexed-tail mouse, which is a mutation in the common house mouse. As the name suggests, the flexed-tail mouse has a rather interesting and distinctive flex in its tail, shown on the next page.

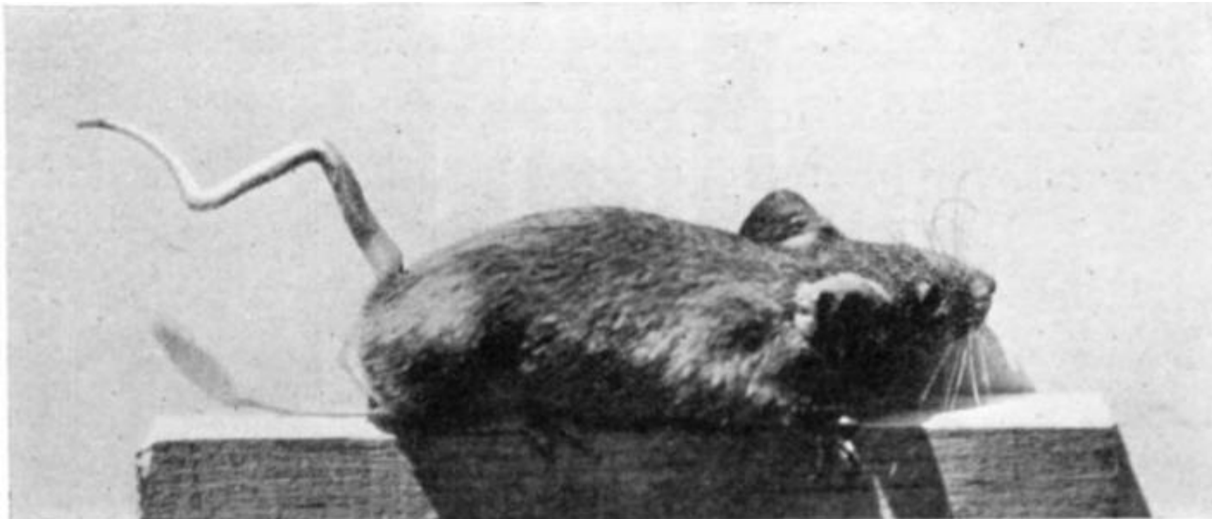

Figure 1. The flexed-tail mouse. Reprinted from "Flexed Tail in the Mouse, Mus Musculus," by H.R. Hunt, R. Mixter, and D. Permar, 1933, *Genetics*, *18*(4), p. 335-366.

Perhaps even more interesting is that the flexed-tail mouse develops anemia through the fetal stage of its life and up to two weeks following birth, after which the anemia mysteriously resolves.[3] Flexed-tail mice have statistically significant lower red blood cell counts than normal mice during these early time points, but they exhibit near normal blood values as adults.[3] A puzzling result like this clearly indicates some sort of erythropoietic defect given the anemic state; however, the specifics were not known at the time. These observations suggested that steady state erythropoiesis was not the issue, as adult flexed-tail mice maintain adequate red blood cell counts under steady state conditions, but they have a defect in fetal erythropoiesis, which continues through the immediate neonatal stage.

A few decades were spent attempting to characterize the curious fetal anemia of the flexed-tail mouse and the anomaly of having erythropoietic defects very early on in life but none thereafter. The posited reason published 10 years later hypothesized that the fetal/early life red blood cells were defective due to a lack of hemoglobin and contributed to the pathology, but ended up being gradually replaced by normal, effective red blood cells as the mice grew into adulthood.[4] At the time, this hypothesis may have seemed reasonable and suggested that fetal

erythropoiesis was different from steady state erythropoiesis. 25 years later, however, experiments showed that adult flexed-tail mice made anemic with phenylhydrazine (PHZ), a drug that causes an acute hemolytic anemia through rapid destruction of red blood cells, had difficulty surviving this challenge and exhibited a long delay in the production of new erythroid precursors and mature erythrocytes that led to a significant lag in reestablishing the normal blood picture (>10 days).[5] Normal mice were able to withstand this challenge and exhibited rapid red blood cell production that far outpaced steady state erythropoiesis and resulted in a return to blood level homeostasis in less than ten days.[5] It became evident that the flexed-tail mouse did not outgrow their erythropoietic defect through gradual replacement of defective red blood cells; but rather, there was a defect in erythropoiesis at times of high erythropoietic need. Once again, these observations pointed to differences between fetal and adult erythropoiesis, with the large growth demand during the fetal stage probably acting as a similar stress stimulus to that of PHZ-induced red blood cell loss.

These new findings laid the foundation for the studies in the 1970s of the response to acute anemia in mice. Wild type mice were given PHZ and studied, and it was noted that the erythropoietic progenitor cells, the BFU-E, were increased in the peripheral blood after the PHZ challenge. This, coupled with a decrease in BFU-E in the bone marrow and increase in the spleen suggested that erythroid precursors moved from the bone marrow into the spleen,[6] where they would proliferate and ultimately differentiate into red blood cells to restore the normal red blood cell picture.

This proposed model stood for around 30 years and was thought to be the mechanism behind erythropoiesis in times of stress, such as a challenge that involved PHZ inducing the significant loss of red blood cells. Of course, some outstanding questions remained. Why would

erythroid precursors migrate to the spleen, when the bone marrow already supports erythropoiesis in the steady state? What are the signals for doing this? What is the connection between PHZ-induced anemia and the fetal/early life anemia seen in the flexed-tail mouse? These unknown questions fueled new studies on stress erythropoiesis, which determined the mechanism behind the rapid red blood cell development pathway.

Chapter 2

The Mechanism of Stress Erythropoiesis: Initial Signals Discovered

Based on the work from the 1970s and into the turn of the 21st century, it was believed that stress erythropoiesis was triggered by insufficient oxygen delivery to tissues, leading to hypoxia, and that high levels of Epo were secreted by the kidney in response. This condition would cause erythroid progenitors (BFU-E) to move from the bone marrow to the spleen, where they would proliferate and ultimately differentiate into red blood cells through Epo's mechanism of action.[6,7] However, the regulation of stress erythropoiesis was unclear and required further investigation, as many aspects of the pathway were still not characterized, especially the signals that direct the response.

New studies in 2005 showed that this existing model had some flaws. These new studies used an adjusted procedure for administering PHZ to induce stress erythropoiesis, which was instrumental in garnering a more consistent response. As opposed to multiple low doses of PHZ (the original protocol used to develop the existing model), a single, large dose of PHZ was used to induce stress erythropoiesis.[7] This change in the treatment protocol enabled more consistent kinetics of recovery and allowed a more accurate way to compare results from different experiments. As a result of this new protocol, no BFU-E were observed in the peripheral blood, which suggested that the progenitors were not migrating from the bone marrow to the spleen.[7] Analysis of spleen BFU-E showed that they grew faster in culture and generated larger colonies than bone marrow BFU-E. Furthermore, the cloning of the mutant gene at the flexed-tail locus was also accomplished at this point, which revealed that a mutation in the Madh5 gene was responsible.[7] Madh5 or Smad5 is a protein involved in the bone morphogenetic protein (BMP) signaling pathway. Given the flexed-tail mouse's poor stress erythropoiesis response, this

suggested that BMP signaling through Smad5 had an essential role in stress erythropoiesis. Further studies identified BMP4 as the BMP family member that acted on stress erythroid progenitors. The erythroid progenitors in the spleen are responsive to BMP4, but bone marrow BFU-E are not affected, which further reinforces the idea that splenic stress erythropoiesis is distinct from bone marrow steady state erythropoiesis.[7] If erythroid progenitors from the bone marrow are not moving into the spleen and not even receptive to the BMP4 signal, this suggests a different mechanism for how stress erythropoiesis works, with the spleen and its resident erythroid progenitors being the central component of the response.

Although the exact role of BMP4 in the stress erythropoiesis response is still not known, what is known is that it is a key component of the pathway. BMP4 is a growth factor that induces the expansion of the BFU-E erythroid progenitors in the spleen, committing them to the stress erythropoiesis pathway as a “stress BFU-E”. The target of BMP4 signaling is the downstream transcription factor Smad5, a Smad-series protein that transduces the BMP4 signal received by moving to the nucleus and turning on gene expression.[8] In a properly functioning BMP4 response, BMP4 binds to a Type I or Type II receptor at the plasma membrane. These two receptors have kinase activity and phosphorylate receptor activated Smads, Smads 1, 5, and 8. These Smads can then dimerize with a common Smad, Smad 4, and the complex migrates to the nucleus and activates gene transcription.[9] The net result is that BMP4 induces erythroid progenitors in the spleen to activate genes that promote the rapid proliferation of stress BFU-E. The importance of BMP4 in stress erythropoiesis is exemplified by the flexed-tail mouse, which exhibit a defect in BMP4 signaling. The mutation in flexed-tail mice affects the splicing of Smad5 mRNA, leading to mis-spliced mRNAs. The mutant proteins encoded by the mutant mRNAs inhibit the function of Smad1 and Smad8 as well as affect the function of Smad5,

blocking their ability to activate genes in a dominant-negative effect.[7] As BMP4 is also involved with bone morphogenesis (as the name of the protein suggests), this impairment in the BMP4 response explains the unusual flexed-tail of the flexed-tail mouse. Defects in BMP4 signaling affect the development of cartilage, which leads to a fusion of vertebrae in the tail.

With the discovery of the role of the BMP4 pathway in stress erythropoiesis, the mystery of the flexed-tail mouse's inability to recover properly from PHZ-induced anemia was solved. Incorrectly produced Madh5 protein antagonizes the transduction of the BMP4 signal such that flexed-tail mice exhibit a delay in BFU-E proliferation in the spleen of up to 4 days,[7] consequently causing a delay in the generation of red blood cells to resolve the anemia. Contrasting this, wild-type (normal) mice exhibit significant BFU-E expansion in around just 36 hours (1.5 days) after PHZ-induced anemia[7] due to a successful BMP4 response.

After the discovery of the critical role of BMP4 in the stress erythropoiesis pathway and the erythroid progenitors in the spleen poised to respond to it, the next logical question was, is BMP4 the only signal necessary in the mechanism of inducing stress erythropoiesis? If BFU-E erythroid progenitors from the spleen were taken and cultured in the presence of BMP4, can a successful expansion and stress erythropoiesis response be induced?

It turns out that the answer is no, indicating that other signals are involved in the stress erythropoiesis response. The discovery of this next signal once again highlights the importance of mouse genetics in uncovering the stress erythropoiesis mechanism. Two mutant mouse strains, the Dominant white spotting or W mouse and the Steel (Sl) mouse, both exhibit anemia at the steady state, but are also unable to respond to anemic stress. The defects in erythropoiesis of W and Sl mice is not a problem with BMP4 expression, as it is more or less the same as normal mice.[10] However, this same study found that the W mice exhibit a remarkable lack of BMP4

responsive cells, despite expressing normal amounts of BMP4. This clearly indicated that there is another signal in play that allows sufficient amounts of BMP4 responsive cells to develop so that BMP4 can induce them to become stress BFU-Es, proliferate, and create a successful stress erythropoiesis response.

This signal in question is stem cell factor (SCF), which was already known to be crucial in the development and expansion of erythroid progenitors.[10] The receptor for SCF is known as Kit, and the two are referred to together as the Kit/SCF pathway. The W mouse has a defect in Kit, and the Sl mouse has a defect in SCF.[11] Given that W/Sl mice express BMP4 but don't have many BMP4 responsive cells, this suggested that SCF plays a vital role in promoting the Kit-dependent expansion of BMP4 responsive cells. This likely accounts for the similarity in phenotype between the W and Sl mouse. The importance of SCF to the stress erythropoiesis pathway does not end here, however. Data in 2007 showed that SCF can increase the size of BFU-E's, as BFU-Es still express the Kit receptor.[10] Larger BFU-Es allows for greater production of erythrocytes, so that SCF would be expressed in large quantities to assist in stress erythropoiesis, further supporting the idea that the splenic microenvironment plays a key role in promoting the expansion and differentiation of Kit+ stress erythroid progenitors.

Although Kit/SCF signaling plays an essential role in stress erythropoiesis, addition of SCF and BMP4 to *in vitro* spleen cultures failed to recreate the same stress erythropoiesis response seen *in vivo*,[10] indicating there was still another signal in the pathway missing. This signal was already known to affect erythropoiesis. Hypoxia is the stimulus that induces stress erythropoiesis and consequently the expression of BMP4. With the addition of hypoxia to the *in vitro* culture (as 2% O_2, low oxygen conditions) along with BMP4 and SCF, this nearly recreated the *in vivo* expansion.[10] Furthermore, under low oxygen conditions, SCF also gains the ability to

increase the number of stress BFU-Es formed, supporting its crucial role in the stress erythropoiesis pathway. Perhaps even more interesting is that hypoxia alone can still induce a very significant BFU-E expansion, without the need for BMP4 and SCF supplementation.[10]

This unique observation drove the next question in uncovering the mechanism of stress erythropoiesis: what does hypoxia specifically do that enhances this stress erythropoiesis response and can induce significant expansion of BFU-Es *in vitro* without the need for BMP4 and SCF (although their addition certainly enhances the response). It was already known at the time that hypoxia affects gene expression through the action of hypoxia-inducible factors (HIFs). HIFs, as the name suggests, are transcription factors induced by hypoxia, but under normal oxygen (atmospheric conditions, ~20% O_2) they are unstable proteins that are readily destroyed. Turnover of HIF proteins is regulated through the action of the Von Hippel Lindau (Vhl) protein with its ubiquitin ligase activity, which polyubiquitinates and tags HIFs to be targeted by the proteasome for destruction. However, under hypoxic conditions, Vhl's activity is inhibited, and as a result, HIFs are stable and will translocate to the nucleus, where they bind to hypoxia-responsive elements (HREs) and activate gene expression of those genes containing the HREs.[12]

Perhaps unsurprisingly, it was found that the BMP4 gene contains multiple HREs that lead to its transcription and ultimately production to induce the stress erythropoiesis response. The transcription factor bound at these HREs is the hypoxia-inducible factor HIF2α, which promotes the activation of the BMP4 gene.[12] This finding explains how hypoxia alone can induce a significant expansion of BFU-E, as BMP4 is consequently made afterward; supplementing with additional BMP4 probably accounts for the further increase in the stress BFU-E response seen in Perry et. al, 2007.[10] Compared to BMP4, SCF is constitutively

expressed; however, it is plausible that BMP4 signaling could lead to increased production of SCF to assist in the stress erythropoiesis response, as SCF is a very potent growth factor.

Although the addition of these three key factors to spleen cell cultures could recapitulate the expansion of stress BFU-E observed *in vivo*, other outstanding questions remained to be answered. In particular, what regulated the ability of mice to respond to anemic stress? Following recovery from the initial stimulus, mice cannot immediately respond to a second anemic stimulus. If mice were made anemic with PHZ and allowed to recover for 7 days before being immediately rechallenged with PHZ, they could not survive the second challenge. However, if a second dose of PHZ was given 21 days after recovery, the mice did survive and responded like an unchallenged mouse.[13] Transplant experiments showed that the precursors of the stress BFU-E originated in the bone marrow. The migration of bone marrow progenitors into the spleen made them competent to respond to BMP4.[13]

To determine this mechanism behind replenishing the stress erythropoiesis response, a very clever approach was taken, involving a look back to the original function of BMP4 and its role in bone and cartilage morphogenesis. The development of chondrocytes that form cartilage cells requires BMP4 signaling. However, precursors to chondrocytes failed to differentiate via BMP4 if they were not sensitized to the BMP4 signal beforehand. The signal that was required to make the progenitors responsive to BMP4 was sonic hedgehog (Shh).[14] The connection was made that perhaps the spleen contains this Hedgehog signal to sensitize the bone marrow progenitors that will generate stress erythroid progenitors to the BMP4 signal, so that they are poised to respond when BMP4 expression is induced via hypoxia and stress erythropoiesis is needed.[13] When these bone marrow cells were treated with Shh and BMP4 they were induced to become stress BFU-Es.[13] Furthermore, using an inhibitor of BMP4, Noggin, prevented the

formation of stress BFU-Es, emphasizing the role of both Shh and BMP4 in replenishing the population of BMP4 responsive stress BFU-Es.[13] While Shh was used for the *in vitro* studies to come to this conclusion, there are two other hedgehog family members, desert hedgehog (Dhh) and Indian hedgehog (Ihh), which are expressed in the spleen.[13] Ihh is most likely the family member to induce incoming HSCs from the bone marrow to become BMP4 responsive, as Dhh exhibits an inhibitory effect on stress erythropoiesis.[15]

Further studies looking at the signaling of Hedgehog (Hh) confirms its role in regenerating the population of BMP4 responsive, spleen BFU-E. Signaling through Hh is primarily dependent on two receptors: Patched (Ptc) and Smoothened (Smo). Ptc inhibits Smo, preventing it from signaling. However, the ligand for Ptc is Hh, which frees the inhibition of Smo, and allows for Smo signaling.[16] Smo signaling makes progenitors competent to respond to BMP4 signals. If Smo is mutated, BMP4 responsive cells cannot be generated.[13] Conversely, if Ptc is mutated, Smo signaling is now constitutively active, making the cell constitutively responsive to BMP4. This is highlighted by the fact that BMP4 responsive cells were found in the bone marrow upon mutation of Ptc, where they typically would not be found.[13] These findings clearly shed light on the observation mentioned earlier of bone marrow erythroid progenitors being unresponsive to the signal of BMP4; unlike the spleen, the bone marrow lacks Hh in its environment, preventing sensitization of its respective cells to BMP4. In summary, Hh closes the cycle of the stress erythropoiesis pathway through the Ptc/Smo signaling pathway, so that stress erythropoiesis can occur again. However, it takes time for the HSCs of the bone marrow to migrate to the spleen, where Hh and BMP4 can then act upon and replenish the population of BMP4 responsive BFU-E.

To close out this chapter, we again return to the tale of the flexed-tail mouse. The flexed-tail mouse had normal steady state erythropoiesis, but struggled under times of stress. Stress is a very broad term that can apply to sudden red blood cell loss, as in the case of PHZ-induced anemia, but it can also apply elsewhere. Fetal development represents a stress, as in utero, the fetus is in an oxygen poor environment and is rapidly growing with an increasing need for oxygen. Flexed-tail mice are anemic during fetal development and in the immediate post-natal period. Is stress erythropoiesis involved in fetal erythropoiesis?

This important connection was verified in 2008, when it was discovered that the fetal liver of the mouse (this is the primary erythropoietic organ until the bone marrow/spleen fully develop and can take over) contains stress BFU-E, and supplementing it with BMP4 results in a marked expansion of these stress BFU-E.[17] As expected, flexed-tail mouse embryos have a delay in the expansion of stress BFU-E, just as adult flexed-tail mice have when undergoing PHZ-induced anemia.[17] The main takeaway from this connection is the emphasis of the importance of mouse genetics in uncovering the real mechanism of stress erythropoiesis. The unusual fetal anemia of the flexed-tail mouse gave a clue into a type of erythropoiesis that is different from the steady state, and the fundamental similarities of fetal erythropoiesis to stress erythropoiesis in adult mice confirms the mechanism discussed in this chapter. Furthermore, this connection is even extended to humans, where erythrocytes in the developing fetus express fetal hemoglobin (HbF), containing γ-globin instead of β-globin. After birth, humans exhibit globin expression switches to adult hemoglobin (HbA). As adults, humans still express a small percentage of HbF containing erythrocytes; however, anemia in adults often leads to induction of HbF. This increase in HbF can be therapeutic in some anemias as patients with sickle cell disease that

express high levels of HbF mitigate the effects of sickle erythrocytes as γ-globin inhibits polymerization of the faulty β globin.[18]

Chapter 3

The Mechanism of Stress Erythropoiesis: Later Signals Discovered

BMP4, SCF, Hedgehog, hypoxia, and Epo represent the minimum necessary signals to induce and maintain a successful stress erythropoiesis response *in vivo*. As discussed in the previous chapter, an *in vitro* culture system was also developed that recapitulates the expansion of stress erythroid progenitors. However, *in vitro* studies noted that the cells arriving to the spleen to replenish the exhausted stock of stress progenitors, CD34+Kit+Sca1+Lin- HSCs, needed a microenvironment of macrophages in the culture to undergo stress erythropoiesis properly. When these macrophages were depleted, the stress erythroid progenitors failed to develop.[19] There were two implications of these results. The immediate one suggested there were important cells (and not just signals) that supported the stress erythropoiesis response, with macrophages being one such group. A subtler implication was that the involvement of macrophages in stress erythropoiesis hinted at some connection of the pathway to the immune response. This will be discussed at great length in the next chapter.

Recall that in steady state erythropoiesis, macrophages are part of the bone marrow microenvironment and assist erythroid precursors in their growth and development stages to become red blood cells. The spleen (the primary site of stress erythropoiesis) also has a population of these tissue-resident macrophages, known as the red pulp macrophage (RPM). However, during homeostasis, the RPM has the opposite role of a bone marrow macrophage: its main task is to assist in the clearance of senescent red blood cells as opposed to helping generate new ones.[20] Originally it was thought that the RPM did this through erythrophagocytosis; however, there is significant evidence showing that the RPM only phagocytoses a remnant of the red blood cell known as an “erythrocyte ghost,” and that the splenic microenvironment actually

induces the red blood cell to lyse before being engulfed by a RPM.[20] For convenience, this process will still be referred to as erythrophagocytosis.

This discovery not only highlighted the role of the RPM in red blood cell turnover, but also the idea of the great importance of a surrounding microenvironment for cellular responses. Based on the observation discussed in Xiang et. al, 2015,[19] it is apparent the RPM plays a vital role in the stress erythropoiesis response by constituting this microenvironment. Further studies helped elucidate this crucial role. The anemic stimulus that precedes stress erythropoiesis increases the phagocytic activity of RPMs,[21] allowing for heme-dependent signaling, as heme is released when red blood cells are degraded (the importance of this response to stress erythropoiesis will be discussed later on in the chapter). Furthermore, although this is not known, increased phagocytic activity of RPMs could also potentially allow for recycling of heme, which will be highly demanded as a result of the incoming rapid erythropoiesis. The change in activity of the RPMs also results in increased expression of Ccl2, a chemokine and ligand for the receptor Ccr2. Ccr2 is expressed by monocytes, the precursors to macrophages. The high concentration of Ccl2 ligand attracts these monocytes to the spleen, where they can differentiate into RPMs[21] and just like in the bone marrow, have an active role in coordinating the growth and development of erythroid precursors to red blood cells. The only difference this time is that the spleen-resident macrophages are coordinating the growth and development of stress progenitors, which will undergo much more rapid growth and proliferation. This requires a different set of signals which are expressed by the RPMs and spleen microenvironment.

To determine what it is RPMs exactly do to enhance the stress erythropoiesis response, the role of tissue-resident macrophages in other organs was consulted. It was known that in the recovery from liver injury, macrophages resident in the liver expressed Wnt, a ligand that

promotes receptive cells to proliferate. In this particular instance, the Wnt-expressing macrophages of the liver induced hepatic cells to proliferate in an effort to regenerate the liver.[22] It was thought that perhaps the RPMs of the spleen may have a similar role in the stress of red blood cell deficiency by inducing erythroid progenitors to proliferate and ultimately restore red blood cell homeostasis. Results that induced stress erythropoiesis through bone marrow transplant (BMT) uncovered that the canonical Wnts (those that lead to activation of β-catenin), Wnt2b and Wnt8a, were highly expressed in the spleen following this procedure, with mRNA peaking at day 4.[23] This pointed to the RPMs expressing the Wnt ligand, primarily Wnt2b and Wnt8a, to promote the proliferation of erythroid progenitors. This is achieved by Wnt2b/8a binding to its receptor, Frizzled, which activates β-catenin, a transcription factor that can translocate to the nucleus and turn on genes required for cell proliferation.[24] One implication of this result is that Wnt2b and Wnt8a mRNA expression can be measured as markers to determine if a successful stress erythropoiesis response was induced.

Furthermore, the same 2020 study also noted that Wnt expression decreased when Epo expression was at its peak. While Epo is known to induce red blood cell differentiation, this suggested it may have a connection with also regulating the switch from proliferation to differentiation. The *in vitro* culture system described above allowed for the identification of Epo as the transition signal. Splenic macrophages that were treated with Epo rapidly repressed the expression of Wnt2b and Wnt8a,[23] adding support to this idea. Further experiments ironed out this mechanism: RPMs in the spleen express high levels of Wnt2b and Wnt8a to induce stress progenitors to undergo rapid proliferation. When serum levels of Epo rise high enough, this acts as the switch to turn off proliferation by repressing Wnt signaling and turn on differentiation by inducing red blood cell formation to resolve the anemic state. It is important to note here that it is

the macrophages, not the stress erythroid progenitors, that receive and transmit the Epo signal. This is primarily done by macrophages increasing their production of lipid messengers (eicosanoids) that inform the red blood cell to differentiate.[23] These discoveries emphasized the great importance of splenic macrophages in coordinating the stress erythropoiesis response. Without these macrophages in the microenvironment, it is no wonder the HSCs failed to undergo successful stress erythropoiesis *in vitro*, as a key regulator of their activity had been lost. Furthermore, the method of Epo signaling in stress erythropoiesis highlights yet another one of its differences with steady state erythropoiesis.

Wnt signaling through splenic macrophages is not the only pathway that supports stress erythropoiesis. Interestingly, another important pathway that supports stress erythropoiesis was found in a similar vein to Wnt. The protein known as Yap1, or Yes-associated protein 1, was already known to be a very growth inducing and anti-apoptotic signal, to the point where its overexpression is implicated in various cancers.[25] Yap1 stimulates cell proliferation just as Wnt does. This observation suggested it may have a role in the stress progenitor expansion seen in stress erythropoiesis. Using the same BMT procedure as in the discovery of Wnt's importance to stress erythropoiesis, it was found that Yap1 mRNA reached peaks of expression during the same period that stress erythroid progenitors were amplifying and proliferating.[26] Furthermore, Yap1-knockout mice had extreme difficulty generating a successful stress erythropoiesis response, and Yap-1 overexpression led to enhanced expansion of stress erythroid progenitors,[26] highlighting the importance of Yap1 in stress erythropoiesis.

Yap1 enhances stress erythropoiesis by positively interacting with the familiar transcription factors of β-catenin, the downstream signaling molecule of Wnt, and Smads 1 and 5, the downstream signaling molecules of BMP4, which both translocate to the nucleus to

activate genes for cell growth and proliferation. This explains why Yap1 expression is correlated with increased mRNA expression of Gls1 (glutaminase) and Myc.[26] Glutamine is an energy source very often used by cancer cells, which need to undergo the same rapid growth and proliferation as stress progenitors, and Myc is a proto-oncogene whose loss of regulation is also implicated in cancers, as it stimulates entry and progression through the cell division cycle.[27] Conversely, knocking out Yap1 leads to much lower expression of genes like Gls1 and Myc, presumably because a key protein is no longer present to enhance β-catenin and Smad 1/5 transcriptional activation of cell proliferation genes. In summary, much like the other proliferation-inducing signals of stress erythropoiesis (BMP4, SCF, Wnt), Yap1 helps support stress erythropoiesis by turning on genes that enable the required rapid cell growth, such as usage of glutamine and stimulation of cell division.

There is one last signal that rounds out the set of signals involved in the stress erythropoiesis response and helps emphasize its difference with steady state erythropoiesis. This is growth differentiation factor 15, or Gdf15. Gdf15 is from the same family as BMP4 – the transforming growth factor – beta (TGF-β) family. *In vitro* studies displayed that Gdf15 is needed for the proliferation of stress erythroid progenitors.[28]

To determine the importance of Gdf15 in stress erythropoiesis, it must be observed what happens when it is not present. Interestingly, Gdf15 knockout mice had no problems with steady state erythropoiesis, highlighting yet another difference in the signals of the two pathways.[28] The problems arose when Gdf15 knockout mice were injected with PHZ to induce stress erythropoiesis; here, they did not fare so well. In fact, the response was so poor so that the dose of PHZ had to be reduced so that the mice could survive. This was the result of reduced stress BFU-E colonies and sizes, which would severely impair the stress erythropoiesis response.[28]

However, this was not the result of the CD34+Kit+Sca-1+Lin- HSCs from the bone marrow failing to home to the spleen; rather, there is a defect in something else very vital to the stress erythropoiesis response discussed in this chapter: the importance of the splenic macrophage and niche. Gdf15 knockout mice were found to have lower amounts of Ccl2+ monocytes in their spleen, the same monocytes that differentiate into the aforementioned red pulp macrophages in order to assist the stress erythropoiesis response.[28] Furthermore, more Ccl2+ monocytes were found in the peripheral blood of Gdf15 knockout mice, suggesting they are lost and failing to home where they are most needed. This would explain the poor development of stress BFU-Es and further underscores the importance of both the macrophages and splenic niche in stress erythropoiesis.

However, Gdf15 is not just important for homing of monocytes to the spleen. Because an increase in Gdf15 mRNA expression is observed much earlier than an increase in BMP4 mRNA expression after inducing stress erythropoiesis, it was thought that Gdf15 controls BMP4 in some particular manner.[28] This would not be surprising, given BMP4's critical importance in the stress erythropoiesis response, and also the fact that Yap1, another signal discussed in this chapter, interacts with BMP4 downstream targets. A variety of data showed that Gdf15 helped maintain BMP4 expression by inhibiting the Von Hippel Lindau (Vhl) protein, the same protein that polyubiquitinates HIF2α for destruction, preventing its binding to HREs in the BMP4 gene.[28] By inhibiting Vhl, Gdf15 enables sustained expression of BMP4, as there is plenty of HIF2α to activate transcription of BMP4.

As its name would suggest, Growth Differentiation Factor 15 is also involved in the regulation of growth genes needed for stress erythropoiesis. Gdf15 knockout mice express lower levels of Gls1, the same enzyme needed for rapid cell growth discussed earlier, as well as Glut1,

the transporter for glucose,[28] also required for rapid cell growth and the sole source of energy used by red blood cells. In summary, the role of Gdf15 in stress erythropoiesis is three-fold: 1) it is needed for correct development of the splenic niche, particularly in attracting sufficient amounts of monocytes to become RPMs; 2) it stabilizes and sustains expression of BMP4, whose key role in stress BFU-E expansion has already been discussed; and 3) it upregulates expression of key enzymes and transporters needed for rapid cell growth.

While the role of the splenic niche and its resident macrophages, Wnt, Yap1, and Gdf15 were discovered much later than the earlier discovered signals of BMP4, SCF, Hedgehog, hypoxia, and Epo, they are no less important in the stress erythropoiesis response. However, what is perhaps an even more impressive discovery is what the true role of the stress erythropoiesis pathway is as a physiological response. This will be discussed in the next chapter.

Chapter 4

The Connection of Stress Erythropoiesis to the Inflammatory Response

It is important to note that up until this point, studying stress erythropoiesis was always done in the context of anemia. From a scientific perspective, this makes sense: because anemia is a lack of oxygen delivery to the tissues due to an insufficiency of red blood cells, stress erythropoiesis would naturally be activated to make up for the deficit. Phenylhydrazine (PHZ), as mentioned many times, was used for much of the early work on studying the pathway because of its reliable and robust induction of stress erythropoiesis via acute red blood cell loss. Later techniques included the aforementioned bone marrow transplant (BMT), where the bone marrow of mice was lethally irradiated and transplanted with new stem cells. This caused anemia as the typical steady state erythropoiesis in the bone marrow was interrupted through irradiation, forcing stress erythropoiesis through the newly transplanted stem cells. The drawback with these methods is that they don’t exactly model clinical or real-life scenarios. Sudden severe blood loss and BMT are quite rare events. It was evident that something was missing in the puzzle regarding stress erythropoiesis’ practical purpose.

Briefly discussed in the first chapter, anemia of inflammation is a common condition. Chronic diseases often contain a constant inflammatory stimulus that results in anemia, as many of the cytokines produced in inflammation inhibit steady state erythropoiesis in the bone marrow. Tumor necrosis factor – alpha (TNF-α) is a classic proinflammatory cytokine and can inhibit erythropoiesis by inducing apoptosis of erythroblasts, the precursors to red blood cells found in the bone marrow.[29] Constant production of another pro-inflammatory cytokine, interferon-gamma (IFN-γ), inhibits erythropoiesis and can also shorten red blood cell lifespan.[30] IL-1β, also a pro-inflammatory cytokine, inhibits erythropoiesis by skewing bone marrow hematopoiesis

towards myeloid (innate immune cell) production.[31] IL-6, another hallmark pro-inflammatory cytokine, leads to increased production of hepcidin, which sequesters iron and severely restricts erythropoiesis.[32] More recently, cytokines such as IL-33 have been shown to cause anemia during inflammation by decreasing Epo-mediated erythroid progenitor differentiation.[33] These observations suggested that steady state erythropoiesis is impaired in times of an inflammatory response and this loss of erythroid output must be replaced.

This idea that stress erythropoiesis was quite involved in the inflammatory response was reinforced further by the observation that a variety of experimental systems inducing inflammation through infection, such as with the bacteria *Brucella abortus* or *Salmonella*, as well as the parasite *Plasmodium*, induced a large expansion of erythroid progenitors in the spleen. Furthermore, the fungal cell wall component β-glucan caused an increase in stress BFU-E, and this production was unaffected by IFN-γ that inhibits steady state erythropoiesis.[34] Yet more interesting is that other pro-inflammatory cytokines, such as TNF-α and IL-1β, actually increase stress BFU-E, and they are even expressed in the spleen of these infection models.[34] Not only did this highlight the continuing idea that stress erythropoiesis and steady state erythropoiesis are much different processes, but also set the stage for determining the clear linkage between inflammation inducing stress erythropoiesis in the absence of a hypoxic stimulus.

The fungal β-glucan, Zymosan, stimulates the Toll-like receptor 2 (TLR2), which creates an inflammatory signal.[34] Despite not inducing anemia initially, it was found that mice treated with Zymosan not only have a large increase in stress BFU-E 24-72 hours after administration as discussed earlier, but this is concomitant with exceptionally large increases in both BMP4 and Gdf15 expression in the spleen.[34] Recall that BMP4 and Gdf15 are the classic markers for a

stress erythropoiesis response. Furthermore, mice with defects in Gdf15 or BMP4 signaling, such as Gdf15-/- and flexed-tail mice respectively, exhibited delays in stress erythropoiesis as well as significant lethality, highlighting that Gdf15 and BMP4 are just as necessary for successful stress erythropoiesis in inflammation as they are in hypoxia. Despite being named after its role in the hypoxic response, inflammation via Zymosan administration also led to accumulation of Hif2α,[34] bolstering the idea that inflammation could induce a "hypoxia-like" state that activates stress erythropoiesis. The accumulation of Hif2α accounted for the precipitous rise in BMP4; however, the signals that increased Gdf15 expression were still unknown.

To determine what signals regulated Gdf15, it needed to be addressed how inflammation induces stress erythropoiesis signals when there is no apparent hypoxia. An important observation was that Zymosan induces increased erythrophagocytosis by RPMs.[34] Furthermore, only F4/80+ macrophages that were phagocytosing erythrocytes in response to Zymosan and were accumulating heme exhibited increased Gdf15 expression.[34] This observation suggested a signal that is activated by heme and mediates Gdf15 expression. SPI-C appeared to be the likely candidate for a variety of reasons: 1) it is dependent on heme for its activation, as heme induces degradation of the transcriptional repressor BACH1, which leads to increased SPI-C expression;[34] 2) SPI-C is required for the development of RPMs;[35] and 3) SPI-C has the very important role in stress erythropoiesis of expanding the splenic niche by inducing incoming monocytes to differentiate into RPMs in the spleen.[34] Thus, it made sense that the accumulation of heme by the macrophages led to activation of SPI-C, which activated expression of the very important stress erythropoiesis signal Gdf15, as SPI-C is a transcription factor. SPI-C activating Gdf15 expression was confirmed when it was found that SPI-C knockout mice could not increase their Gdf15 expression and as a result did not undergo successful stress erythropoiesis.[34]

While Zymosan administration and the ensuing inflammatory stimulus leads to induction of stress erythropoiesis activating factors, as discussed earlier, it is also accompanied by expression of pro-inflammatory cytokines. Stress BFU-E production is not affected by the pro-inflammatory cytokine IFN-γ despite its effects on erythroid progenitors in steady state erythropoiesis. In addition, other pro-inflammatory cytokines, such as TNF-α and IL-1β, while inhibiting steady state erythropoiesis, actually help the stress erythropoiesis response. This was evidenced by the fact that stress erythroid progenitors plated in these two cytokines led to a subsequent increase in stress BFU-E. TNF-α was especially effective at doing this, while IL-1β's effect was more muted.[34] The question here is whether TNF-α and IL-1β are acting directly on the stress erythroid progenitors, or indirectly through signaling via the splenic macrophages, which are receptive to these cytokines and potentially occurring in a very similar vein to Epo-mediated differentiation of stress erythroid progenitors. Nevertheless, there is more than enough evidence that inflammatory stimuli, like Zymosan, induce stress erythropoiesis not only through induction of BMP4 and Gdf15, but also through production of pro-inflammatory cytokines that enhance stress erythropoiesis by inducing stress BFU-E expansion. This of course was all done in the absence of an anemic stimulus, which sheds light on the true role of stress erythropoiesis, to be discussed for the rest of this chapter.

While stress erythropoiesis can certainly help return to red blood cell homeostasis as a result of sudden blood loss or bone marrow transplant (it is important to note here that both PHZ and BMT also induce inflammation and all the pro-inflammatory cytokines), with the findings discussed earlier, it is apparent that the most probable role of stress erythropoiesis is to act as a mechanism to prevent anemia associated with inflammation. This hypothesis is supported by the fact that inflammation turns off steady state erythropoiesis and activates stress erythropoiesis,

and makes sense from an immunological perspective: inflammation often signifies that some kind of infection or tissue damage has occurred, and immune cells must be deployed to fight the infection and promote wound healing. In response to inflammation, bone marrow hematopoiesis is skewed to produce myeloid effector cells at the expense of steady state erythropoiesis. Without the bone marrow making red blood cells, the dangers of anemia are very real. However, stress erythropoiesis in the spleen, with its rapid red blood cell output, can temporarily maintain red blood cell homeostasis until the inflammatory stimulus is cleared and the bone marrow can resume making erythrocytes. This makes sense as the mice treated with Zymosan mentioned earlier prevented anemia for at least 7 and up to 9 days as the inflammatory stimulus was dealt with.[34] The main takeaway here is that there is coordination between the erythropoiesis in the spleen and bone marrow hematopoiesis, and that inflammation highlights this coordination.

To emphasize this idea, recall that the final stage of stress erythropoiesis involves high Epo levels that cause the switch from proliferation to differentiation and production of a new bolus of erythrocytes. However, Epo is known to be a potent anti-inflammatory signal, as it reduces NF-κB signaling,[36] the hallmark pro-inflammatory transcription factor. As discussed earlier, in mice, stress erythropoiesis can delay the onset of anemia for around 7-9 days. At this point, in a properly functioning immune response, the inflammation should have led to successful defeat of the inflammatory stimulus, at which point Epo arrives and adjusts inflammatory signaling, which is required for the differentiation of stress erythroid progenitors and generation of a new wave of red blood cells. Furthermore, with the stock of stress erythroid progenitors in the spleen exhausted at this point, the bone marrow can replace the spleen in erythropoiesis, as well as supply the next round of HSCs to replenish the depleted stress erythroid progenitors. Given how common inflammation is, this only bolsters the idea that

inflammation and stress erythropoiesis would be tightly linked processes. Future studies are needed to gather the concrete evidence, which will be discussed in the next chapter.

The idea that stress erythropoiesis prevents anemia during inflammation begs the question: how does anemia of inflammation exist if stress erythropoiesis is specifically attuned to preventing it? There are a variety of plausible reasons. Perhaps the most obvious reason is one previously discussed: after a successful stress erythropoiesis response is mounted, it takes time to replenish its capability. On average, it takes about 21 days to replenish the progenitors in the spleen.[36] If the inflammatory stimulus continues to persist before this can be accomplished, anemia is the result. The bone marrow is tied up with addressing the inflammatory stimulus and cannot deliver the erythroid progenitors to the spleen fast enough to allow stress erythropoiesis to maintain and/or restore the normal red blood cell picture. This is why anemia often results due to chronic inflammation, as the persistent stimulus does not allow the stress erythropoiesis response to recover in time. Furthermore, new studies have been released showing a type of innate immunity memory with regards to inflammation, known as trained immunity. Constant stimulation of cells with the TNF-α pro-inflammatory cytokine leads to them becoming much more sensitive to the stimulus.[37] This can potentially have far reaching implications for stress erythropoiesis, as increasing sensitivity to inflammation may prevent the full cycle of stress erythropoiesis from occurring before another inflammatory response is activated. It needs to be noted that erythroid progenitors must differentiate into red blood cells to actually resolve the anemic state; this is not possible if the anti-inflammatory signal of Epo, which directs this action, cannot be afforded. Furthermore, if the inflammation is dominated by TNF-α, this can also have grave consequences for stress erythropoiesis, as TNF-α promotes proliferation of stress BFU-E. Constant TNF-α responses induced by inflammation prevents erythroid differentiation by

promoting proliferation, making resolution of the anemic state impossible. Future studies should have a look at this impact.

To conclude, while stress erythropoiesis can compensate and maintain normal red blood cell homeostasis during times of severe blood loss or bone marrow transplant, the pathway's likely real purpose is preventing anemia during inflammation, which happens on a much more common basis. This is done through inflammation inducing stress erythropoiesis factors such as BMP4 and Gdf15, as well production of pro-inflammatory cytokines that enhance stress BFU-E formation. With the new realization of the tight linkage between inflammation and stress erythropoiesis, many future studies are both warranted and necessary, and will be discussed in the upcoming final chapter.

Chapter 5

Future Studies in Stress Erythropoiesis: Where to From Here?

From the examination of the literature on stress erythropoiesis, there appear to be three outstanding questions, all pertaining to the recent discovery of the connection between stress erythropoiesis and the inflammatory response. The first is finding and developing a suitable model of human chronic inflammatory anemia. The second is, what is the potential role of trained immunity and stem cell memory in affecting the stress erythropoiesis response? The third and final question is, what are some potential anti-inflammatories that can resolve the inflammatory stimulus and rectify anemia of chronic inflammation? For the final chapter, future studies and experiments will be discussed that could uncover new information to answer these questions.

Perhaps the most important next step to characterize the linkage between stress erythropoiesis and inflammation is to develop a suitable model that can emulate a real-life inflammatory response. As discussed in the previous chapter, Zymosan was originally used to assess the stress erythropoiesis response in response to inflammation. However, there are some issues with this model. For starters, mice needed to be desensitized to the heavy inflammatory stimulus of Zymosan through pretreatment with lipopolysaccharide (LPS), or else they would die due to complications of a massive inflammatory response.[34] This suggests that Zymosan is too powerful of an inflammatory stimulus that would normally be encountered in real life. As a result, if knowledge involving the stress erythropoiesis and inflammatory linkage is to be used for applications to humans, the corresponding animal model should be as similar to human chronic inflammatory anemia as possible. Thus, this is another reason why Zymosan is not a great model for inflammation, as humans are not readily exposed to large quantities of fungal

antigen. A model is needed that utilizes an inflammatory stimulus that humans would encounter on a daily basis, improving applications of the ensuing stress erythropoiesis response.

One promising avenue involves the bacterium *Brucella abortus* in its heat-killed form (HKBA). It is known that HKBA makes a robust anemia of inflammation model in mice.[38] However, what is not characterized is the ensuing stress erythropoiesis response. Given that anemia results, it is likely there is a good stress erythropoiesis response; however, this needs to be verified to certify it as a model that can induce stress erythropoiesis in the context of anemia of inflammation. If this is the case, HKBA would make an excellent model, as humans come into contact with various bacteria on a daily basis. This would make the data obtained from future studies using this model very transferable, as it emulates the actual physiological response to a pathogen, stress erythropoiesis and all included.

To assess the stress erythropoiesis response in this model, HKBA would be injected into C57BL/6 mice. Multiple mice should be used for various time points, which would span hours, days, and a week. After this, the mice are euthanized, and their spleen cells cultured for the presence of BFU-Es. Furthermore, expression of the stress erythropoiesis signals BMP4, Gdf15, Wnt2b, and Wnt8a should be measured using qRT-PCR. Finally, Epo expression should be measured via analysis of kidney RNA for the molecule, as well as blood serum levels using ELISA.

The prediction is that HKBA will induce a more moderate but also more true-to-life anemia of inflammation response, leading to marked expansion of stress BFU-E, especially in the earlier time points. BMP4, Gdf15, Wnt2b, and Wnt8a should all see increases in expression, particularly in the earlier time points as they drive the proliferation of stress BFU-E. Epo expression in the kidney and presence in the blood serum should be quite increased in the later

time points (6 days, 8 days) as they induce the switch from proliferation to differentiation of stress BFU-Es into red blood cells. Ideally, if this is all seen, it is clear that HKBA is inducing a significant stress erythropoiesis response via inflammation, and should be used for future studies addressing the linkage between the two pathways for its applicability to the human realm.

In addition to characterizing a suitable model that emulates human anemia of chronic inflammation, the next major question to be answered is the role of trained immunity, or innate immune memory, in affecting the stress erythropoiesis response. As discussed in Chapter 4, a paper was published in November 2020[37] finding that constant TNF-α stimulation can greatly enhance the sensitivity of future inflammatory responses. Given the interplay between inflammation and stress erythropoiesis, it is important to see what implications this inflammatory memory would have for the stress erythropoiesis response.

The hypothesis is that inflammatory memory acts as a double-edged sword for stress erythropoiesis. On the one hand, a more rapid, robust, and sensitive inflammatory response could certainly enhance the responsiveness of stress erythropoiesis, given that proliferation of stress BFU-Es in the pathway is enhanced by pro-inflammatory cytokines like TNF-α. However, this inflammatory memory could also be pathological. One potential problem resulting from inflammatory memory is that even the slightest inflammatory stimulus could lead to rapid activation of stress erythropoiesis, where it would be completely unnecessary, as steady state erythropoiesis could easily compensate. Another problem is that stress erythropoiesis could be activated to maintain red blood cell homeostasis during an instance of inflammation, only for the sensitivity of the immune response to respond to another bout of inflammatory stimulus, restarting the stress erythropoiesis pathway and ultimately preventing the resolving stage of

differentiation of the stress progenitors into red blood cells. This could propagate the anemia, as the stress progenitors are never able to differentiate into the functional red blood cells.

Investigating these possibilities could explain why anemia results due to chronic inflammation. Fortunately, using the HKBA model discussed earlier, it can be discovered if this is actually the case. In a similar vein to characterizing the HKBA model, mice can be sensitized to the HKBA inflammatory stimulus through injection. After both a successful inflammatory and stress erythropoiesis response, the HKBA stimulus can be applied again, and the quickness and magnitude of the stress erythropoiesis response can be characterized, by measuring the expression and time of induction of the stress erythropoiesis signals BMP4, Gdf15, Wnt2b, Wnt8a, and Epo. If these signals are being expressed at higher levels and quicker than the initial response, it is probable that stress erythropoiesis is responding quicker and more robustly than before, and likely that trained memory has a role in modulating it. Given the dependency of stress erythropoiesis on inflammatory signaling, it is predicted that inflammatory memory would likely result in stress erythropoiesis being induced faster in response to a second bout of the same inflammatory stimulus.

Furthermore, a new dose of HKBA can be applied in the midst of a response to HKBA to see if the stress erythropoiesis response breaks down, measured via deregulated timing or expression of the signals and disordered stress BFU-E proliferation and differentiation. This can assess the potential problems manifested in stress erythropoiesis due to inflammatory memory. It is anticipated that a second HKBA dose in the midst of an inflammatory HKBA response would result in increased expression of stress erythropoiesis genes such as BMP4, Gdf15, Wnt2b and Wnt8a, as this would restart the pathway back into the initial proliferation stage of stress BFU-Es. RNA-seq should be employed here to measure induction and expression of these signals.

Furthermore, ATAC-seq can also be employed to assess the impact of changes in chromatin accessibility on gene expression of various stress erythropoiesis elements (BMP4, Gdf15, etc.) – are these elements becoming more accessible as the inflammatory stimulus is repeated?

This hypothesis leads into the final suggested future study, and really the ultimate question – why is stress erythropoiesis not working in anemia of inflammation? The previous suggested future study may uncover a role of trained memory in exacerbating the quickness and robustness of an inflammatory response that completely deregulates stress erythropoiesis. However, pinpointing the exact reason why stress erythropoiesis breaks down in anemia of inflammation can identify the therapeutic approach to treat this disease. One interesting avenue is using Epo – given its anti-inflammatory properties, it can reduce the inflammatory insult and also drive differentiation of stress progenitors into red blood cells to resolve the anemic state in a sort of two-for-one mechanism. However, Epo administration is inconsistent – sometimes it helps, sometimes it does not. Uncovering why it doesn't work may provide insight into the breakdown of the stress erythropoiesis response. Ultimately, identifying and resolving the underlying source of the inflammation is probably the best course of action to restore stress erythropoiesis. However, are there any ways to modulate the stress erythropoiesis response to provide temporary relief of anemia through an anti-inflammatory, pro-erythropoietic signaling component of the pathway as the primary insult is corrected? Epo should be one such avenue to explore.

Experiments addressing this can be combined in conjunction with the studies on inflammatory memory. After the mice have become anemic due to the chronic inflammatory insult of HKBA, giving them Epo should be attempted to rectify their anemia. If the mice get better, this should be measured both qualitatively and quantitatively – are they visually getting

better but also, are they making more red blood cells (measured via a complete blood count)? If the mice do not get better, this should also be investigated – why isn't there new red blood cell differentiation? Because stress erythropoiesis is needed to make up for the red blood cell deficit, one area to investigate may be in the splenic niche – are enough RPMs present and downregulating Wnt signaling to induce the erythroid progenitors to the differentiation stage? Furthermore, is Epo tapering off the inflammatory signaling? This would be measured by decreases in TNF-α and IL-1β signaling, for example. If this is not the case, this data would suggest that the constant inflammatory signaling and resulting sensitization is too strong and hyperactive for Epo to turn off. Finally, it suggests that the only surefire way to resolve anemia of inflammation is to correct the inflammatory insult, and that the constant inflammatory signaling dysregulates the stress erythropoiesis pathway to be bogged down in the proliferation stage. This is especially true if proliferation signals like BMP4, Gdf15, and Wnt remain high despite Epo administration.

In closing, over the course of the past 20 years, great strides have been made to characterize the stress erythropoiesis response and reveal an incredibly important pathway to maintain red blood cell homeostasis in times of great need, whether it be in hypoxia/anemia or more likely, in taking over red blood cell production for the bone marrow while it shifts to support the immune system during inflammation. This new realization invites new studies to characterize this linkage further, including exploration of a potential human model of anemia of chronic inflammation, the role of trained immunity in stress erythropoiesis, and investigation of Epo administration as a potential anti-inflammatory to assist in resolving anemia of chronic inflammation.

BIBLIOGRAPHY

1. May A, Forrester LM. The erythroblastic island niche: modeling in health, stress, and disease. Exp Hematol. 2020;91:10-21.
2. Camaschella, C. Iron deficiency. Blood. 2019;133(1):30-39.
3. Mixter R, Hunt HR. Anemia in the Flexed Tailed Mouse, Mus Musculus. Genetics. 1933;18(4):367-87.
4. Gruneberg, H. The anaemia of flexed-tailed mice (*Mus musculus* L.). Journal of Genetics. 1942;43:45-68.
5. Coleman DL, Russell ES, Levin EY. Enzymatic studies of the hemopoietic defect in flexed mice. Genetics. 1969;61(3):631-42.
6. Hara H, Ogawa M. Erthropoietic precursors in mice with phenylhydrazine-induced anemia. Am J Hematol. 1976;1(4):453-8.
7. Lenox LE, Perry JM, Paulson RF. BMP4 and Madh5 regulate the erythroid response to acute anemia. Blood. 2005;105(7):2741-8.
8. Song B, Estrada KD, Lyons KM. Smad signaling in skeletal development and regeneration. Cytokine Growth Factor Rev. 2009;20(5-6):379-88.
9. Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, Zhang Q, Ye J, Yan Z, Denduluri S, Idowu O, Li M, Shen C, Hu A, Haydon RC, Kang R, Mok J, Lee MJ, Luu HL, Shi LL. Bone Morphogenetic Protein (BMP) signaling in development and human diseases. Genes Dis. 2014;1(1):87-105.
10. Perry JM, Harandi OF, Paulson RF. BMP4, SCF, and hypoxia cooperatively regulate the expansion of murine stress erythroid progenitors. Blood. 2007;109(10):4494-502.

11. Bernstein A, Forrester L, Reith AD, Dubreuil P, Rottapel R. The murine W/c-kit and Steel loci and the control of hematopoiesis. Semin Hematol. 1991;28(2):138-42.
12. Wu DC, Paulson RF. Hypoxia regulates BMP4 expression in the murine spleen during the recovery from acute anemia. PLoS One. 2010;5(6):e11303.
13. Perry JM, Harandi OF, Porayette P, Hegde S, Kannan AK, Paulson RF. Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. Blood. 2009;113(4):911-8.
14. Murtaugh LC, Chyung JH, Lassar AB. Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. Genes Dev. 1999;13(2):225-37.
15. Lau CI, Outram SV, Saldaña JI, Furmanski AL, Dessens JT, Crompton T. Regulation of murine normal and stress-induced erythropoiesis by Desert Hedgehog. Blood. 2012;119(20):4741-51.
16. Cohen MM Jr. The hedgehog signaling network. Am J Med Genet A. 2003;123A(1):5-28.
17. Porayette P, Paulson RF. BMP4/Smad5 dependent stress erythropoiesis is required for the expansion of erythroid progenitors during fetal development. Dev Biol. 2008;317(1):24-35.
18. Akinsheye I, Alsultan A, Solovieff N, Ngo D, Baldwin CT, Sebastiani P, Chui DH, Steinberg MH. Fetal hemoglobin in sickle cell anemia. Blood. 2011;118(1):19-27.
19. Xiang J, Wu DC, Chen Y, Paulson RF. In vitro culture of stress erythroid progenitors identifies distinct progenitor populations and analogous human progenitors. Blood. 2015;125(11):1803-12.

20. Klei TRL, Dalimot J, Nota B, Veldthuis M, Mul FPJ, Rademakers T, Hoogenboezem M, Nagelkerke SQ, van IJcken WFJ, Oole E, Svendsen P, Moestrup SK, van Alphen FPJ, Meijer AB, Kuijpers TW, van Zwieten R, van Bruggen R. Hemolysis in the spleen drives erythrocyte turnover. Blood. 2020;136(14):1579-1589.
21. Liao C, Prabhu KS, Paulson RF. Monocyte-derived macrophages expand the murine stress erythropoietic niche during the recovery from anemia. Blood. 2018;132(24):2580-2593.
22. Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, Ridgway RA, Seo SS, Spee B, Van Rooijen N, Sansom OJ, Iredale JP, Lowell S, Roskams T, Forbes SJ. Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. Nat Med. 2012;18(4):572-9.
23. Chen Y, Xiang J, Qian F, Diwakar BT, Ruan B, Hao S, Prabhu KS, Paulson RF. Epo receptor signaling in macrophages alters the splenic niche to promote erythroid differentiation. Blood. 2020;136(2):235-246.
24. Pai SG, Carneiro BA, Mota JM, Costa R, Leite CA, Barroso-Sousa R, Kaplan JB, Chae YK, Giles FJ. Wnt/beta-catenin pathway: modulating anticancer immune response. J Hematol Oncol. 2017;10(1):101.
25. Fernandez-L A, Northcott PA, Dalton J, Fraga C, Ellison D, Angers S, Taylor MD, Kenney AM. YAP1 is amplified and up-regulated in hedgehog-associated medulloblastomas and mediates Sonic hedgehog-driven neural precursor proliferation. Genes Dev. 2009;23(23):2729-41.

26. Hao S, Matsui Y, Lai ZC, Paulson RF. Yap1 promotes proliferation of transiently amplifying stress erythroid progenitors during erythroid regeneration. Exp Hematol. 2019;80:42-54.e4.
27. García-Gutiérrez L, Delgado MD, León J. MYC Oncogene Contributions to Release of Cell Cycle Brakes. Genes (Basel). 2019;10(3):244.
28. Hao S, Xiang J, Wu DC, Fraser JW, Ruan B, Cai J, Patterson AD, Lai ZC, Paulson RF. Gdf15 regulates murine stress erythroid progenitor proliferation and the development of the stress erythropoiesis niche. Blood Adv. 2019;3(14):2205-2217.
29. Zamai L, Secchiero P, Pierpaoli S, Bassini A, Papa S, Alnemri ES, Guidotti L, Vitale M, Zauli G. TNF-related apoptosis-inducing ligand (TRAIL) as a negative regulator of normal human erythropoiesis. Blood. 2000;95(12):3716-24.
30. Libregts SF, Gutiérrez L, de Bruin AM, Wensveen FM, Papadopoulos P, van Ijcken W, Ozgür Z, Philipsen S, Nolte MA. Chronic IFN-γ production in mice induces anemia by reducing erythrocyte life span and inhibiting erythropoiesis through an IRF-1/PU.1 axis. Blood. 2011;118(9):2578-88.
31. Pietras EM, Mirantes-Barbeito C, Fong S, Loeffler D, Kovtonyuk LV, Zhang S, Lakshminarasimhan R, Chin CP, Techner JM, Will B, Nerlov C, Steidl U, Manz MG, Schroeder T, Passegué E. Chronic interleukin-1 exposure drives haematopoietic stem cells towards precocious myeloid differentiation at the expense of self-renewal. Nat Cell Biol. 2016;18(6):607-18.
32. Gardenghi S, Renaud TM, Meloni A, Casu C, Crielaard BJ, Bystrom LM, Greenberg-Kushnir N, Sasu BJ, Cooke KS, Rivella S. Distinct roles for hepcidin and interleukin-

6 in the recovery from anemia in mice injected with heat-killed Brucella abortus. Blood. 2014;123(8):1137-45.

33. Swann JW, Koneva LA, Regan-Komito D, Sansom SN, Powrie F, Griseri T. IL-33 promotes anemia during chronic inflammation by inhibiting differentiation of erythroid progenitors. J Exp Med. 2020;217(9):e20200164.
34. Bennett LF, Liao C, Quickel MD, Yeoh BS, Vijay-Kumar M, Hankey-Giblin P, Prabhu KS, Paulson RF. Inflammation induces stress erythropoiesis through heme-dependent activation of SPI-C. Sci Signal. 2019;12(598):eaap7336.
35. Haldar M, Kohyama M, So AY, Kc W, Wu X, Briseño CG, Satpathy AT, Kretzer NM, Arase H, Rajasekaran NS, Wang L, Egawa T, Igarashi K, Baltimore D, Murphy TL, Murphy KM. Heme-mediated SPI-C induction promotes monocyte differentiation into iron-recycling macrophages. Cell. 2014;156(6):1223-1234.
36. Paulson RF, Ruan B, Hao S, Chen Y. Stress Erythropoiesis is a Key Inflammatory Response. Cells. 2020;9(3):634.
37. Zhao Z, Zhang Z, Li J, Dong Q, Xiong J, Li Y, Lan M, Li G, Zhu B. Sustained TNF-α stimulation leads to transcriptional memory that greatly enhances signal sensitivity and robustness. Elife. 2020;9:e61965.
38. Kim A, Fung E, Parikh SG, Valore EV, Gabayan V, Nemeth E, Ganz T. A mouse model of anemia of inflammation: complex pathogenesis with partial dependence on hepcidin. Blood. 2014;123(8):1129-36.

Academic Vita of Andrew P. Keane II

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EDUCATION

B.S., Immunology and Infectious Disease, Schreyer Honors College
Minors in Microbiology, Political Science, and Global Health
The Pennsylvania State University, University Park, PA — May 2021

RESEARCH EXPERIENCE

Undergraduate Researcher — September 2019 – Present
Paulson Research Lab, The Pennsylvania State University, University Park, PA

- Researched stress erythropoiesis, a novel pathway being studied for its role in the response to anemia and modulation of inflammation
- Worked with a team of other undergraduate and graduate students in production of lentiviral vectors that would silence particular genes of interest in the stress erythropoiesis response

TEACHING EXPERIENCE

CHEM 210 Guided Study Group (GSG) Leader — August 2019 – December 2020
Penn State Learning, University Park, PA

- Facilitated two instructional sessions per week for the first semester organic chemistry course, working independently and preparing materials and exam reviews for large classes of 350-700 students
- Selected to have sessions recorded to illustrate effective strategies on how to engage students, including engaging demonstrations and the *Who Wants to be a Millionaire* quiz game show
- Mentored and taught new GSG leaders, broke attendance records upon movement to online learning

SKILLS

- Experienced in essential laboratory and microbiology practices: sterile technique, streak plating, making agar (including antibiotic-infused), gram-staining, antibiotic sensitivity tests, and various other assays
- Experienced in identifying medically significant bacteria, including *Streptococcus* (*pyogenes, agalactiae, pneumoniae*), *Staphylococcus* (*aureus, saprophyticus, epidermidis*), *Neisseria* (*gonorrhoeae, lactamica*), and Enterobacteriaceae (*E. coli, Salmonella, P. aeruginosa*).
- Proficient in public speaking, both in-person and online instructional delivery, and innovative teaching

SCHOLARSHIPS, GRANTS, HONORS, AND AWARDS

Overall Student Marshal at Graduation, Penn State College of Agricultural Sciences	Spring 2021
Top Senior, Penn State College of Agricultural Sciences	Spring 2021
College of Agricultural Sciences Research Grant ($3,000)	Spring 2020
Nominated and Endorsed by Penn State University for the Marshall Scholarship	Spring 2020
Evan Pugh Scholar Award – Junior	Spring 2020
Nominated for Outstanding GSG Leader Award	Spring 2020
Hodge Agricultural Science Honors Scholarship ($1,824)	Fall 2019-Spring 2020
Zerbe Scholarship for Agricultural Sciences ($2,939)	Fall 2019-Spring 2020
Baynard & Ethel Kunkle Scholarship ($1,000)	Fall 2019-Spring 2020
President Sparks Award	Spring 2019
Angstadt Family Agricultural Excellence Scholarship ($3,452)	Fall-Spring 2018-2019, 2020-2021
President's Freshman Award	Spring 2018
Dean's List	Fall 2017-Fall 2020
Honorary A+, CHEM 111 and 113B (experimental chemistry lab)	Fall 2017, Spring 2018
University Park 4 Year Provost Award ($16,000)	Fall 2017-Spring 2021