STUDIES OF HEME-REGULATED eIF2α KINASE STRESS SIGNALING ON MATURATION OF MACROPHAGES AND ERYTHROBLASTIC ISLAND FORMATION IN IRON RESTRICTIVE ERYTHROPOIESIS

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**ABSTRACT**

Iron is the most important metal for the human body. Different states of iron deficiency have long existed and remain very common in today's population. The vast majority of cases of iron deficiency are acquired as a result from blood loss. Any condition in which dietary iron intake does not meet the body’s demands will result in iron deficiency.

Iron and heme are both fundamental in hemoglobin synthesis and erythroid cell differentiation. In addition to act as a prosthetic group for hemoglobin, heme regulates the transcription of globin genes and controls the translational activity in erythroid precursors through modulation of the kinase activity of the eIF2α kinase HRI which is regulated by heme.

HRI, the heme-regulated inhibitor of translation, was first discovered in reticulocytes under the conditions of iron and heme deficiencies. During heme deficiency conditions, in the erythroid precursors protein synthesis is inhibited by phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF2α) as the result of the activation of HRI. The role of HRI is to control that the amount of globin chains synthesized are not in excess of what can be utilized for hemoglobin tetramers depending of heme available. In the absence of HRI, during erythropoietic stress, heme-free globins precipitate causing severe damage of cells in iron-deficiency anemia. In addition to inhibiting protein synthesis, eIF2α phosphorylation increases the translation of specific mRNAs, most notably the activating transcription factor 4 (ATF4), a key factor during cell adaptation to stress.

Chen laboratory published new important findings about the role of HRI in murine macrophage maturation. For the first time HRI was studied in a non-erythroid cellular line. HRI is expressed at a lower level in macrophages, but in HRI deficiency, macrophages did not develop the typical morphology and expressed less critical markers for the growth and maturation of macrophages compared to wt mice.

Macrophages are responsible for iron recycling, taking iron from the hemoglobin of senescent red blood cells by phagocytosis and releasing it through the iron transporter ferroportin. During definitive erythropoiesis, erythroblasts are known to express a diverse array of adhesion molecules, these proteins arrange both erythroblast/erythroblast and erythroblast/macrophage interactions forming the erythroblastic islands (EI). These specialized niches within definitive mammalian erythroblasts have the task to facilitate proliferation and differentiation of erythroblasts. EIs can be described as a central macrophage surrounded by erythroblasts in different stages of maturation and are essential for the developing of erythroblasts that are going to enucleate.

In this project we focused on the role of HRI pathway in the specific subset of macrophages that form EIs. We compared macrophages from Hri-/- mice, which lack HRI in all the tissues, with macrophages from an eAA mouse model, which are defective in eIF2α signaling specifically in the erythroid lineage to examine the differences between the two models under erythropoietic stress. We investigated the interactions of macrophages and erythroid precursors (EI) in the bone marrow and spleen of wild type, Hri-/-, and eAA mice using immunofluorescent microscopy to study the morphology of native EI. We also reconstituted EI by using macrophages from the bone marrow of wild type and mutant mice.

We cultured the bone marrow and spleen derived macrophages (BMDMS and SDMS) from Hri-/-, eAA and Atf4-/- mutant mice. We were able to obtain more SDMS from iron deficient mice compared to iron sufficient mice starting with the same number of nucleated cells but there was much less BMDMS and SDMS from Atf4-/- mice and we validated these results with the colonies assay.

EIs from Hri-/- mice showed impaired macrophages when compared to the EI from eAA and Wt mice. The Hri-/- macrophages were smaller with the cell extremities less expanded; in contrast, EIs from eAA mice were well differentiated and they formed large EI with many erythroblast precursors. This finding was validated with the reconstituted islands, using Wt macrophages reconstituted with Hri-/- erythroblasts.
Il ferro è quantitativamente il metallo più importante dell’organismo. La carenza di ferro è spesso conseguenza di perdite di sangue (ad esempio a livello gastrointestinale), richieste eccessive (durante una rapida crescita nell’infanzia) o un inadeguato apporto alimentare. Ferro ed eme sono fondamentali per la sintesi dell’emoglobina durante l’eritropoiesi. L’eme non solo funge da gruppo prostetico nell’emoglobina, ma ne regola anche la sintesi e la traduzione nei precursors eritroidi, modulando l’attività dell’enzima Heme-Regulated eIF2α chinasi (HRI).

HRI, inibitore della traduzione mediata dall’eme, è stato scoperto nei reticolociti in condizioni di carenza di ferro ed eme. Quando la concentrazione di eme è insufficiente per il fabbisogno eritropoietico, si attiva HRI che, fosforilando la subunità alfa di eukariotic Initiation Factor 2 (eIF2alfa), inibisce la sintesi delle globine. HRI in condizioni fisiologiche assicura che non siano prodotte catene globiniche in eccesso per la formazione di tetrameri in base alla quantità di eme disponibile. Nel caso di stress eritropoietico da anemia da carenza di ferro, in assenza di HRI, le catene globiniche libere sintetizzate precipitano e diventano la causa principale di distruzione cellulare. La eIF2alfa, oltre ad essere il bersaglio principale di HRI, aumenta selettivamente la traduzione di Activating Transcription Factor 4 (ATF4), fondamentale nella risposta adattativa cellulare allo stress.

I macrofagi sono responsabili del recupero del ferro dall’emoglobina degli eritrociti senescenti tramite la fagocitosi e del suo rilascio nel siero con la ferroportina. Nelle fasi finali dell’eritropoiesi gli eritroblasti esprimono un insieme di molecole di adesione che subiscono una variazione dinamica durante il differenziamento. Queste proteine mediano le interazioni eritroblasto/eritroblasto ed eritroblasto/mastrofago e sono responsabili della formazione delle Isole Eritroblastiche (EI), microambienti specializzati all’interno dei quali avviene l’eritropoiesi. Le EI, formate da un macrofago centrale circondato da un anello di eritroblasti in via di sviluppo, sono fondamentali per la maturazione degli eritroblasti destinati a perdere il nucleo e a diventare eritrociti. Il gruppo di lavoro della Prof.ssa Chen ha studiato per la prima volta HRI in una linea cellulare non eritroide, pubblicando risultati interessanti sul ruolo di HRI durante la maturazione dei macrofagi nel modello murino. L’enza HRI è meno espresso nella linea macrofagica rispetto a quella eritroide, ma in assenza di HRI i macrofagi non sviluppano la loro morfologia tipica e appaiono meno differenziati.

# INDEX

## 1. INTRODUCTION

<table>
<thead>
<tr>
<th>1.1. Iron Deficiency Anemia</th>
<th>pg. 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2. Heme – Regulated eIF2α Kinase (HRI)</td>
<td>2</td>
</tr>
<tr>
<td>1.2.1. Role of heme in hemoglobin synthesis</td>
<td>2</td>
</tr>
<tr>
<td>1.2.2. HRI structure and activation</td>
<td>2</td>
</tr>
<tr>
<td>1.2.3. eIF2α kinases</td>
<td>5</td>
</tr>
<tr>
<td>1.2.4. Generation of HRI universal Knockout mouse model Hri-/-</td>
<td>6</td>
</tr>
<tr>
<td>1.2.5. Hematological response of Hri-/- mice in steady-state</td>
<td>7</td>
</tr>
<tr>
<td>1.2.6. Hematological response on Hri-/- mice in stress erythropoiesis</td>
<td>7</td>
</tr>
<tr>
<td>1.2.7. HRI/ eIF2αP signaling pathway</td>
<td>9</td>
</tr>
<tr>
<td>1.2.8. Role of HRI in β-Thalassemia intermedia</td>
<td>11</td>
</tr>
<tr>
<td>1.3. Macrophage’s role in erythropoiesis</td>
<td>13</td>
</tr>
<tr>
<td>1.3.1. Macrophage functional adaptability</td>
<td>13</td>
</tr>
<tr>
<td>1.3.2. Function of HRI in macrophage maturation</td>
<td>14</td>
</tr>
<tr>
<td>1.4. Erythroblastic Island (EI); niche for erythropoiesis</td>
<td>15</td>
</tr>
<tr>
<td>1.4.1. Brief story of EI</td>
<td>15</td>
</tr>
<tr>
<td>1.4.2. Erythroblastic Islands composition</td>
<td>17</td>
</tr>
<tr>
<td>1.4.3. Central macrophage characterization</td>
<td>19</td>
</tr>
<tr>
<td>1.4.4. Enucleation of definitive erythrocytes</td>
<td>20</td>
</tr>
<tr>
<td>1.4.5. Different erythroblastic island functions</td>
<td>22</td>
</tr>
</tbody>
</table>

## 2. AIM OF THE PROJECT

| 2. | 24 |

## 3. MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>3.1. Mice model</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2. Hematological analysis</td>
<td>25</td>
</tr>
<tr>
<td>3.3. Macrophages cell cultures</td>
<td>26</td>
</tr>
<tr>
<td>3.3.1. Cell culture of BMDMS and SDMS</td>
<td>26</td>
</tr>
<tr>
<td>3.3.2. Flow cytometry analysis of adherent cells</td>
<td>26</td>
</tr>
<tr>
<td>3.3.3. Methocult media assay for CFU-M</td>
<td>27</td>
</tr>
<tr>
<td>3.4. Erythroblast Island (EI)</td>
<td>28</td>
</tr>
<tr>
<td>3.4.1. EI isolation protocol</td>
<td>28</td>
</tr>
<tr>
<td>3.4.2. Reconstituted EI protocol</td>
<td>29</td>
</tr>
<tr>
<td>3.5. Immunofluorescence microscopy</td>
<td>30</td>
</tr>
<tr>
<td>3.6. Iron staining</td>
<td>32</td>
</tr>
</tbody>
</table>

## 4. RESULTS

| 4. | 33 |
4.1. Unpublished results from Chen’s lab in the characterization of eAA and ATF4 mice during iron restrictive erythropoiesis

4.2. Macrophages
   4.2.1. Growth and differentiation of macrophages defective in HRI stress
   4.2.2. CFU-M colony assay of spleen and bone marrow from Wt and mutant mice in iron restriction erythropoiesis

4.3. Erythroblastic Island (EI) in iron deficit anemia
   4.3.1. Isolation and study of EI morphology
   4.3.2. Comparison of native EI from eAA and Hri-/- mice in iron restriction Erythropoiesis
   4.3.3. Reconstitution of EI using BM macrophages and erythroblasts from Hri-/- mice spleen

4.4. Iron status in spleen sections of eAA mice

5. DISCUSSION

6. WORK IN PROGRESS AND FUTURE PERSPECTIVES

7. BIBLIOGRAPHY

8. ABSTRACTS AND PUBLICATIONS

9. ACKNOWLEDGMENT
1. INTRODUCTION

1.1 Iron Deficiency Anemia

The first cases of people who suffered from iron deficiency were documented during the Middle Ages. These first cases described patients with chlorosis which at the time was a confounding disorder that we now know is due mainly to iron deficiency anemia (1). Various levels of iron deficiency severity have been well documented and are a common occurrence in the population today. Therefore, conditions which lead to abnormal red blood cells (RBCs) may simultaneously be manifested as iron deficiency in disorders such as thalassemia, sickle cell disease, malaria and other infections, or situations such as extreme climatic conditions and ingestion of natural toxins, heavy metals or drugs (1).

Iron deficiency anemia continues to be a major public health problem worldwide, with an estimated 3 billion people affected (1). The vast majority of cases of acquired iron deficiency result from blood loss (1). Any condition in which dietary iron intake does not meet the body’s demands will result in iron deficiency. Children become iron deficient because their rate of rapid growth surpasses iron supply. Similarly, premenopausal women become iron deficient because they regularly experience menstrual blood loss.

In much of the world, chronic parasitosis results in intestinal blood loss that surpasses dietary iron intake. Other conditions associated with blood loss, such as tumors, inflammation, infections, and congenital malformations, may also cause iron deficiency (2). During the last few years it was recognized that rare genetic defects can also be the cause of iron deficiency anemia. In situations of iron deficiency, the increased demand for mature RBCs leads to prioritization of erythroid production at the expense of other tissues (3, 4).
1.2. Heme-Regulated eIF2α Kinase (HRI)

1.2.1 Role of heme in hemoglobin synthesis

The most abundant form of hemoprotein is the hemoglobin, a protein that contains up to 70% of the total iron content in a normal healthy individual. Due to this fact, it is rational to question whether the regulation of both heme synthesis and iron metabolism are different in non-erythroid cells as compared to erythroid cells which are the only cells that synthesize hemoglobin (5). In mammals, mature RBCs contain high concentrations of hemoglobin, at levels up to 5mM. In order for effective erythroid differentiation and maturation to occur, the 3 main components of hemoglobin, consisting of α-globin, β-globin, and heme, must be synthesized in a ratio of 2:2:4 and only under this condition can a stable α₂β₂ hemoglobin complex containing 4 heme molecules be formed (5). The cytotoxic properties of these three components of hemoglobin can potentially have harmful effects on RBCs and their precursors if their concentrations are not in balance. Thalassemia, a common disease of human RBCs, is a prime example of the consequence of imbalances in the concentration of α and β-globin proteins, and the hypochromic anemia in iron deficiency is a good example of the outcome of an unbalanced globin-heme ratio (5).

Iron and heme play critical roles during hemoglobin synthesis and erythroid cell differentiation (6, 7). The heme molecule operates as a prosthetic group for hemoglobin but it is also involved in regulation of the transcription of globin genes through its binding to the transcriptional factor Bach1 during erythroid differentiation (7, 8). Furthermore, the translation of erythroid precursors is regulated by heme via a mechanism in which it modulates the eIF2α kinase activity of the heme-regulated eIF2α kinase (HRI) an important kinase involved during erythropoietic stress (9).

1.2.2 HRI structure and activation

HRI is a heme-regulated inhibitor of translation and it was characterized for the first time in reticulocytes during chronic anemia caused by the conditions of iron or heme deficiencies. The enzyme functions appeared to involve the inhibition of protein synthesis at initiation with disaggregation of polysomes (5, 10, 11). In a situation of heme deficiency, protein synthesis is inhibited by phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF2) and its consequence activation as translational regulator of specific mRNAs targets. This is a fundamental step for the cell adaptive response to stress through the activation of HRI (12).

The Chen laboratory extensively studied the biological function of HRI for years. They studied its protein structure and pathway to elucidate how the dimeric protein becomes active. Biochemical
studies have demonstrated that HRI is inhibited by heme in a concentration-dependent manner (5, 13). 2 different types of heme-binding sites have been characterized on HRI, 2 for each monomer (14) as indicated in the HRI protein structure in Figure 2. One binding site becomes saturated with stably-bound endogenous heme that can be co-purified. The second binding site is the one free to bond with exogenous heme in a reversible manner (Fig. 1) (5) and appears to be responsible for the inactivation of HRI by heme (5).

The HRI protein structure is divided into 3 important regions - the N-terminus, the kinase insert (KI) domain, and the C-terminus (Fig.2). The heme binding domains are positioned in the N-terminus and KI and these are the only sites that can bond with heme (15). The N-terminus site is the first to bind heme and is the most stable. It is also required for achieving higher eIF2α kinase activity. While the N-terminus site is not essential for the kinase activity of HRI, it is essential for the highly sensitive heme regulation of HRI (15). Biochemical studies from Chen suggested that heme may be incorporated into the N-terminus domain of HRI co-translationally or soon after synthesis (Fig. 1), as is the case of assembly of heme into globin chains (5).

In the presence of heme, HRI is inactive and this state is maintained by inter subunit disulfide bond formations supported by the heme binding (16, 17).

The studies conducted by Chen et al. proposed that in heme deficiency situations, the newly synthesized HRI containing the heme molecule bond in its N-terminus domain, rapidly dimerizes and achieves intermolecular multiple autophosphorylation in 3 different stages (Fig.1). In the first stage, the auto-phosphorylation of newly synthesized HRI makes the enzyme more stable against aggregation (6) (Fig1).
Figure 1. HRI regulate heme and globin synthesis by sensing intracellular heme concentrations. During the synthesis of hemoglobin, one molecule of heme is incorporated into each globin chain. When heme concentration is high, heme binds to the second heme-binding domain of HRI and keeps HRI in an inactive state to allow globin protein synthesis and the formation of stable hemoglobin. In heme deficiency, heme is not binding the second site on the molecule and HRI is activated by autophosphorylation (5).

Figure 2. Protein structure of HRI. HRI is divided into 5 domains as indicated. Structure taken from amino acid sequence of mouse HRI. Heme molecules are marked in red; S = stable heme-binding site, R = reversible heme-binding site. Kinase I and II indicate the kinase catalytic domains. The star indicates histidine residues that coordinate the heme molecule (5).

At this stage, HRI is an active auto-kinase, but its eIF2α kinase function is still inactive. The additional auto-phosphorylation in the second stage is essential to the formation of a stable dimeric molecule that senses the concentration of heme (13). As shown in figure 1, because the function of HRI is to prevent an excess of globin synthesis that would interfere with optimal cellular health, when heme concentration is high, HRI stays inactive, and heme is able to bind HRI in a second domain. In heme
deficiency, without secondary heme binding HRI becomes fully active and it undergoes the final stage of autophosphorylation at Thr485 (T485P) which allows the activation of the eIF2α kinase. The fully activated HRI no longer responds to the heme concentration and it is degraded (18).

1.2.3 eIF2α kinases

eIF2α is an important part of a multimeric complex which regulates gene translation in different tissues. Upon its activation, in the phosphorylated form, eIF2αP is important in its role to alleviate cellular injury or alternatively induce apoptosis. Loss of eIF2 kinase pathways can result in important health consequences (19). In mammals, four different eIF2 kinases have been identified: GCN2, PEK, PKR and HRI. Each molecule contains sole regulatory regions that respond to different sets of stress conditions (19). HRI is the most abundantly expressed eIF2α kinase in the erythroid cell lineage and the observation that the pathological consequences of the disruption of the mouse HRI gene are revealed under conditions of diet-induced iron deficiency is consistent with the fact that phosphorylation of eIF2α is part of the cell adaptive response to stress (5). The four eIF2α kinases display widespread homology in their kinase catalytic domains and are able to phosphorylate eIF2α at the same Ser51 residue (20, 5), even if their functions involve different results in vivo. Pkr (RNA-dependent protein kinase) plays a role in the cell's anti-viral defense mechanism; Gcn2 (general control non-depressible-2) senses amino acid deprivation; Perk (pancreatic eIF2α kinase) is activated in response to mis-folded protein in the ER (ER stress); and Hri is regulated by heme (19, 5). All these functions have been verified by generation of a specific mouse knock-out model for each of the 4 eIF2α kinases (5). In addition to eIF2αP's ability to inhibit global protein synthesis, it is also able to reprograms gene translation and transcription activities necessary for adaptation to stress. For this reason, members of the class of kinases which activate eIF2α can be described as homeostatic guardians and ensure cellular adaptation against major environmental stress (21, 22).

Different environmental stresses are involved in the activation of eIF2αP signaling. These include: heat shock, arsenite treatment, osmotic stress, and nutrient starvation (23, 24). All 4 Kinases are expressed in different levels in erythroid precursors, but HRI is the one involved in erythroid survival and development during iron deficiency. Others kinases may have role in other stresses in the erythroid progenitors (25). HRI is activated by various cytoplasmic stresses with ER stress and nutrition starvation being the exceptions (24). Most importantly, analyzing the Hri−/− mouse model, Chen’s findings demonstrated that HRI is the only eIF2α kinase that is activated by arsenite treatment of erythroid cells (24). These results emphasize the importance of HRI in stresses other than heme deficiency, such as the oxidative stress induced by arsenite (24).
1.2.4 Generation of HRI universal knockout mouse model \( Hr^{−/−} \)

In order to better understand the physiological role of HRI in erythropoiesis and the consequences of its activation, the Chen group was able to clone the murine HRI gene to generate the universal \( Hr^{−/−} \) mouse, a model that universally doesn’t express HRI in any tissues. (26).

They started by cloning a 19 kb mouse genomic DNA fragment that contains five exons from the mouse HRI gene (Fig.3). These exons encode the second conserved kinase lobe of HRI with the kinase domains (from VIa to XI in the Fig.3) and the whole C-terminus. The HRI gene was mapped to the very distal end of mouse chromosome 5, which is the 7p13q human chromosome. To date, there have not been any reported human diseases that can be associated with these chromosomal loci (26). The selected construct was prepared by displacing a 5 kb DNA segment containing the three exons that encode kinase catalytic domains (from VIb to X) with the neomycin phosphotransferase gene under the control of the phosphoglycerate kinase promoter (PGK-Neo) (26). The construct targeted the essential gene region for HRI kinase activity. In this model, the protein containing the internal deletion would be completely inactive (26).

Figure 3. Targeted disruption of the HRI gene. HRI wild-type locus (top), targeting construct (middle) and targeted homologous recombination at the HRI locus before and after Cre-mediated excision of the neomycin resistance gene (bottom). Exons were marked by the filled rectangles and labeled with Roman numerals. Abbreviations used for restriction enzymes: N, NdeI; B, BssHII; E, EcoRV; Bg, BglII (26).
1.2.5 Hematological response of \(Hr^{/-}\) mice in steady-state

Without erythropoietic stress, \(Hr^{/-}\) mice showed minimal alterations in the hematological parameters; RBC volume in general was slightly increased (MCV) with a moderate but notable gain in the mean RBC hemoglobin content (CHr). An increase of Heinz body number was also displayed in the RBCs from \(Hr^{/-}\) mice (22). Under normal dietary conditions the \(Hr^{/-}\) embryos phenotype didn’t display any evident alteration in embryonic erythropoiesis (26).

In the reticulocytes of \(Hr^{/-}\) mice, globin synthesis increased by 7-fold when compared to reticulocytes obtained from \(Wt\) mice (26). The increase in protein synthesis was in line with an augmented size of polysomes and no differences were observed in the \(\beta\)-globin mRNA levels between \(Wt\) and \(Hr^{/-}\) reticulocytes (26). These results demonstrated that \(Hr^{/-}\) reticulocytes have an enhanced rate of translation initiation. This is additional evidence that exhibited the involvement of eIF2\(\alpha\) phosphorylation by HRI in the regulation of translational initiation (26). All together these findings showed that in the absence of HRI, the level of protein production was increased in reticulocytes in a heme-independent manner with the main impact on the synthesis of \(\alpha\)- and \(\beta\)-globin chains, the most abundant proteins translated in these cells (26).

1.2.6 Hematological response of \(Hr^{/-}\) mice in stress erythropoiesis

In a state of iron deficiency, the hematological adaptive response to the stress in humans and mice is the hypochromic/microcytic anemia, where MCV and mean cell hemoglobin (MCH) concentrations are decreased in RBCs. In order to induce heme deficiency, \(Hr^{/-}\) mice were placed on a low iron diet because heme is synthesized by the insertion of iron into protoporphyrin IX via ferrochelatase during the last step of heme biosynthesis (26). The Chen laboratory elaborated a specific protocol to induce erythropoietic chronic stress. The state of iron deficiency was achieved by placing 21 day old mice (after weaning) on a low iron diet containing 5 ppm Fe. For controls, some of the littermates were fed a normal diet containing 196 p.p.m. Fe. Complete blood analyses of the peripheral blood to check the hematological/pathological response were carried out. \(Hr^{/-}\) mice developed a very unusual pattern of slight hyperchromic and slight macrocytic anemia with an accentuated decrease in RBC counts (26). When exposed to iron deficiency, the mutant mice displayed a higher reduction of heme levels in the peripheral blood when compared to the \(Wt\), 46.2% and 27.4% respectively, when the free protoporphyrin content was not significantly altered, \(Hr^{/-}\) mice also displayed a more pronounced decrease in heme content compared to the \(Wt\) blood (26).

Bone marrow and spleen analysis showed a marked expansion of erythroid precursors in iron-
deficient Hri<sup>−/−</sup> mice and a significantly elevated reticulocyte counts in the blood. These findings serve as additional evidence for erythroid hyperplasia, an excessive production of erythroid precursors. There was no apparent difference in the lifespan of RBCs from Wt and Hri<sup>−/−</sup> mice whether on normal or iron-deficient diets (26). But Hri<sup>−/−</sup> mice have been shown to have an increased rate of apoptosis in late erythroblast precursors compared to Wt mice. This discovery was a major step in revealing the fact that HRI was also essential for the survival of erythroid precursors in iron deficiency (26). Multiple variably sized globin inclusions were observed inside reticulocytes, and in a lesser number also in mature RBCs from iron-deficient Hri<sup>−/−</sup> mice (Fig.4) (26). The hematological response of Hri<sup>−/−</sup> mice during stressed erythropoiesis is summarized in figure 5. Studies in vivo of Hri<sup>−/−</sup> mice in iron deficiency revealed the function of HRI in coordinating the synthesis of globins in RBC precursors for heme availability (26). As previously stated, working HRI ensures that no globin chains are translated in excess of what can be used to form the hemoglobin tetramers for the amount of heme available. When HRI is absent or not working properly, heme-free globins precipitate in iron deficiency and become a major cell destruction component in the pathophysiology of the iron-deficiency anemia (26). HRI is responsible for the adaption of microcytic hypochromic anemia in iron deficiency and have been shown to play a role in the determination of red blood cell size, cell number, and hemoglobin content per cell (5).

Figure 4. Precipitation of globins in RBCs of Hri<sup>−/−</sup> mice in iron deficiency diet. Heinz bodies are stained in blue. Cell size is slightly larger than the Wt. Blood from mice at day 92 after being kept in a low iron diet was collected and stained with crystal violet for the presence of Heinz bodies. (26).
Figure 5. HRI is responsible for the adaptive response to an erythropoietic stress developing microcytic hypochromic anemia. During iron deficiency several things happen: heme concentration declines, which leads to HRI activation and inhibition of globin synthesis. The Hri\(^{−/−}\) mouse model displayed a different and stronger response with decreased hemoglobin and total protein content. Because in the absence of HRI (Hri\(^{−/−}\)) protein synthesis continues in the face of heme deficiency, resulting in excess globins. These heme-free globins are unstable and precipitate as inclusions (marked in green) in RBCs and their precursors, causing destruction of these cells. Hri\(^{−/−}\) mice have a decreased level of RBC numbers, reticulocytosis, and splenomegaly (5).

1.2.7 HRI/eIF2αP signaling pathway

Phosphorylation of eIF2 leads to a reduction in global translation, allowing cells to conserve resources and to initiate a reconfiguration of gene expression to effectively manage stress conditions (19). Translation initiation factor 2 (eIF2) is a heterotrimeric protein that transfers methionyl-initiator tRNA\(^{\text{Met}}\) to the small ribosomal subunit (40S) in a ternary complex consisting of Met-tRNA\(^{\text{Met}}\), the heterotrimeric initiation factor 2 (eIF2), and GTP (27). The resulting 43S pre-initiation complex binds to the mRNA and scans the AUG start codon. After the hydrolyzation of the eIF2-GDP complex, the 60S ribosomal subunit joins to form the 80S initiation complex (27). The recycling of eIF2 for another round of translational initiation is required to regenerate eIF2-GTP. This recycling reaction is stimulated by eIF2B while eIF2αP served as its competitive inhibitor. In summary, when eIF2α is phosphorylated by HRI at Ser51, the phosphorylated eIF2αP–GDP binds much more tightly to eIF2B than eIF2-GDP does and this prevents the GDP/GTP exchange activity of the eIF2B/eIF2αP complex, thereby inhibiting translation initiation (27).

In addition to inhibiting protein synthesis of highly translated mRNAs, eIF2α phosphorylation also increases translation of certain selected mRNAs such as ATF4 for cell adaptation to stress (Fig.6) (28). ATF4 is a transcription factor with multiple target genes that are transcriptionally activated by the ER and other stresses that induce eIF2 phosphorylation. Therefore, ATF4 translation increases with
increasing eIF2αP (Fig.6, 29). As shown in figure 7, ATF4 translation is controlled by a strict and regulated mechanism (28). Its mRNA bears two upstream open reading frames (uORF1 and 2) in the 5’ leader sequence before the main coding ORF (Fig.7, 28). In steady state conditions, these uORFs restrict the flow of translating ribosomes from the 5’ mRNA cap to the AUG initiation codon of ATF4 (28). Upon stress, eIF2αP reduces the pool of functional eIF2s and slows down the initiation to permit translation at the coding sequence of ATF4 mRNA (28).

ATF4 upon activation acutely increases the transcription factor C/EBP homologous protein-10 (CHOP), which is up-regulated transcriptionally in a wide variety of cells upon many stresses. Another key component of the cellular response to stress is GADD34, which interacts with protein phosphatase 1 to dephosphorylate eIF2α, which alleviates the inhibition of translation (28, 30). Both CHOP and GADD34 mRNAs are only translated for the duration of the stress response or period of elevated eIF2 phosphorylation and they are translationally controlled with uORFs programmed into the 5’-leaders of their mRNAs (30, 31). eIF2α phosphorylation appears to be fundamental for translation attenuation, transcriptional induction and cellular survival during ER stress (29) and increased ROS levels were observed in cells bearing mutations in eIF2α phosphorylation (28, 29).

Figure 6. Integrated stress response of eIF2αP signalling. In mammalian cells, there are four eIF2α kinases that are activated in response to four major stresses: nutrient starvation, viral infection, heme deficiency and ER stress. Activation of eIF2α kinases that phosphorylate eIF2α and inhibit protein synthesis to prevent proteotoxicity of unfolded protein occurs during ER stress and globin inclusions in heme deficiency. In addition, eIF2αP selectively enhances the translation of ATF4 mRNA. ATF4 then initiates an adaptive gene expression to mitigate stress (28).
Figure 7. Up-regulation of the translation of ATF4 mRNA by eIF2αP upon stress. In the 5'UTR of ATF4 mRNA, there are two uORFs that are preferentially translated under non-stressed conditions and prevent the downstream translational initiation at the coding sequence of ATF4 mRNA. As initiating 40S ribosomal subunits scan from the cap structure, translation starts at uORF1. After termination of translation, the 40S subunit remains associated with mRNA and reinitiates efficiently at uORF2 under non-stressed conditions. Upon stress, elevated eIF2αP impairs the reinitiation of 40S at uORF2 due to limiting functional eIF2. Thus, 40S continues to scan downstream and initiates at the AUG codon of the coding sequence of ATF4 mRNA permitting the synthesis of ATF4 protein (28).

1.2.8 Role of HRI in β-thalassemia intermedia

As discussed previously, HRI can be activated by non-heme stress to protect erythroid cells and plays a critical role in the physiological response to RBC disorders beyond iron and heme deficiency (5). The Chen group in 2005 demonstrated that HRI reduced the severity of murine β-thalassemia by decreasing free α-globin accumulation and inclusion body formation (32).

β-thalassemia is one of the most common autosomal recessive disorders worldwide (33). Annually, on average, 300,000 to 400,000 infants are severely affected or born with thalassemia and require treatment throughout their life (34). This disease is chronic and is in large part due to an increase in oxidative stress and ineffective erythropoiesis. The causes of the disease are well known but the proteins and the roles that they play in the process of attenuating oxidative stress and ineffective erythropoiesis are still quite a mystery to science (1). Thalassemia is characterized by the reduced or absent synthesis of the β-globin chains, respectively of $\beta^0$ and $\beta^+$, of the hemoglobin (Hb) tetramer, while the synthesis of α-chains remains unchanged. The clinical severity of β--thalassemia is related to the imbalance between the alpha globin and β globin chains (33). Three different clinical conditions are recognized: the β-thalassemia carrier state, β-thalassemia intermedia, and β-thalassemia major
The β-thalassemia carrier state, which results from heterozygosity for β-thalassemia, is clinically asymptomatic. Thalassemia major is a severe transfusion-dependent type of anemia and Thalassemia intermedia, which includes a clinically and genotypically heterogeneous group of thalassemia-like disorders, ranging from asymptomatic state to the severe transfusion-dependent type (33). Genotypically the β-thalassemias are very heterogeneous because the altered production of β-globin chains is caused by different mutations or small deletions at the level of the β-globin cluster on chromosome 11 (33).

The murine β-thalassemia model (Hbb–/–), which had a deletion of the murine β-globin major gene (35), showed a severe hypochromic microcytic anemia and reticulocytosis similar to humans with β-thalassemia intermedia (32). Hbb–/– mice had a more severe anemia when compared to Hri–/– mice (32). To understand the physiological prominence of HRI in the evolution of β-thalassemia, the authors created a double knockout mouse model defective for HRI and β globin (Hri–/–Hbb–/–), but their results showed that no double knockouts were carried through to gestation (32). To determine when and why double mutant embryos die, they analyzed the litters at E15.5. The study showed that the double-knockout embryos were very pale and smaller in size, and they died uniformly by E18.5 (Fig. 8) (32). Hematocrit results confirmed that Hri–/–Hbb–/– double-knockout embryos had a severe decrease in volume of the percentage of RBCs compared to Hri+/+Hbb–/– mice and died of severe anemia (32).

Lacking 1 copy of HRI (Hri+/–Hbb–/– mice) affected the hematological response more seriously compared to Hbb–/– mice: Hri+/–Hbb–/– mice showed a decreased MCV (mean corpuscular volume), decreased RBCs number with more inclusion bodies in the reticulocyte, and decreased hemoglobin content (32) (Fig. 8 panel B). The study’s results supported the notion that HRI is activated by denatured globins and contributes to the microcytosis seen in thalassemia (32), and accumulation of α-globin inclusions in β-thalassemia might normally be restricted through translational inhibition by activated HRI (32). In fact, the inclusions analyzed in the Hbb–/– blood samples were composed primarily of α-globin chains, despite the presence of β-minor globin chains in the soluble hemoglobin. This demonstrates that activation of HRI is important in minimizing the accumulation of denatured α-globin aggregates, and both copies of HRI are required for this function (32). Furthermore, Hri+/–Hbb–/– mice had more severe splenomegaly and cardiomegaly than Hbb–/– with a reduced lifespan and impaired fertility (32).

The conclusions from this work were that the presence of HRI reduced the severity of β-thalassemia features and the maximal opposite effect is gained when both copies of HRI are deleted but HRI haploinsufficiency is enough to cause more severe clinical and pathological manifestations in β-thalassemia (32).
Figure 8. HRI Deficiency Exacerbates β-Thalassemic Syndrome. (A) E17.5 embryos with different β-major genotypes. (B) Wright-Giemsa stained peripheral blood smears from adult mice. Inclusions are indicated by arrows. Original magnification, x1000 (32).

1.3. Macrophage’s role in erythropoiesis

1.3.1 Macrophage functional adaptability

Macrophages can produce an impressive array of cytokines, chemokines, enzymes, arachidonic acid metabolites and reactive radicals upon activation (36). Many of these functions appear to antagonize or counter each other. Macrophage multiple functions can both enhance or suppress adaptive immune responses. Macrophages display both pro-inflammatory and anti-inflammatory functions, respectively classic activation (M1) or alternative activation (M2). They can also produce tissue debriding metalloproteinases and inhibitors of these metalloproteinases, and produce toxic radicals that contribute to tissue cell destruction or cytokines that promote tissue regeneration and wound healing (36). It is not possible to express all of these functions simultaneously but they seem to be regulated so that macrophages display a harmonious balanced pattern of functions (36). It is interesting to highlight that for several decades it has been argued that the macrophage lineage possibly contains several developmentally distinct sub-lineages and to date the M1/M2 classification of macrophages can be consider oversimplified to describe the wide spectrum of macrophage populations (36).

Macrophages play an important role in iron recycling, taking iron from the degraded hemoglobin of senescent RBC by phagocytosis and releasing it through ferroportin (37).
About a quarter of the total body iron (0.5–1.0 g) is stored in macrophages and hepatocytes as a storage that can be easily accessible for erythropoiesis. These stores, however, can become depleted when iron uptake is less than iron usage and loss, such as with decreased dietary iron uptake and excessive blood loss eventually resulting in the development of iron deficiency anemia (38, 39).

1.3.2 Function of HRI in macrophage maturation

HRI, as mentioned previously, plays an important role in the production of hemoglobin and formation of the RBC, which contain nearly 70% of total body iron, and macrophages are pivotal part in the iron recycling mechanism (40). In 2007, the Chen laboratory published new important findings about the role of the HRI role in murine macrophage maturation. This was the first time HRI was studied in a non-erythroid cell (37).

They examined the expression of HRI in bone marrow–derived macrophages (BMDMs) cultured in vitro. Although HRI is expressed at a lower level in macrophages, those obtained from Hri–/– mice showed an impairment of macrophage morphology (Fig.9 panel A) with reduced expression of the maturation marker CSF-1R and cell-surface markers F4/80 and CD11b (Fig.9 panel B, C) (37).

These results were clear evidence of the role of HRI in macrophage maturation. In fact, the level of eIF2Pα were also reduced in Hri–/– BMDMs, compared with BMDMs macrophages from Wt, meaning that HRI is responsible for a significant part of eIF2α phosphorylation in macrophages (37).

Furthermore they investigated HRI role in iron metabolism by analyzing iron level in mutant and Wt mice. In steady state condition Hri–/– mice had splenic iron reduction with higher levels of iron in the serum when compared to the Wt, but in a state of iron deficiency, mutant mice showed more splenic iron and less serum iron than the control (37). In vitro Hri–/– macrophage also phagocytosed less RBCs than the Wt macrophages, at both baseline and after treatment with the macrophage stimulator Lipopolysaccharide (LPS) (37). The authors of the study also demonstrated the HRI involvement in macrophages during erythropoietic stress, inducing chronic hemolytic anemia by treating Hri–/– mice with Phenylhydrazine (PHZ), a hematotoxic compound. PHZ-treated Hri–/– mice exhibited impaired erythrophagocytosis of damaged RBCs by splenic and hepatic macrophages (37). These findings provide new evidence of the role of HRI in macrophages, revealing a previously unknown important function of HRI in macrophage differentiation (37).
1.4 Erythroblastic island (EI): niche for erythropoiesis

1.4.1 Brief story of EI

Hematopoiesis is the process by which a population of stem cells that continuously self-renew can provide replenishment of differentiated blood cells by generating progeny with a sequential changed pattern of gene expression (41, 42). During the first stage of this complex process there is the evolution of lineage-committed progenitors that are still microscopically not recognizable (43, 44). During the second stage of erythroid differentiation the nucleated precursors are morphologically recognizable, which are usually bigger cells if compared to more mature cells from the later stages of differentiation. The nucleated precursors progress from the proerythroblastic to basophilic, polychromatophilic, and orthochromatotic stages (Fig.10) (43). In mammals, erythropoiesis can be divided into four distinguished and fundamental steps that can be briefly summarized: the accumulation of hemoglobin that contributes to the passage from basophilic to acidophilic cytoplasm in more mature cells, the expansion of erythroblast numbers through a limited number of cell divisions, a progressive and visible decrease of the cell size, and the progressive condensation of the nucleus until the cell enucleates to form mature red blood cells that enter into the blood flow (39). From the Colony Formation Unit (CFU-E) to the orthoerythroblast (OrthoEB) stage, immature erythroblasts are, for the entire time, in physical contact with a macrophage (45) (Fig.10).
Figure 10: Starting at the colony-forming unit (CFU-E) stage, cells begin to attach and interact with the central macrophage to form the erythroblastic island and begin hemoglobin synthesis starting at the basophilic erythroblast stage until the formation of the reticulocyte, which then enters the circulation (45).

The others steps of erythroid differentiation involve the maturation of the reticulocytes in circulation to become mature red blood cells (RBCs). In the blood stream, reticulocytes, which are already without nucleus and organelles, go through major alterations; they change their ribosomal system and assume a biconcave discoid shape to facilitate the passage into small capillaries. The mature erythrocytes circulate in the vascular flow until senescent, which usually occurs after 120 days and when they are removed by the macrophages within the reticuloendothelial system (46). Marcel Bessis was the first to study the erythroblastic islands in 1958 (47). Bessis originally characterized and described the erythroblastic island as developing erythroblasts surrounding a central macrophage. His studies were based on important analysis of transmission electron micrographs of sections of rat bone marrow (48). A published EI photo taken with scanning electron micrography is provided in Fig. 11 (48). The interesting characteristic of niches in general are the multiple cell-cell and cell–extracellular matrix interactions. These microenvironment compartments, such as the erythroblastic islands subset, are recently being considered responsible for both positive and negative regulation during cell proliferation and differentiation in many tissue types (43). Recent studies show that some aspects of erythroid niches can be important contributors during the normal erythroid development and a fundamental aspect of EI seems to effect altered erythropoiesis and diseases. These findings correlate the macrophage involvement in the ineffective erythropoiesis in thalassemia and in the disordered erythroid maturation in the myelodysplasia (MDS) (49).

During the past 50 years continuous improvements in the field of erythroblastic islands research has made it possible to better understand their role in erythropoiesis, and offer new insights into the possible role of erythroblastic niches both in steady-state or altered situations.
1.4.2 Erythroblastic Island composition

Erythroblastic islands (EI) have been recognized in vivo in the mammalian sites for definitive erythropoiesis: fetal liver, bone marrow, and splenic red pulp. After the development of the long-term bone marrow cultures in vitro it was possible to further investigate the niche composition (50). As already mentioned above, EI are pivotal for the maturation of erythroblasts that are destined to enucleate. Erythroblastic islands have never been found in the yolk sac where there is a different kind of niche, the yolk sac blood islands, which have long been recognized as a first blood emergency site during embryonic development (43). Even if the two islands have different functions, it is interesting to notice that cells forming yolk sac niches all derive from the same precursors and it would be interesting to investigate if there are any similarities with the erythroid niches in this aspect (43). Some studies speculated that the erythroblastic islands would be most abundant adjacent to the sinusoids, allowing easily access of newly formed reticulocytes into the vasculature (43, 51). However, an equal predominance of islands have been found in other locations, presenting the interesting theory of islands migrate closer to capillaries when erythroblasts become more mature and ready to leave the macrophage. Different experiments with quantitative light and electron microscopy of rat bone marrow showed a difference between the composition of islands adjacent and nonadjacent to the sinusoids (52). In brief, it appears that nonadjacent islands contain more proerythroblasts, an earlier precursor than the orthochromatophilic erythroblasts, which are more abundant in the islands adjacent to the sinusoids. The numbers of basophilic and polychromatophilic erythroblasts appeared
comparable in both sinusoids adjacent and nonadjacent niches (51). Erythroblastic islands can be described as a differentiated macrophages positioned centrally from multiple erythroblast in several stages of differentiation (Fig. 12).

![Graphic image of an erythroblastic island.](image)

**Figura 12.** Graphic image of an erythroblastic island. Erythroblasts are in different stages of maturation attached to a central macrophage (different size cells). Blue cells are early erythroid precursors which are also bigger compare to the others erythroblasts, during cell differentiation erythroblast becomes smaller and because they increase the hemoglobin content they turn to red. The reticulocytes (red small cells with an irregular shape) are detached from the macrophage before leaving the marrow to enter the blood stream. Extruded nuclei are created when reticulocytes are formed from orthoerythroblasts and remain inside the central macrophage because they have been phagocytosed (adapted from 52).

Since EI are easily disrupted when bone marrow smears are prepared for examination, it has been difficult to elaborate a protocol to isolate correctly the erythroid niches. Bessis started studying the islands by dissolving a drop of fresh bone marrow in blood and with this process he studied the island composition without many alteration of the original or native structure (43). Following his method, Le Charpentier isolated and examined EI by treating the bone marrow with a combination of gentle physical and enzymatic disruption (53). Nowadays, different methodologies have been tested and are currently in use (in modified versions) of the enzymatic protocol described in the Crocker and Gordon paper in 1985, to isolate niches from bone marrow clusters (54, 55). Cell clusters are groups of cells rich in myeloid precursors and erythroblasts connected together. The islands resulting from the incubation of cell clusters can be considered a native erythroblastic island. It is possible to obtain reconstituted niches by adding erythroblasts to mature macrophages already in culture (55, 56).
Erythroid niches contain a variable number of erythroblasts, depending on the origin of tissue or species (43).

1.4.3 Central macrophage characterization

Although it serves as a fundamental element of the erythroid niche, the central macrophage’s complete genetic and proteomic profile remains a puzzle that continues to be assembled. Bone marrow is rich in heterogeneous monocyte/macrophage populations, and it seems that the island macrophages originating from monocyte precursors, is a specific macrophage subset, with certain characteristics, and it is present in the hematopoietic tissue (43). It has been reported that central macrophages collected from human bone marrow clusters highly express CD4, CD31, CD11a, CD11c, CD13, CD14, CD16, CD18, FcRI, and HRL-DR that are different monocyte/macrophage markers (57). To date characterization of central macrophage is still incomplete for both murine and human and the function of different markers is still unknown. It has being speculated that certain of these adhesive linkages may initiate signaling pathways coordinating gene expression and cell-cell adhesion (49). Mouse central macrophages are characterized by a high expression of the macrophage markers F4/80, a surface glycoprotein and Forssman glycosphingolipid, which share a cell surface homology to the G–protein-linked transmembrane 7-hormone receptor family (43, 58). Observations from the early Crocker and Gordon studies in 1985 reported important findings; macrophages isolated from cell clusters in mouse bone marrow were compared to the resident macrophages from the peritoneal cavity and the circulating monocytes. The results showed that niche macrophages had some peculiar morphology outlines. They are very large cells with diameters frequently more than 15 μm and have a nuclear/cytoplasmic ratio of much less than 1 (54). Other than being intensely F4/80 avid, they have developed plasma membrane processes. The articulated arms appear to connect directly with the attached erythroid cells. It was also interesting to note the difference in smaller macrophages, with a rounded shape and less developed extremities that showed a weak stain of the marker F4/80. These cells appeared to be morphologically closer to pre-monocytes and monocytes, with a more elongated kidney shape of the nuclei and a nuclear/cytoplasmic ratio greater than 1 (43, 54). Other than the unique morphology of the island macrophages that appear to support the surrounded immature erythroblast, there are other important features to report. In fact, niche macrophages showed a high phagocytic activity and the absence of a respiratory burst with a potent stimulator. The multiple erythroblasts associated with the central cell were actively rotating. This action may indicate that they are not only scavengers of dead cells, but with their pronounced adhesive properties and ability for greedy endocytosis, they are a perfect candidate to also function as nurse cells (43). The absence of respiratory burst and the consequent release of toxic oxidative species are perhaps to avoid damaging the surrounding erythroblasts (43).
Macrophages isolated from splenic and hepatic erythroblastic islands have similar profiles to the described bone marrow-derived central macrophages (59, 60). In contrast, the monocytes and peritoneal macrophages with their complement receptors and strong respiratory burst activity play a primary role in pro-inflammatory states (43) to prevent infections and sustain tissue inflammation. When these differences are highlighted the intriguing hypothesis that these macrophages may be 2 different cellular subsets appears plausible.

1.4.4 Enucleation of definitive erythrocytes

Enucleation of definitive erythrocytes is the result of numerous phases where the erythroid precursors go through several cell divisions and a progression of morphologically identifiable forms. This results in the accumulation of hemoglobin, and increased expression of transferrin receptor and membrane glycoprotein glycophorin A until the nucleus condensates, ready to be expelled (43). The loss of intermediate filaments allows the nucleus to change position and move to the periphery of the cell to occupy an acentric position prior to expulsion. Many studies show few similarities between the enucleation and the cytokinesis (43, 60) and have suggested that F-actin is organized in a ring that creates pressure, similar to the cleavage groove of mitotic cells (61). The nucleus is pinched off by the combination of the cleavage line formed by merging multiple vacuoles with new membranes and the constriction of the actin ring (Fig.12). The nucleus remains covered with a thin layer of cytoplasm surrounded by a flap of membrane from the reticulocyte. The process of loss of nuclei involves the isolation of cytoskeletal and cell surface proteins between the plasma membrane remaining on the nucleus and the newly synthesized reticulocyte (43). The groove contains several mitochondria, which are seen also in a similar location during cytokinesis and these considerations help to support the concept that erythroid enucleation might be a specialized type of cell division (61). This entire process, briefly described above regarding the different steps of erythroblast enucleation, occur in the definitive erythroblastic islands, so that the central macrophage can easily be in close proximity to the pyrenocyte for phagocytosis and digestion (43). Many cell adhesion molecules and different interactions found in the erythroid niche have been considered critical for the correct erythroid maturation progress and island integrity. The precise role of these adhesive interactions is still uncertain but it has been speculated that the integrin–actin cytoskeleton interactions might regulate intracellular signaling and they may coordinate adhesion and gene expression in the erythroblastic islands (43). When the bonds between α4β1 integrin expressed on erythroblasts and its vascular receptor adhesion molecule-1 (VCAM-1) on macrophage cells, are compromised using monoclonal antibodies, the island integrity is destroyed in vitro (63). The enucleation process and separation of pyrenocyte from the reticulocyte remain a rarely occurring phenomenon in vitro (64).
Figure 12. Red blood cell (RBC) formation in mammals requires the enucleation of late-stage erythroblasts that became reticulocytes before going into the blood flow. This process is now recognized to be a multi-step process that includes the acentric positioning of the nucleus associated with a microtubule network, the pinching of the erythroblast associated with a localized actin-myosin ring and lipid rafts, as well as endocytic vesicle formation and the formation of 2 cells: a pyrenocyte that is rapidly engulfed by macrophage cells and a reticulocyte that continues to mature into a biconcave RBC (adapted from 62).

A recent study described a detailed model for the engulfment of pyrenocyte using an in vitro system (65). In summary, the following model for erythropoiesis in erythroblastic islands was proposed. Erythroblasts are bound to the central macrophage within the island by using the interaction between integrin-α4β1 on erythroblasts and Vcam1 expressed on the macrophage. Immature erythroblasts proliferate in association with the macrophages, and progress through maturation followed by enucleation, which is escorted by a progressive down regulation of integrin-α4β1 (65). It is important to note that pyrenocytes showed a low affinity for macrophages but they were engulfed by the macrophages in the presence of methylcellulose in vitro. This suggests that the consistent viscosity created by using methylcellulose in cultures may have been sufficient to maintain the close proximity of pyrenocytes and macrophages, reproducing a similar environment found in the matrix protein of bone marrow and providing the optimal stress required for the dissociation between pyrenocytes and reticulocytes (65, 49). The remaining pyrenocyte expressed the phosphatidylserine (PtdSer) on the membrane surface (65). This molecule is also exposed on the apoptotic cell membranes when they are incorporated by macrophages in a PtdSer dependent action. Different studies have proposed that protein MerTK, a protein expressed on the macrophage membrane, as being essential during the phagocytosis process (65, 66). The model illustrated by Toda et al. showed that the central macrophages engulf pyrenocytes and might use a MerTK- dependent mechanism similar to the one associated with phagocytosis (65).
1.4.5 Different erythroblastic Island functions

Numerous questions regarding the many potential roles of EI and other erythroid niche functions remain unanswered. In contrast with what has been described above, erythroblasts can differentiate and fully mature in culture in vitro but the entire process appears to be less efficient with lower numbers of enucleated cells (67). The generation of mature erythrocytes is enhanced by co-culturing them with macrophages (67) or other accessory cells such as murine stromal cell lines or human mesenchymal cells (68). This evidence suggests that erythroblastic islands are essential during early and late stages of erythroid maturation.

It has been proposed that the macrophages in early stages of erythroid maturation can provide nutrients and proliferative and survival signals to support the erythroblasts (49). In fact, co-culture results showed that erythroblasts in contact with macrophages increased their proliferation for a reduced transit time of G0/G1 phase (69).

Different studies suggested that macrophages secrete cytokines that promote erythroblast proliferation and maturation, including burst-promoting activity and insulin-like growth factor-1, factors that can stimulate the growth of BFU-E and CFU-E (70, 71). Although many in vitro studies described progenitor niches as a site where autocrine factor and extracellular inputs are secreted, the precise role of macrophages remains unclear.

While the in vivo contributions of the erythroblastic island leaves many unanswered questions, a recent study explored the role of macrophages during stress and pathological erythropoiesis using murine models of β-thalassemia and polycythemia vera (72). The new findings highlight a prominent role for erythroblastic island in situations under different stress conditions such as anemia, epo administration, polycythemia vera and β-thalassemia (72, 73). Interestingly, macrophage-depletion studies in mouse models of polycythemia vera or β-thalassemia reversed some key features linked to both diseases. The two mice models were treated with clodronate, which depletes macrophages and showed a significant improvement of anemia with a reduced erythropoietic activity, even if less RBCs were produced. These cells had a better quality with a longer lifespan. β-thalassemic mice also had a decrease of the spleen size with complete recovery from splenomegaly (72). In summary, the results of the study showed that macrophage-dependent erythropoiesis leads to a more severe progression of the β-thalassemia, which is caused by enhanced erythropoiesis and large precipitation of α globin chain. The erythroblastic island independent erythropoiesis clearly ameliorated the features of β-thalassemia in the mouse model (74). They extended these observations to human samples and presented evidence that co-cultures with macrophages and erythroblasts showed an increased percentage of cell cyclins, supporting the need for direct contact between macrophages and erythroid progenitors in erythroblast maturation (72). Another important yet unclear question is whether macrophages supply iron for hemoglobin synthesis (43). Over the last few years, our understanding of
mammalian iron transport and homeostasis has advanced. Some of the key players in iron homeostasis, such as the circulating peptide hormone hepcidin, and ferroportin, which are the major transmembrane transporters of iron out of enterocytes, macrophages, and, to a lesser extent, hepatocytes and their signal pathway has been studied (75-78). Hepcidin expression is altered in response to the stimuli that affect iron homeostasis. For example, in conditions of stress erythropoiesis such as hypoxia or iron deficiency anemia, levels of hepcidin are decreased, resulting in lower levels of ferroportin inactivation (79).

As previously reported, macrophage depletion improved the ineffective erythropoiesis in a mouse model of β-thalassemia, caused by high level of erythropoietin and iron absorption. The elevated levels of erythropoietin were due to increased erythropoiesis and the iron absorption was caused by low levels of hepcidin. In a recent article it was advanced the idea that macrophage depletion discontinue this downward cycle by regulating splenic erythroblast numbers and increasing hepcidin levels, which leads to less intestinal iron absorption (74). These findings have helped to reach a more comprehensive understanding of the role of macrophages during stress erythropoiesis. To address different erythroblastic island functions, it would be necessary to use quantitative systems approach to include all the various elements where EIs are involved: iron homeostasis, adhesion interactions, effects on cell differentiation (73).

Recent advances in the field of EI provided valuable new insights into the mechanism that support definitive erythropoiesis. HRI is fundamental for the erythroblast differentiation during erythropoietic stress and furthermore, macrophages that have an absence of the signaling pathway, revealed an impairment of cell maturation. These findings present the idea of a possible link between the HRI pathway and the central macrophage of the niche and the possibility that the ineffective erythropoiesis developed in the Hri−/− mouse model during erythropoietic stress can also be caused by the alteration of the EI, which themselves lack the HRI signaling pathway.
2. AIM OF PROJECT

In the last few years the studies of erythroblastic niches have become increasingly important to understand their role in normal and pathological erythropoiesis in vivo. Within the EI, the macrophages have a key role to support the proliferation and differentiation of immature erythroblasts and recent studies have shown that HRI is an important contributor in macrophage maturation. The principal target of HRI is the translational regulator, eIF2α, involved in the development and proliferation of erythroblasts, especially under stress. Phosphorylation of eIF2α by HRI not only inhibits translation of most mRNAs, but also enhances translation of ATF4 mRNA. Despite many studies which have demonstrated the importance of HRI in erythroid cells, it remains unknown the contribution of HRI in the specific subset of macrophages that form erythroblastic niches. In this project we investigated the components of HRI stress signalling pathway in functions of bone marrow and splenic macrophages as well as specifically EI macrophages in stress erythropoiesis of chronic iron deficiency. We accomplished this by examining the interactions between macrophages and erythroid precursors within EI in the bone marrow and spleen of wild type, Hri-/-, eAA mice and reconstituted EI by using macrophages from the bone marrow of wild type and mutant mice with erythroblasts isolated from the spleen.

This research is an important step forward in understanding the role of the EI macrophage under conditions of iron deficiency anemia and their response in a state of HRI deficiency. The outcome of this thesis may be important in better understanding new aspects of macrophage involvement in stress erythropoiesis and helping to find new targets for the treatment of anemia.
3. MATERIALS AND METHODS

3.1 Mice model

Hri^{−/−} mice of an inbred B6.129 mixed genetic background (19). Atf4^{−/−} mice were bred as described in a previous study (23). The Chen lab was interested in investigating the role of the HRI substrate eIF2P in the development of early erythroid progenitors. They recently determined the contribution of eIF2α P in mediating HRI function in oxidative stress and stress erythropoiesis by specifically ablating eIF2α P in the erythroid precursors. Mice with universal eIF2α Ser51Ala knockin in all tissues (A/A mice) died shortly after birth due to a defect in gluconeogenesis (81). To rescue the neonatal lethality of A/A homozygous mice, a constitutively expressed eIF2α transgene (Tg) flanked by LoxP sites was introduced into A/A mice to generate A/ATg mice. Erythroid-specific eIF2αAla51 knockin mouse lines were generated in Dr.Chen’s laboratory. An erythroid specific CRE-expressing mouse line was used in order to make the erythroid-specific deletion of the eIF2α transgene in A/ATg mice. The erythroid-A/A mice should have the eIF2α transgene deleted only in the erythroid lineage, so the mice showed the erythroid-specific knockin phenotype of eIF2αSer51Ala (Chen’s lab unpublished results). Mice were housed in a specific pathogen-free facility at the Department of Comparative Medicine (DCM) at MIT University. All animal experiments were performed in accordance with protocols approved by the Animal Care and Use Committee at MIT. Mice on a normal diet were fed with water and commercial mouse chow provided by DCM. We induced stress erythropoiesis in mice by keeping them on an iron deficiency diet after weaning at 21 days after birth. Mice were kept on a special diet provided by the Chen laboratory for 2 months prior to sacrifice by CO₂ inhalation.

3.2 Hematological analysis

Blood, liver, and spleen samples were collected from 2 month old mice. Peripheral blood cell morphology was examined on Wright-Giemsa staining. The hematological analyses of peripheral blood and reticulocyte counts were performed at the MIT facility.

Hemoglobin concentration (Hb), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), red blood cell count (RBC), reticulocyte count (Retic), content of hemoglobin in reticulocytes (CHr), mean cell volume of reticulocytes (MCVr), platelets (PLT), and white blood cell (WBC) counts were assessed in whole blood EDTA.

The hematocrit (Ht or HCT) or packed cell volume (PCV) or erythrocyte volume fraction (EVF) is the volume percentage (%) of red blood cells in blood. The mean corpuscular volume, or “mean cell volume” (MCV), is a measure of the average red blood cell size. The MCV is calculated by dividing
the total volume of packed red blood cells (hematocrit) by the total number of red blood cells. It is the MCV measurement that allows classification as either a microcytic anemia (MCV below normal range), normocytic anemia (MCV within normal range) or macrocytic anemia (MCV above normal range). The mean corpuscular hemoglobin, or "mean cell hemoglobin" (MCH), is the average mass of hemoglobin per red blood cell in a sample of blood. A red blood cell (RBC) count is the number of red blood cells per volume of blood and is reported in either millions in a microliter or millions in a liter of blood.

3.3 Macrophages Cell Culture

3.3.1 Cell culture of BMDMS and SDMS

Bone marrow cells were flushed from the femur and tibia of 8-week-old male or female mice and resuspended in medium by gentle pipetting. Spleens were dissected from the abdominal cavity and placed in 1 ml of pre-warmed 0.05% collagenase (Sigma) and 0.002% DNASE (Sigma) DMEM solution to be digested for 1 hour at 37°C with gentle shaking. Digested cells were washed twice with PBS plus 2% FBS and filtered through a 40μm nylon strainer. Before seeding, nucleated cells were counted with crystal violet that stains the nucleus. We seeded the same number of cells. Spleen macrophages were present in a smaller percentage of total cells, so 2-3 times more cells were seeded as compared to the bone marrow. Cells were cultured in DMEM medium with high glucose, glutamine, 15% heat-inactivated FBS, 25 ng/ml Macrophage Colony-Stimulating Factor (M-CSF) (Pepro-Tech), penicillin/streptomycin (Invitrogen), at 37°C, 5% CO². Three days after seeding, half of the medium was changed every 2 days until day 7 or 8.

3.3.2 Flow cytometry analysis of adherent cells

Culture of bone marrow-derived and spleen-derived macrophages (BMDMS and SDMS respectively) were detached from the culture dishes by treating with Accutase solution (Stemcell) for 15 minutes at 37°C. Cells were washed twice with PBS and cell numbers were then counted. Macrophages were resuspended in PBS containing 0.5% BSA. Non-specific Ab binding was blocked with addition of Fc blocked Ab, then fluorochrome-labelled Abs against macrophage surface markers were added in different concentrations according to the Abs; cells were stained for 20 min on ice and washed 3 times with cold PBS (Table 1). Unstained samples were prepared for cell size assessment, and single color control samples were used to calculate the compensation. Data were collected with Flow Cytometer LSRII and analysed with Flow Jo software.
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Table1: antibody markers used for the Flow Cytometry analysis: all antibodies are anti-mouse from rat (BioLegend).

3.3.3 Methocult media Assay for CFU-M

All mice were cultured for both BM and spleen (Spl) CFU-M. To perform the colony assay we seeded the same number of nucleated cells from spleen (668000 cells) and bone marrow (33400 cells) of Wt, Hri<sup>-/-</sup> and Atf4<sup>-/-</sup> mice in the methylcellulose medium. Total bone marrow cells or spleen cells were plated in duplicate at a density in 1.1 ml of MethoCult (Stemcell 04100) with IMDM medium plus 2% FBS with 25ng/ml M-CFS. The correct amount of cells were resuspended in 100ul of IMDM 2% FBS and added to the bottom of a 1.5ml plastic tube. On the top of the cells we added 1.1ml of MethoCult medium using a 1ml syringe without needle. Tubes were vortexed for 5-10 seconds in order to homogeneously mix the medium with the cells. Before seeding the cells on 12 well plates, tubes were left at RT for a few minutes to eliminate the bubbles inside (Fig.4). The last step was to seed the samples in duplicate: 500ul in each well with a total of 2 wells per sample. To do this operation, we completely filled a 1ml syringe, trying to avoid creating bubbles and seeded the correct amount per well.
Figure 4. Methocult colony assay protocol: (A) Wash cells with IMDM 2%FBS; (B) Add cells to the methocult medium and vortex; (C) Let tube stand to allow bubbles to dissipate; (D) Plate and incubate cells into culture dishes for 8-10 days. The colonies are macroscopically counted.

3.4 Erythroblast Island (EI)

3.4.1 EI isolation protocol

Erythroblastic islands were prepared from spleens and bone marrow using a modified protocol from the original papers by Crocker et al. and Toda et al. (54, 65) To analyse native EI I collected clusters of mixed cells by using the same gravitational principle described in the paper and I cultured them on a small glass coverslip for different intervals of time to allow island macrophages to adhere. The difference was that I concentrated the cells in a very small volume to be able to repeat experiments without using large amounts of medium for each sample. In detail, spleens from mice were minced and digested with 0.075% collagenase and 0.004% DNase in RPMI for 1 hour at 37°C on a shaker, passed through nylon mesh, and resuspended at a density of 100X10^6 cells in 2.5ml RPMI 10% FBS. Bone marrow cells was collected from femur, tibias and humerus, flushed with a syringe and gently resuspended in 2.5 ml RPMI 10% FBS. Usually around 70-80X10^6 cells are collected. Cells were then layered over 5 ml RPMI 30% FCS in a15 ml falcon tube. After 1 hour at room temperature (RT), the
sedimented cells were washed twice with RPMI by centrifugation at 3X100 g for 10 minutes, and allowed to adhere on a 10 mm glass rounded coverslip inside a 24 well plate with DMEM 10% FBS for 90 minutes at 37°C (Fig.5). Coverslips were dipped 3 times in a PBS buffer solution to remove loosely adherent cells. For microscopic examination, the erythroblastic islands were fixed with 4% paraformaldehyde (PFA) for immunofluorescence microscopy analysis (Table 2) and fixed with ethanol or methanol for wright Giemsa staining or crystal violet (65). Coverslips were also collected at 5 hour intervals and overnight: cells were left in culture with IMDM 10% FBS, 3U/ml epo and 200ug/ml transferrin. Common histology stains such as May-Grunwald Giemsa and Wright-Giemsa were both valid solutions to check cell morphology, the erythroblasts turned very dark and are recognized by their rounded shape with a large compact nuclei.

Figure 5. EI protocol. Total cells from BM or Spl in RPMI 10% FBS medium were layered on RPMI 30% FBS medium. After 1 hour at RT we obtained a supernatant rich with single cells. The cell clusters were accumulated at the bottom of the falcon tube. The clusters were seeded on a glass round coverslip.

3.4.2 Reconstituted EI protocol

The method of Crocker and Gordon was slightly modified to obtain purified macrophages for reconstitution of erythroblastic islands (54). Macrophages were isolated from bone marrow as previously described. The cell suspension was layered to obtain cell clusters. 0.5x10^6 clusters were seeded on glass coverslips and allowed to attach for 3 hours. To strip erythroblasts from
macrophages, cells were incubated in PBS lacking calcium and magnesium for ten minutes and flushed five times with PBS to remove erythroblasts and obtain “stripped” macrophages. Adherent macrophages were allowed to spread again for 2 hours in complete medium before receiving fresh 0.25x10⁶ erythroblasts (“reconstituted” clusters) from Hn⁻/⁻ mice spleen. To prepare erythroblasts from total spleen, cells were magnetically labelled with a cocktail of biotinylated antibodies against a panel of so called “lineage” antigens (Miltenyi Biotec) to obtain a “clean” population of erythroblast precursors. Purified erythroblasts were added to the macrophages and they were allowed to incubate overnight at 37°C, 5% CO₂. Finally, the reconstituted clusters were dipped 3 times in PBS buffer solution to remove the unbound cells before being processed for double F4/80, V-cam and TER119 immunofluorescence using standard procedures (Fig.6) (Table 2).

Figure 6. Reconstituted EI protocol. As previously described, cells clusters were seeded on 10 mm coverslips. Macrophages were allowed to attach to the glass for 3 hours. We stripped macrophages from the attached erythroblasts using PBS –Mg²⁺ –Ca²⁺, leaving macrophages alone for 2 hours before adding purified erythroblasts from HRI-Fe spleen. We collected coverslips, fixed and stained them.

3.5 Immunofluorescence microscopy

The immunofluorescence study was done according to the standard procedures. Stripped macrophages or EIIs were cultured on coverslips as described above and were fixed in 4% PFA for 15 minutes at room temperature (RT). After washing 2 times with PBS, excessive pfa was quenched with 0.2M glycine in PBS for 10 minutes at room temperature. Fixed cells were further washed 2 times with
PBS for 5 min at RT and were blocked for 1 hour in PBS with 1% BSA plus 5% normal rat serum, and IgG in a concentration of 1:50. ELs were incubated overnight at 4°C with different concentrations of primary antibodies conjugated with Alexa-Fluors: anti ter-119 Alexa Fluor 488, anti V-cam Alexa Fluor 594, Texas Red, and anti F4-80 Alexa Fluor 647 (Table 2). After washing with PBS, coverslips were briefly air-dried and then mounted with anti-fading mounting reagent plus DAPI (Life Technology) and examined by fluorescent microscope (Leica microscope). Pictures were elaborated using Fiji program.

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Table 2: F4/80 and V-cam1 antibodies were used to detect macrophages. Ter119 was used to detect erythroblasts. All antibodies were labeled with Alexa-Fluor dyes (BioLegend).

The immunofluorescence study of BMDMS was done according to the standard procedure already described above with small modifications. After 7 days in culture, the cells were detached with Accutase solution and seeded for 24h in a 96 well black plate. In each well no more than 20,000 cells were seeded. The cells were first fixed with 4% PFA for 30 min and quenched. Then macrophages were stained with primary antibody CD11b in a concentration of 1:50 overnight at 4°C. The cells were washed twice with PBS-BSA 1% and stained with the secondary antibody (Alexa Fluor 594) and DAPI (1ug/ml) at the concentration of 1:500 for 90 minutes. Cells were washed twice and quickly analyzed under the microscope without using mounting solution.
3.6 Iron staining

Perls’ Prussian Blue Iron Kit (Sigma-Aldrich) was used to detect and identify ferric (Fe3+) iron and non-heme iron in tissue preparations and blood smears. Minute amounts of ferric iron are commonly found in bone marrow and in the spleen. Prepared tissue sections cut 5–6 microns in thickness and fixed in neutral buffered formalin is satisfactory for iron staining. Blood smears were prepared in the usual fashion, air dried at least 30 minutes and fixed in absolute methanol for 7 minutes. After deparaffinization, slides were hydrated with deionized water. Slides were placed in Working Iron Stain Solution for 10 minutes and then rinsed with deionized water. To obtain contrast color against the blue, we used Working Pararosaniline Solution for 3–5 minutes. Slides were rinsed in deionized water and rapidly dehydrated with different concentrations of alcohol and xylene solutions and mounted with a xylene-based mounting medium (Thermo scientific).
4. RESULTS

4.1 Unpublished results from Chen lab in the characterization of eAA and ATF4 mice during iron restrictive erythropoiesis

Recent results from the Chen lab have shown that eAA mice did not have a significant hematological change during steady-state erythropoiesis. After two months of iron deficiency, these mice developed anemia similar to that of Hri−/− mice. It was slightly macrocytic and hyperchromic (Chen’s unpublished results).

Phosphorylation of eIF2α by HRI not only inhibits translation of most mRNAs, but also enhances translation of ATF4 mRNA. Atf4−/− mice were observed to have serious microphthalmia because the crystalline lens of their eyes are degenerated by abnormal developmental differentiation of lens fiber cells (82). During normal adult steady-state erythropoiesis, Atf4−/− mice, similar to eAA mice, did not exhibit significant hemathological changes (Chen’s unpublished results). Two months after being on the special diet (without iron), Atf4−/− mice developed microcytic and hypochromic anemia (Chen’s unpublished results).

4.2 Macrophages

4.2.1 Growth and differentiation of macrophages defective in HRI stress signaling

Macrophages are essential for definitive erythropoiesis. Interactions between erythroblasts and macrophages play a critical role during erythropoiesis, especially under stress. Previous results showed that HRI is predominantly expressed in the erythroid lineage and also expressed at a lower, yet significant level in macrophages (37).

To better understand how HRI signalling may regulate splenic and bone marrow macrophages, I have examined the bone marrow and splenic–derived macrophages (BMDMS and SDMS) cultured in vitro in the presence of M-CSF. I cultured the BMDMS and SDMS from Wt, Hri−/−, eAA and Atf4−/− mutant mice, and monitored their growth on a daily basis. The number of cells seeded and the plate size used changed for different experiments depending on the available cell number from the Hri−/−, eAA and Atf4−/− mutant mice. However, the cell number seeded was identical in all the samples of the same experiment. The spleen has less monocyte/macrophage cells so we seeded twice the number of cells than the corresponding bone marrow. The mutant mice analyzed for this set of experiments were the BMDMS of 3 Atf4−/− mice on iron deficiency diet, 2 eAA mice on iron deficiency diet and 2 eAA on normal diet, 1 Hri−/− mouse on iron deficiency and 1 Hri−/− mouse on normal diet. The SDMS analyzed were from 2 Atf4−/− mice on iron deficiency diet, 2 eAA mice on iron deficiency diet, 2 eAA on normal diet, 1 Hri−/− on iron deficiency diet and 1 Hri−/− on normal diet.
The spleen from the Hri−/− mouse on iron-restricted diets generated significantly more SDMS in culture than the iron sufficient diet mouse (Fig.5). In addition, we were able to obtain more BMDMS and SDMS from eAA iron deficient mice compared to eAA iron sufficient mice, starting with the same number of nucleated cells and SDMS from Wt mice from normal diet were less than the macrophages from eAA in the same condition (Fig.6).

![Figure 5](image)

**Figure 5.** Pictures of SDMS cells after 10 days in culture. 10X10⁶ cells from the spleen were seeded in a 6 well plate. SDMS from Hri−/−Fe had more cells compared to Wt+Fe and Hri−/−Fe. Original magnification x20 (Evos microscope).

However, this is not the case for Atf4−/− in the iron deficient mice that we analyzed. In fact, there was much less BMDMS and SDMS taken from Atf4−/− mice (Fig.7). Flow cytometry analysis of the total bone marrow and spleen cell population showed that the 3 Atf4−/− mice analyzed didn’t have large expansions of erythroid cells and had a significantly higher percentage of myeloid lineage cells. None the less, I didn’t obtain many macrophages. These results suggested that ATF4 might be required for the production of myeloid precursors that may grow and differentiate into macrophages in vitro in the presence of M-CSF (Fig. 7). These findings regarding Atf4−/− macrophages can be considered early preliminary results and it is important to further investigate the role of ATF4 in macrophages.
Figure 6. Pictures of BMDMS and SDMS cells after 12 and 14 days in culture. SDMS from eAA-Fe had significantly more cells compared to eAA+Fe. SDMS from Wt-Fe are less than eAA-Fe. Similar results were found in 2 different experiments. The photos above show eAA cell cultures seeded 4X10⁶ for BM and 10X10⁶ for the spleen in a 6 well plate. Original magnification x20 (Evos microscope).

Figure 7. Cell density of BMDMS (BM) and SDMS (Spl). Because the Atf4⁻/⁻ mouse was very anemic 1X10⁶ bone marrow cells were seeded for each sample in a 12 well plate and 10X10⁶ spleen cells in a 6 well plate. Cultures were monitored daily. These pictures were taken on day 4. Original magnification x20 (Evos microscope).

Flow cytometry analysis of cultured macrophages from the spleen and bone marrow using macrophage surface markers F4/80, CD169, Cd11b and CD68 didn’t reveal any difference between Wt and Hri⁻/⁻, eAA and Atf4⁻/⁻ mutant mice at any time (Fig.8). To obtain enough macrophages for the flow cytometry analysis, cells were kept in culture for more than one week. This extended period in culture made it difficult to lift the cells from the plate without using mechanical methods. It was
interesting to note that BMDMS from Wt and eAA mice developed a similar elongated morphology in culture with M-CSF (Fig.9). Therefore, we further investigated these observations regarding cell growth by performing methylcellulose colony assays for macrophages in the presence of M-CSF.

Figure 8. Flow Cytometry analysis of BMDMS cells after 10 days in culture with M-CSF. No significant differences were detected between mutants and Wt mice. It was obtained a lower number of macrophages to analyse from the ATF4 culture. The same number of cells were seeded from both the mutant and Wt mice and the number was normalized depending of the cell availability. F4/80, CD169, CD11b are macrophage markers usually expressed in mature macrophages.
Figure 9. BMDMS from Wt+Fe, eAA+Fe and eAA-Fe. After 7 days in culture, cells were stained with CD11b (red) and DAPI (blue) to reveal the nuclei (right). On the left there are the corresponding bright-field pictures. Original magnification x40, Leica immunofluorescent microscope.

4.2.2 CFU-M colony assays of spleen and BM from Wt and mutant mice in iron restriction erythropoiesis

The mice analyzed for the colony assay consisted of wild type and mutant mice given a normal diet (+Fe) or iron deficient diet (-Fe): 5 Wt+ Fe, 5 Wt-Fe, 3 Hri+/+Fe, 3 Hri+-Fe, 2 Atf4+/+Fe, 2 Atf4+/-Fe and 4 Atf4-/-Fe. An assay using cells from eAA mice could not be performed because we did not have eAA cells available for analysis due to contaminated cells. We were not able to perform the assay with cells from Atf4-/- mice on a normal diet due to insufficient knockout mice to test both iron sufficient and deficient conditions. During the year spent in Chen’s lab we obtained only 7 Atf4-/- mice. These mice were usually much smaller than the other mutants and suffered from severe anemia. Very often we couldn’t collect enough cells to perform all the experiments.

After 10 days in culture, CFU-M from bone marrow didn’t show any significant difference between the Wt, Hri+/+ and Atf4+/+ mice both from normal and iron deficiency diets (Fig.10). CFU-M from spleen in iron deficiency diet of Hri+/+ and Atf4+/+ mice showed a higher number of colonies compared to the Wt, The Hri-/- mice fed an iron deficient diet also developed more macrophage precursors in the spleen compared to the Hri+/+ mice on a normal diet (Fig.10). It would be necessary to repeat the CFU-M colony assays using cells from Atf4+/+ mice placed on a normal diet and iron deficient diet before reaching any definitive conclusion about the role of ATF4 in the macrophage maturation, but my preliminary ATF4 results showed that we have a decrease of macrophage precursors compare to the Hri+/+ and eAA sample. It would be important to include eAA samples in colony assay to be able to
compare them with $Hri^{/-}$ mice which have an increased number of splenic macrophage precursors then the Wt, especially during iron restriction erythropoiesis.

Figure 10. Macrophage colonies (CFU-M) in Wt and mutant mice. It shows the number of colonies obtained / seeded cells. Samples used to obtain data for the graph: 3 mice from Wt on normal diet and iron deficient diet, 3 mice from $Hri^{/-}$ on normal and iron deficient diet, 2 mice from $Atf4^{/-}$ in iron deficiency.

4.3 Erythroblastic Island (EI) in iron deficit anemia

4.3.1 Isolation and study of EI morphology

My CFU-M results suggested that iron deficiency increased the presence of splenic macrophages, which may facilitate their interaction with erythroblasts leading to ineffective erythropoiesis. I investigated this hypothesis by modifying the protocol described by Crocker and Gordon (54). I used immunofluorescence assays to analyze the morphology of native EI. As described in the introduction, the central macrophage arises from a monocyte precursor and can be distinguished from other stromal cells in hematopoietic tissues by the high expression of F4/80 and V-cam markers, so these two antibodies were used to view the central macrophages and to detect the erythroblast, the Ter119 antibody was used.
To study the EIs I seeded the same number of nucleated cells (2x10^6 cells) on the top of glass coverslips and incubated them overnight with M-CSF, Epo and transferrin allowing time for the macrophages to adhere to the surface. During the long incubation, macrophages formed cell “clusters” of differentiated macrophages and erythroblasts (Fig.11 panel A). However, in the presence of M-CSF macrophages become more elongated and are different from the nursing macrophage in EI. Additionally, no classic native islands were observed and I didn’t see any differences among Wt and mutant samples which is consistent with the flow cytometry results of cell culture previously described.

We therefore sought to isolate native EI directly from BM and spleen of wt and mutant mice. Erythroblastic islands can be easily dissociated when bone marrow or spleen cells are dispersed for examination. To successfully isolate the native EI, we modified a protocol that was recently described by Toda et al. (65). As described in the method section, the cell clusters obtained from the sedimentation were counted, analyzed using flow cytometry and stained with Wright-Giemsa (Fig. 11 panel C). I was able to observe native EIs on coverslips. Macrophages were present at the center of the erythroblastic islands and several erythroblasts were associated with each macrophage (Fig.11 panel C and D). In figure 11, I show the May-Grunwald Giemsa and Wright-Giemsa stains (panel C and D) on the cell clusters and macrophages niches. The May-Grunwald Giemsa stained the nuclei of macrophages or white cells blue, with their cytoplasm in light blue in contrast with the pink-red erythroblasts (Fig.11 panel C). To stain the erythroblastic niche I used the Wright Giemsa coloration. The macrophage, with extended lamellipodia, turned light purple and it is in contrast with the erythroblast precursors that are very dark and the pink reticulocytes (Fig.11 panel D).

To better characterize cell nature and the niche composition in the EI, it was necessary to perform immunofluorescent microscopy study. In my experiments I wanted to investigate the erythroblastic niches from Hri^-/- and eAA mutant mice placed on a normal diet or iron deficiency. Under normal conditions, in absence of erythropoietic stress, it was difficult to isolate sufficient islands for my study. During Iron deficiency, Hri^-/- and eAA mice developed erythroid expansion with increasing numbers of erythroblastic islands.

EIs were collected at three different time points (90 minutes and 5 hours) to study the morphology and architecture of the native EI, and further incubation overnight with Epo and transferrin to follow the erythroblast differentiation in the islands. EIs from Hri^-/- mice, the experiments were repeated 3 times and for eAA mice 2 times. The slides stained with F4/80, V-cam and Ter119 were made in triplicate or duplicate depending on cell availability.
Figure 11. (A) May-Grunwald Giemsa stain of adherent macrophages from total bone marrow cells \((2 \times 10^6)\) from a Wt incubated overnight with 25ng/ml M-CSF on glass coverslip. The nuclei of cells appear in dark blue, whereas the cytoplasm in light blue. Original magnification x20. (B). Wright-Giemsa stain of erythroblast islands isolated from Wt mouse BM. I purified cell clusters by sedimentation protocol, seeded them on a coverslip \((0.25 \times 10^6\) cells) and incubated 90 minutes without M-CSF; dye stains the cytoplasm of cells a pink color and the nucleus varying shades of blue to purple. Original magnification x20. (C). May-Grunwald Giemsa stain of clusters by sedimentation protocol; central macrophages that interacts with erythroblasts and neutrophils. (D). Example of native erythroblastic island adherent to glass coverslip, Wright-Giemsa stain. Macrophage appears large and well adherent to the glass with a lot of cytoplasm inside (light purple colour) and it is in contrast with erythroblasts on the top which are rounded with a big centric nuclei. The 2 reticulocytes are pink. Original magnification x100.

4.3.2 Comparison of native EI from eAA and Hn\(^{-/-}\) mice in iron restriction erythropoiesis

There was no difference between bone marrow EIs collected at 90 minutes and those collected at 5 hours and I also noticed that a longer incubation time was necessary to isolate EIs from the spleen. So, I focused on the results after 90 minutes for BM and overnight intervals for both spleen and BM. Native EIs isolated from bone marrow and spleen of Hn\(^{-/-}\) mice appeared smaller and numerically inferior compared to the Wt (Fig.12).
Figure 12. Wright-Giemsa staining of bone marrow native erythroblast islands from iron deficiency. EIs were collected after 90 minutes of incubation. 0.25×10⁶ cells were seeded on the coverslips and slides were made in duplicate both for Hri⁻/⁻ and Wt. Hri⁻/⁻ islands are less numerous in abundance and smaller than the Wt. Original magnification x20

Analysis of EI isolated from BM of Hri⁻/⁻ and eAA mice revealed considerable differences. As expected, eAA islands have normal macrophages since the eAA mouse model is eIF2αP deficient only in erythroid lineage. Most importantly, the EIs from eAA were larger and more robust than islands isolated from Hri⁻/⁻ mice, which had defective macrophages (Fig.13).

Figure 13. Wright-Giemsa staining of bone marrow native erythroblast islands from iron deficiency after 90 minutes incubation. Macrophages are under the erythroblasts but we can see their cytoplasmic extensions in pink-red. These in vitro EIs can contain more than 1 macrophage per island. The macrophages of the islands from Hri⁻/⁻ mice seem incapable of spreading and forming bigger islands. Original magnification x100.
The immunofluorescence staining of EIs further substantiate the above results from the Giemsa staining (Fig.14). The EIs from the Wt and eAA sample have multiple erythroblasts (green) in different stages of differentiation (varying cell sizes) that interact with many macrophages. However, clusters of cells isolated from Hri\textsuperscript{−/−} mice were different. Macrophages were not surrounded with many erythroblasts, and seem less differentiated (Fig. 14 blue arrows). The Hri\textsuperscript{−/−} islands displayed a decrease in F4/80 and V-cam expression compared to the eAA sample (Fig.14).

I noticed that native EIs can be formed by more than 1 central macrophage. In fact, in figure 13, we can see that some islands are formed with multiple macrophages (purple-red cells) which interact with each other and with the erythroblasts (green).

![Native EI from Bone Marrow](image)

**Figure 14.** Native erythroblast islands from bone marrow of Wt, Hri\textsuperscript{−/−} and eAA mice in iron deficiency collected after 90 minutes of incubation. Erythroblastic islands were stained with DAPI (blue) for the nucleus, Alexa-conjugated Ter-119 antigen (green) for the erythroblasts, F4/80 antigen (purple) and V-cam antigen (red) for the macrophages. Blue arrows point at early erythroid precursors (Ter 119 positive cells with a large nucleus). Original magnification x40. Leica immunofluorescence microscope.
Significant differences in Wt, Hri\textsuperscript{\textdagger} and eAA erythroblastic islands were also observed after incubation overnight. The Hri\textsuperscript{\textdagger} island’s architecture was visibly impaired because macrophages didn’t seem to be well differentiated and the macrophages that were “alone” displayed weakly expressed F4/80 and V-cam (MØ and bone marrow panel Fig.15). In Wt EI, overnight incubation resulted in erythroblast differentiation into reticulocytes (smaller green cells without blue DAPI stain) and the engulfment of pyrenocytes (small DAPI stained nuclei) by central macrophages in the spleen samples (Fig.15 panel Wt spleen). In the Hri\textsuperscript{\textdagger} and eAA samples, fewer reticulocytes were observed consistent with the inhibition of erythroid differentiation observed in the spleen of these mice, suggesting defects in erythroblasts for erythroid differentiation in the case of eAA EIs. In contrast, macrophages from the Hri\textsuperscript{\textdagger} spleen also expressed less markers as compared to the Wt and eAA samples and were not able to interact with erythroblasts. In summary, EI macrophages from spleen and bone marrow of Hri\textsuperscript{\textdagger} mice couldn’t interact with erythroblasts (Fig.14 panel Hri\textsuperscript{\textdagger} spleen and BM).
Figure 15. Erythroblastic islands. The upper panel shows native EI from bone marrow of mice on iron deficiency diet. The lower panel shows native EI from mice spleen on iron deficiency diet. Red arrows point to the reticulocytes which are small and irregular cells, both extremely Ter119 positive and dapi negative. The blue arrow points to pyrenocytes inside spleen macrophages that have a small nucleus. Coverslips were collected after overnight incubation. Original magnification x40. Leica immunofluorescence microscope.
4.3.3 Reconstitution of EI using BM macrophages and erythroblasts from Hri⁻/⁻ mice spleen.

To extend our findings, I attempted to reconstitute erythroblastic islands by using macrophages from BM of Wt and Hri⁻/⁻ mice and erythroblasts from spleen of Hri⁻/⁻ mice as described in the methods. Cell clusters were isolated from bone marrow of Wt mouse fed an iron deficient diet and from Hri⁻/⁻ mice kept on normal and iron deficient diets. Of note, adherent island macrophages stripped from the erythroblasts did not survive. No viable cells remained on the coverslips following incubation overnight. Iron-deficient Wt macrophages formed well-developed EIs with Hri⁻/⁻ erythroblasts but I didn’t observe any EIs on the coverslips with macrophages from Hri⁻/⁻ mice (Fig.16) and It is most likely that erythroblasts which didn’t interact with macrophages stayed in the supernatant. In fact when I analyzed Hri⁻/⁻ samples, most of the coverslips of iron deficient macrophages had very few macrophages still adherent. Furthermore, the Hri⁻/⁻ macrophage phenotype is more severe in an iron deficient condition when HRI is needed and should be active. The result from this experiment further clarifies that macrophages which lack HRI are less differentiated than the Wt macrophages and can’t form EI. Furthermore the phenotype aggravates for the  Hri⁻/⁻ macrophages in an iron deficient condition.

Figure 16. Reconstituted erythroblastic islands, macrophages (purple and red) were isolated from bone marrow and erythroblasts (green) were purified from the spleen of Hri⁻/⁻ mice on iron deficient diet: 0.5x10⁶ cell clusters were seeded on the coverslip, then stripped and reconstituted with 0.25x10⁶ erythroblasts. Original magnification x20. Leica immunofluorescence microscope.
4.4 Iron status in spleen sections of eAA mice

Previous studies demonstrated the involvement of HRI in iron homeostasis (37). Using a commercial Iron kit that stained deposits of non-heme iron, I examined the iron levels in cross sections of gut, bone marrow, liver, kidney and spleen from Wt and eAA mice. Tissue stained an intense blue when treated with acid ferrocyanide according to the Prussian blue reaction. Tissue sections from iron deficient mice didn’t have visible blue staining after approximately 2 months on an iron deficient diet since iron was completely utilized (Fig.17 panel A). Bone marrow and spleen from mice kept on a normal diet displayed iron deposits and the slides from eAA mice spleen showed more staining when compared to the Wt spleen samples (Fig.17 panel A). Non-heme iron in the spleen is mainly localized in the macrophages which were observed with larger cytoplasms than the erythroblasts (Fig.15 panel B). The iron deposits are distinguishable in diffused scattered granules or clumps in the cytoplasm. Previous studies from the Chen lab showed that the splenic iron was significantly reduced in $Hri^{-/-}$ mice (37). It would be interesting to further investigate why eAA spleen sections seem to have more iron compared to the Wt samples. Macrophages from eAA mice are supposed to be functional compared to the $Hri^{-/-}$ macrophages, which lack an HRI signalling pathway. My histology data are just a starting point and we need to further investigate the differences between $Hri^{-/-}$ and eAA mice by analyzing more samples.
Figure 17. Iron staining of spleen tissue sections. (A) From normal diet (+Fe) eAA has more iron staining than the Wt. The sample from iron deficiency (-Fe) didn’t show iron deposit. (B) enlarged image of eAA section. The red circles highlight macrophages, many of them are positive for iron. The green arrow points to an erythroblast that is rounded and dark pink, while the black arrow points to iron deposit granules.
5. DISCUSSION

HRI was first identified as an inhibitor of protein synthesis in reticulocytes in 1960. The Chen lab uncovered the physiological function of HRI by analyzing the phenotype of mice with a targeted disruption of the HRI gene (19). It has been demonstrated that HRI has an essential protective role in anemia to reduce ineffective erythropoiesis during iron/heme deficiency as well as in β-thalassemia. Furthermore, previous findings showed that HRI is also involved in the maturation of macrophages (40).

Macrophages are present in different tissues, including hematopoietic and lymphoid organs and they have always been considered a key component in physiological iron metabolism and immune response against infection. During the last 50 years, many studies have been conducted that intended to elucidate the role of macrophages in erythropoiesis and their relationship with erythroblasts precursors in the definitive hematopoietic sites, particularly in distinct units called erythroblastic islands. Although erythroblastic islands have been well characterized morphologically, the complete functional roles of macrophage-erythroblast interactions and the central macrophages, especially under stress condition, remain poorly understood. The model mouse eAA used in this project, during iron deficiency stress, has a functional HRI but completely lacks eIF2αP signalling pathway in the erythroid lineage. The comparison between the well described Hri−/− mouse model that is in deficit of HRI in all tissues and the eAA new mouse made it possible to better understand the impact of the HRI pathway not only in erythroid cells but also in the macrophages. In this thesis I have shown that there were more macrophages from the spleen of eAA and Hri−/− mice under iron deficit stress conditions than under normal conditions and the colony assays showed that cells from Hri−/− mice on an iron deficient diet were able to form more colonies than the Wt. The mechanism behind these findings is still unclear. However it is consistent with the findings that Hri−/− and eAA mice have developed ineffective erythropoiesis and more severe anemic conditions in iron deficiency compared to Wt mice. Since macrophages are important for erythroid maturation, it is interesting to speculate how impaired or ineffective erythropoiesis might result from an enrichment of macrophage precursors in the myeloid lineage of the spleen in an attempt to help produce more RBCs. A recent study reported that macrophages might be responsible for magnifying ineffective erythropoiesis in vivo in a mouse model of β-thalassemia (72). We still don’t know if increased macrophage precursors can aggravate the anemic condition of Hri−/− and eAA mice models and it would be necessary to perform more experiments to further investigate this hypothesis. It would be interesting to see if Hri−/− mice depleted of macrophages may actually have an improvement in their anemic condition under iron deficiency.

This study shed light on the role of HRI signaling during stress erythropoiesis in macrophages forming EI. Erythroblastic islands from Hri−/− mice showed impaired macrophages if compared to the EI from
eAA and Wt mice. We obtained less EI from Hri\textsuperscript{−/−} bone marrow and spleen and the macrophages were smaller with the cell extremities less expanded. Conversely, erythroblastic island macrophages from eAA mice, which do not carry any genetic deletions, were well differentiated and they formed large EI with many erythroblast precursors. This finding can reinforce the supportive role of HRI in macrophage maturation in the subset of macrophages that are involved in the formation of erythroblastic islands. The hypothesis that HRI supports macrophage maturation was validated with the reconstituted islands. Wt macrophages had well-formed islands with HRI defective erythroblast, suggesting that the EIs impairment noticed in Hri\textsuperscript{−/−} EIs might come exclusively from macrophages that can't interact with erythroblasts. It was also interesting to see that the defection of HRI might cause the reduction of EIs in vitro and this would be an interesting aspect to focus on for further studies in vivo. I was only able to perform the experiment twice and we would need to substantiate these results by adding eAA samples to determine if we can obtain EIs as seen in the native EI.

In the literature it is still not clear how many membrane proteins are involved in the interactions between central macrophage and erythroblasts but V-cam was recently described as an important membrane protein on the macrophage that binds integrin-α4β1 expressed on the erythroblast (65). Central macrophages that lack HRI seem to express less F4/80 and V-cam markers. This aspect may help to better understand why macrophages from Hri\textsuperscript{−/−} don't interact with erythroblasts as well as eAA macrophages.

The aim of this thesis was to elucidate if EI macrophages with a defective HRI signaling pathway may be directly involved in the block of differentiation in our transgenic mouse models. Even if Hri\textsuperscript{−/−} mice clearly carry macrophage alteration in the EIs that can’t be seen in eAA mice, it is hard to say if it has a negative impact on the development of the anemia. In fact, both mice share similar severe anemia under iron deficiency stress, so further investigation should be done to understand how much impact the HRI deficit in macrophages, which leads to impaired EIs, can have on the erythropoiesis.
6. WORK IN PROGRESS AND FUTURE PERSPECTIVES

The biological significance of erythroid islands has been actively under investigation recently. However, further work is necessary to clarify the true relevance of this system in normal and pathological hematopoiesis. The well-documented ability of erythroid precursors to complete terminal differentiation in vitro in the presence of Epo but without other accessory cells demonstrates that an autonomous differentiation program exists in erythroblast committed progenitors (49). However, in vitro data from different studies has shown that it is not possible to reproduce the cell numbers at the same proliferation and differentiation as we have in vivo (49), which means that erythroblastic niches are probably necessary to help or increase the speed of erythroid maturation and maintain the correct quantity of RBCs. This balance is altered during ineffective erythropoiesis.

The results within this thesis represent an initial study of a potential role of the HRI pathway in the proliferation and differentiation of macrophage subset forming erythroblast islands. Due to time constraints I was not able to study in my thesis other important aspects that would be interesting to investigate to better understand the role of erythroblastic niches in anemia. One important aspect that should be evaluated is the relevance of the HRI pathway in the erythroblastic niches of the β-Thalassemic mouse model. Previous results from the Chen lab showed the protective role of HRI in the β-thalassemic mice model against ineffective erythropoiesis (32). It would be interesting to compare the Els of Hbb$^{-/}$ mice to Els from Hri$^{-/-}$ mice under an iron restricted state and to also develop ineffective erythropoiesis in the Hri$^{-/-}$ mouse and study the morphology of central macrophages in both models. Hbb$^{-/}$ mice should not carry any mutations that impair macrophages, but I would expect to see more islands than the Hri$^{-/-}$ mouse with a severe accumulation of abnormal erythroblasts.
7. BIBLIOGRAPHY


8. ABSTRACT AND PUBLICATIONS

Poster:
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