MicroRNA Function in Cellular Stress Response

by

Carolyn Sangokoya

University Program in Genetics and Genomics
Duke University

Date:_____

Approved:

_____
Jen-Tsan Ashley Chi, Supervisor

_____
Jack Keene

_____
Salvatore V. Pizzo

_____
Bruce Sullenger

_____
Marilyn Telen

Dissertation submitted in partial fulfillment of
the requirements for the degree
of Doctor of Philosophy in
the University Program in Genetics and Genomics
in the Graduate School
of Duke University

2011

ABSTRACT

MicroRNA Function in Cellular Stress Response

by

Carolyn Sangokoya

University Program in Genetics and Genomics
Duke University

Date:_____

Approved:

_____
Jen-Tsan Ashley Chi, Supervisor

_____
Jack Keene

_____
Salvatore V. Pizzo

_____
Bruce Sullenger

_____
Marilyn Telen

An abstract of a dissertation submitted in partial
fulfillment of the requirements for the degree
of Doctor of Philosophy in
the University Program in Genetics and Genomics
in the Graduate School
of Duke University

2011

Abstract

MicroRNAs are key post-transcriptional regulators that have been found to play critical roles in the regulation of cellular functions. There is an emerging concept that microRNAs may be just as essential for fine-tuning physiological functions and responding to changing environments and stress conditions as for viability or development. In this dissertation, two studies are presented: The first study demonstrates a role for microRNA in the regulation of oxidative stress response in erythroid cells and the functional consequences of dysregulated microRNA expression in Sickle Cell Disease (SCD) pathobiology. The second study examines a functional role for microRNA in the cellular response to changes in cellular iron concentration. Together these studies illustrate the scope of importance of microRNAs in the coordination of cellular responses to diverse stresses.

Homozygous Sickle Cell (HbSS) erythrocytes are known to have reduced tolerance for oxidative stress, yet the basis for this phenotype has remained unknown. Here we use erythrocyte microRNA expression profiles to identify a subset of HbSS patients with higher miR-144 expression and more severe anemia. We reveal that in K562 erythroid cells and primary erythroid progenitor cells, miR-144 directly regulates NRF2, a central regulator of cellular response to oxidative stress, and modulates the oxidative stress response. We further demonstrate that increased miR-144 is associated with the reduced NRF2 levels, decreased glutathione regeneration, and attenuated antioxidant capacity found in HbSS erythroid progenitors, thereby providing a mechanism for the reduced oxidative stress tolerance and increased anemia severity seen in HbSS patients.

Iron is an essential nutrient that is necessary for all cells. The maintenance of cellular iron homeostasis involves RNA-binding proteins (RBPs) in the post-transcriptional coordination

of iron uptake, utilization, and storage in order to prevent toxicity to the cell while ensuring availability when appropriate. The post-transcriptional regulation of the IRP1/2 (iron regulatory protein) regulon in the cellular response to iron deficiency is well characterized. Here we identify iron-dependent microRNA expression and examine the potential role for microRNA-mediated regulation in the coordinated response to cellular iron deficiency.

Dedication

For my parents, Samuel and Helen Sangokoya

For my brothers, Fred, Phillip, and David

that we will continue to ‘bend in strange winds,

to respond to the warmth of other suns,

and, perhaps, to bloom.’

Thy hand doth still my table spread,

May all my work be praise.

Contents

List of Tables

List of Figures

List of Abbreviations

3'UTR	3'-untranslated region
5'UTR	5'-untranslated region
Ago2	Argonaute 2
ALAS2	erythroid-specific 6-aminolevulinate synthase
ARE	antioxidant response element
CAT	catalase
CLIP	cross-linking and immunoprecipitation
DFE	desferrioxamine
DNA	deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	erythropoietin
FAC	ferric ammonium citrate
FACS	fluorescence-activated cell sorting
FBXL5	F-box and leucine-rich repeat protein 5
FPN	ferroportin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCLC	glutamate-cysteine ligase, catalytic subunit
GCLM	glutamate-cysteine ligase, modifier subunit
GCS	glutamate-cysteine ligase
GFP	green fluorescent protein
GPX1	glutathione peroxidase 1
GSH	glutathione
GYPA	glycophorin A
HbAA	homozygous hemoglobin A
HbF	fetal hemoglobin
HbSS	homozygous hemoglobin S
HEK293	human embryonic kidney-293 cell line
HEL	human erythroleukemia cell line
HepG2	human hepatocellular cell line

hsa-miR-	human microRNA
IRE	iron regulatory element/ iron response element
IRP	iron regulatory protein
K562	human erythroleukemia cell line
MCS	multiple cloning site
NQO1	NAD(P)H dehydrogenase, quinone 1
NRF2	nuclear factor (erythroid-derived 2)-like 2
PAM	Prediction Analysis for Microarray
PBS	phosphate-buffered saline
qRT-PCR	quantitative real-time polymerase chain reaction
RBP	RNA-binding protein
RIP	RNA immunoprecipitation
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
ROS	reactive oxygen species
SCD	Sickle Cell Disease
SCF	stem cell factor
SEM	standard error of the mean
siRNA	Short-interfering RNA
SOD	superoxide dismutase
TFRC	transferrin receptor
TLDA	Taqman Low Density Array

Acknowledgments

I would like to thank my advisor Dr. Chi for his mentorship, for providing a supportive research environment, and for his patience in allowing me some autonomy before seeking guidance. I have found myself in the right place at the right time. I am grateful for my committee members: Drs. Jack Keene, Bruce Sullenger, Marilyn Telen, and Salvatore Pizzo for their guidance, intellectual contributions, and encouragement. I would like to thank Dr. Telen, without whom a great deal of this work would not have been possible, for her helpful discussions and ability to help make things happen. I would especially like to thank Dr. Pizzo, whose leadership of the MSTP along with the enthusiasm of Dr. Timothy A. Fields brought me to Duke. My formative years in the program were colored warmly by candid conversations over lunch. I would like to thank Dr. Nancy Andrews for her time, interest, and feedback on my side project. Additionally I am grateful to Drs. Mardee Delahunty of the Telen Lab, Tomasa Barrientos De Renshaw and Jackie Lim of the Andrews Lab, and Nisha Philip formerly of the Chi Lab for their help with protocols, reagents, materials, and feedback. The post-docs and scientists in the Nevins lab past and present: among them Drs. Guang Yao and Janet Leung, have been encouraging and knowledgeable lab neighbors. I would like to thank fellow MSTP students who have gone before me, especially Drs. Gabriel Howles, Karen Winkfield Walker, Quentin Quinones, Kafui Dzirasa, Gustaaf DeRidder, Erin Wilfong, and Marisa Buchakjian for their examples, advice, encouragement, and camaraderie. I am fortunate for the lunchtime think-tank discussions with Greg LaMonte, Taylor Herbert, Jeff Mito, and Jonathan Kotula, for computational advice from Stoyan Georgiev, and for daily career advice from Dr. David Hsu. I am indebted to my parents, Samuel and Helen Sangokoya, for their vision, sacrifice, and courage, which allowed me to grow up in this country, and for their dedication over these many years in support of my education.

1. MicroRNAs: Biogenesis and Mechanism of Post-transcriptional Gene Regulation

MicroRNAs play critical roles in coordination of a variety of complex biological processes, including proliferation, metabolism, stress response, and differentiation. There is evidence that this novel layer of gene regulation plays an integral role in the pathophysiology of human disease from diabetes (Poy, Eliasson et al. 2004; Kolfschoten, Roggli et al. 2009; Regazzi 2010) to cardiovascular disease (Srivastava 2006; Ji, Cheng et al. 2007; Yang, Lin et al. 2007; Naga Prasad, Duan et al. 2009; Small and Olson 2011) to neurogeneration (Bushati and Cohen 2008), to cancer (Calin, Dumitru et al. 2002; Calin, Ferracin et al. 2005; Garzon, Fabbri et al. 2006; Croce 2009; Garzon, Calin et al. 2009). MicroRNAs are 21- to 25-nucleotide small regulatory RNAs that regulate protein synthesis by binding to complementary sites in the 3' untranslated region (3' UTR) of target mRNAs and driving translational repression and/or mRNA degradation (Ambros 2004; Bartel 2004). In mammalian cells, biogenesis of microRNAs begins with transcription by RNA polymerase II or III and expression as an approximately 80 nucleotide RNA hairpin that is part of a larger 'pri-miRNA' primary transcript (Lee, Kim et al. 2004). The upper part of this RNA hairpin is excised by an RNase III enzyme called Drosha to produce an approximately 65 nucleotide intermediate 'pre-microRNA' (Lee, Jeon et al. 2002; Zeng and Cullen 2003). The pre-microRNA is then bound by the nuclear export factor Exportin5 and transported to the cytoplasm, where the RNase III enzyme Dicer removes the terminal loop of the pre-miRNA to generate a 20 base pair RNA duplex. Finally, mature microRNA is formed from one strand of this duplex and is incorporated into the RNA-induced silencing complex (RISC), where it functions with the RNA-binding protein Argonaute 2 (Ago2) to guide RISC to complementary mRNA targets and effect translational repression and/or mRNA degradation (Schwarz, Hutvágner et al. 2003; Gregory, Chendrimada et al. 2005; Diederichs and Haber 2007).

When bound to RNA, RNA-binding proteins (RBPs) can function to promote or inhibit target availability and binding, leading to the enhancement or inhibition of mRNA stability and translation. The cooperative contribution of different RBPs, including the microRNA-guided RISC, to the functional outcome of stability or translation of target RNAs constitutes a major regulatory layer of gene expression (Keene 2007; Tenenbaum, Christiansen et al. 2011). Dynamic cellular responses to stress and other environmental perturbations can be coordinately regulated at the post-transcriptional level by the RISC and other RBPs. Several examples involving RBP regulation and the relief of microRNA-mediated repression have been reported (Bhattacharyya, Habermacher et al. 2006; Kedde, Strasser et al. 2007; Vasudevan and Steitz 2007). Chapters 7-8 of this dissertation will examine the potential role for microRNA-mediated regulation in cooperation with RBPs within a well-characterized model: the post-transcriptional regulation of mammalian cellular iron homeostasis.

2. MicroRNAs in Erythroid Biology

2.1 MicroRNA Expression and Function in Erythroid Cells, Erythrocytes, and Erythroid Disorders

MicroRNAs have been found to be expressed in erythroid cells, with changes in expression during erythroid differentiation and maturation (Bruchova, Yoon et al. 2007; Masaki, Ohtsuka et al. 2007; Zhan, Miller et al. 2007). Further findings have shown that overall microRNA expression levels are increased throughout erythroid differentiation (Zhan, Miller et al. 2007). Argonaute-2 , a key component of the RISC complex, is a key regulator of microRNA homeostasis in erythroid cells (O'Carroll, Mecklenbrauker et al. 2007) and loss of Argonaute-mediated control of microRNA biogenesis and subsequent altered expression of microRNA can play a major role in the development of erythropoietic defects, including severe anemia and ineffective erythropoiesis.

Of the microRNAs expressed during human erythropoiesis, miR-451 is the most significantly upregulated during late stages of erythroid maturation and miR-144 has also been found to be significantly increased at this developmental stage (Zhan, Miller et al. 2007). The sequences encoding human miR-451 and miR-144 are located on human chromosome 17 (Griffiths-Jones, Saini et al. 2008) and transcribed as one highly conserved transcriptional unit (Altuvia, Landgraf et al. 2005). The genomic arrangement of these two microRNAs is highly conserved across species (Nelson, De Planell-Saguer et al. 2007), and their expressions have been found to be erythroid-specific (Rathjen, Nicol et al. 2006; Chen, Wang et al. 2008; Merkerova, Belickova et al. 2008). Although the miR-144/451 locus has been identified a target of GATA-1 (Dore, Amigo et al. 2008), an important transcription factor in erythropoiesis, little is known regarding the function of these erythroid-specific microRNAs.

MicroRNAs have been profiled in erythroid disorders such as Polycythemia Vera (Bruchova, Yoon et al. 2007; Bruchova, Merkerova et al. 2008), Myelodysplastic Syndrome (Bousquet, Quelen et al. 2008), and homozygous Sickle Cell Disease (HbSS) (Chen, Wang et al. 2008), yet the functional roles that microRNAs play in these and other erythroid disorders have yet to be elucidated.

2.2 Erythrocyte Biology and Sickle Cell Disease

The structural, biochemical, and physiological features of erythrocytes have been studied extensively. The mature red blood cell has a limited lifespan of 120 days and is the product of a complex process of differentiation and maturation in the bone marrow, followed by enucleation and circulation in the peripheral system as an immature erythrocyte (reticulocyte) undergoing terminal differentiation to the mature form. Once mature, the cell is devoid of a nucleus and organelles, including mitochondria for metabolism, ribosomes for translational capability, and a nucleus to direct regenerative processes. The mature erythrocyte contains the share of enzymes, phospholipids, and cytoskeletal proteins expected to last throughout its lifespan (Hoffman 2005).

These features make the circulating human red blood cell a remarkably durable cell that is set in an exceedingly hostile environment, where it encounters mechanical (shear, hydrostatic) and biochemical (oxidative, osmotic) stresses as well as rapid changes in oxygen pressure as is necessary for its role in oxygen delivery and gaseous waste disposal in the body. Unique and efficiently adaptive membrane structures and antioxidant defense system allow the cell to persist in the circulation and maintain its cytoplasmic contents in the soluble and nonoxidized state which allows for its normal function. Deficiencies in these systems contribute to the shortened lifespan of the cell seen in human anemia disorders (Hoffman 2005).

Sickle Cell Anemia is a hemolytic anemia of autosomal recessive inheritance resulting from a single gene amino acid substitution in the beta-globin gene of the hemoglobin molecule

that renders the cell susceptible to the formation an insoluble 'sickle' shape in the deoxygenated state and subsequent intracellular polymerization (Neel 1949; Pauling and Itano 1949; Ingram 1959). Individuals homozygous for the sickle mutation have the most common and most severe form of Sickle Cell Disease (SCD), and the sequellae of this disorder are chronic and lifelong, including painful episodic vaso-occlusive events, acute chest syndrome, splenic sequestration, aplastic crises, stroke, and chronic end-organ deterioration, together contributing to the overall shortened life expectancy in affected men and women (Platt, Brambilla et al. 1994).

Although Sickle Cell Anemia results from a single gene mutation, both the clinical manifestations and severity of the disease are remarkably heterogeneous. SCD patients experience variable clinical courses in terms of incidence of painful events, severity of anemia, incidence of acute complications, and frequency of end-organ damage (Castro, Brambilla et al. 1994; Gill, Sleeper et al. 1995; Vichinsky, Styles et al. 1997).

2.3 Erythrocyte MicroRNA Expression Profiling in the Study of SCD Heterogeneity

Current approaches to study of the molecular basis for phenotypic heterogeneity in SCD use candidate genes, quantitative trait loci, and genome-wide association studies that identify DNA-based genetic variants associating with particular phenotypes (Sebastiani, Solovieff et al. 2010; Solovieff, Milton et al. 2010). These approaches require large sample sizes in order to detect significant associations. A novel approach developed in this laboratory employs the use of erythrocyte microRNA expression profiles (Sangokoya, LaMonte et al. 2010). This method uses easily accessible peripheral blood and has been shown to demonstrate abundant, diverse, and disease-specific microRNA expression profiles in a pilot study where samples from normal erythrocytes or from anemia disorders grouped into their respective types simply based on microRNA expression (Figure 1). Since erythrocytes do not contain DNA or larger RNAs, the

RNA extracted from these cells is enriched for microRNAs, thus only a small amount is needed for robust analysis. Erythrocytic microRNA expression can give insight into the total molecular picture of the life of the red blood cell and further illustrate temporal, developmental, stress-responsive, and otherwise functionally meaningful relationships. The use of erythrocytic microRNA expression profiles is thus a tool which can be used to further identify novel disease modifiers and potential therapeutic targets.

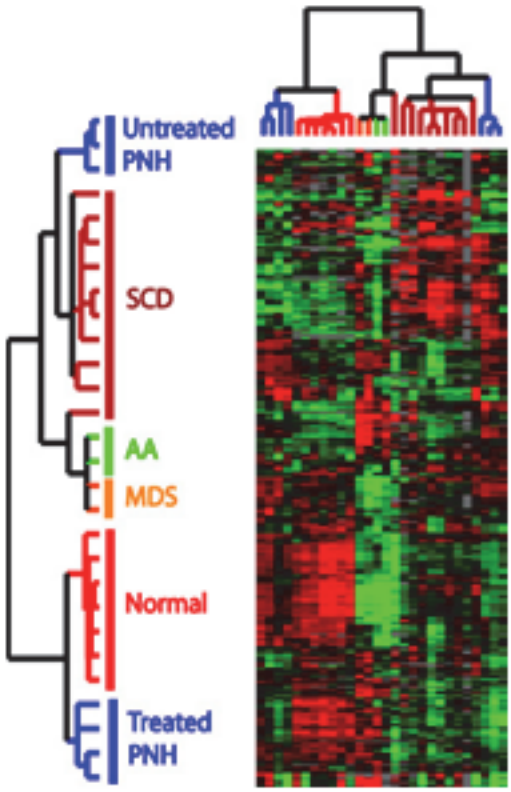


Figure 1: Erythrocyte microRNA expression profile: unsupervised analysis separates normal erythrocytes from erythrocytes of anemia disorders

Heatmap representation of microRNA expression analysis from the purified erythrocytes from patients with Paroxysmal Nocturnal Hemoglobinuria (PNH)(n=7), Sickle Cell Disease (SCD) (n=9), Aplastic Anemia (AA) (n=2), Myelodysplastic Syndrome(MDS) (n=2), and normal (n=7) samples.

3. Association of MicroRNA-144 expression with a severe anemia phenotype in SCD

3.1 Identification of SCD Heterogeneity based on Erythrocyte MicroRNA Expression

Since global analysis of gene expression has uncovered novel subtypes of cancers, (Alizadeh, Eisen et al. 2000; Perou, Sorlie et al. 2000) it was reasoned that similar analysis of erythrocyte microRNA might also identify clinically relevant subtypes within erythrocyte disorders. To test this possibility, we performed an unsupervised analysis of 12 HbSS individuals based on a hierarchical clustering of their microRNA expression. Two distinct groups of HbSS patients were readily identified based on the expression of the 141 microRNAs with greater than 1.6 fold variation in at least two samples (Figure 2A). Four HbSS samples consistently grouped into one branch (HbSS group I), while the remaining eight samples grouped into a separate branch (HbSS group II).

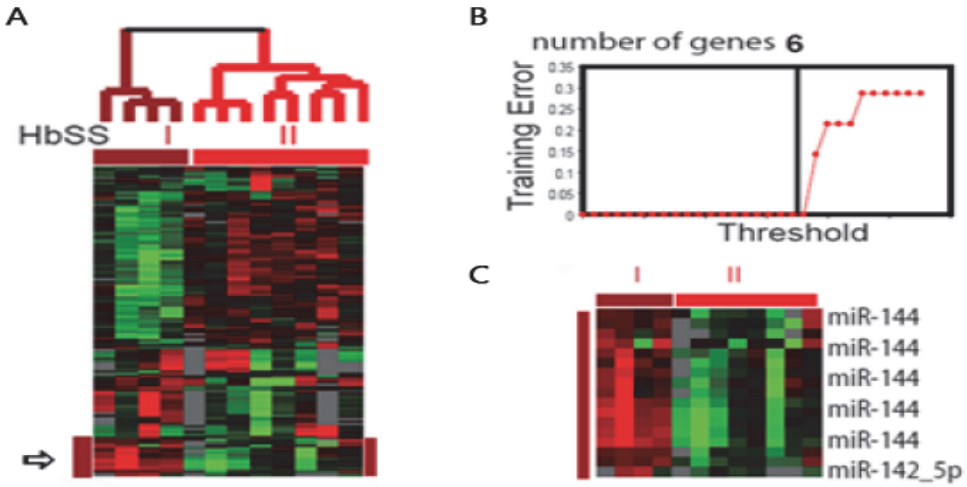


Figure 2: Erythrocyte microRNA expression identifies SCD subtypes

(A) HbSS patient samples separate into 2 groups (HbSS group I: brown; HbSS group II: red) based on unsupervised analysis of the erythrocyte microRNA expression pattern. (B,C) PAM analysis selects a subset of 6 microRNAs whose expression alone will classify each sample into the correct subtype.

3.2 Severely Anemic Clinical Phenotype among SCD subtype characterized by high MicroRNA-144 Expression

To identify the microRNA probes required to accurately separate these two HbSS groups based on erythrocyte microRNA expression, we used Prediction Analysis of Microarray (PAM) to prioritize the differentially expressed microRNAs from array analysis (Tibshirani, Hastie et al. 2002) PAM is a class prediction tool based on the shrunken centroids of gene expression, which computes a standardized centroid for each class and selects the number of genes required to characterize each assigned class with a defined error rate. PAM analysis showed that only 6 probes were needed to achieve 100% accuracy of class prediction (Figure 2B). The top five probes selected by PAM were all miR-144 homologues from different species with identical sequences (Figure 2C). Since miR-144 is not reticulocyte-specific (Chen, Wang et al. 2008), it was concluded that these two HbSS groups were not identified based on reticulocyte contamination. Additionally, due to the fact that the miR-*144/451* locus is co-transcribed and known to be essential for erythroid homeostasis, we also noted the expression of miR-451 in our erythrocytic microRNA microarray dataset. We found that while miR-451 was also expressed at a significantly higher level in the erythrocytes of group I (high miR-144, more severely anemic) HbSS patients ($p=0.013$), the ability of miR-451 as a predictor to accurately separate the two HbSS groups was not as significant as using miR-144.

In a separate dataset , the significant enrichment of miR-144 expression in HbSS (compared with HbAA) erythrocytes as well as the heterogeneity of miR-144 expression among HbSS patients was confirmed using quantitative real-time PCR (Figure 3, Table 1). Interestingly, the HbSS 'low miR-144' group was not significantly different from HbAA ($p=0.95$) in miR-144 expression, while the HbSS 'high miR-144' group showed significantly higher ($p=0.002$) miR-144 expression compared to HbAA.

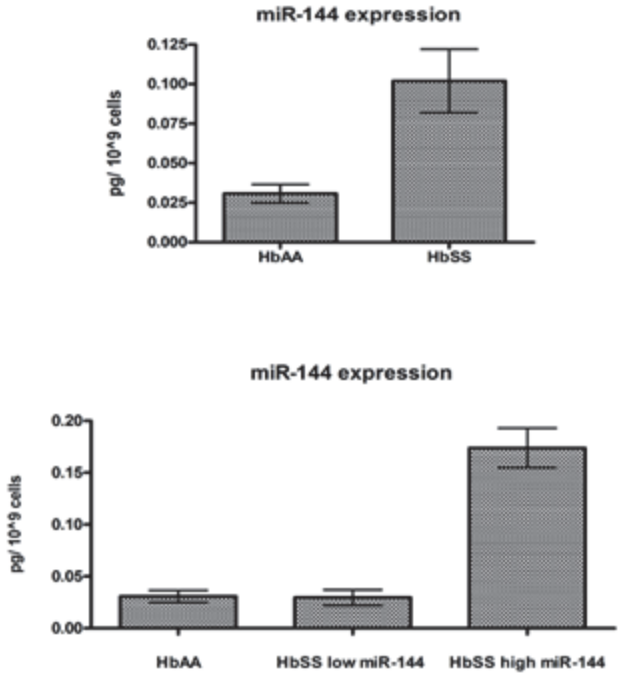


Figure 3: Erythrocytic MicroRNA miR-144 demonstrates HbSS heterogeneity

(top) MiR-144 expression in HbSS (n=18) vs HbAA (n=3) using quantitative Real-time PCR. **(bottom)** The HbSS samples in this dataset were separated into 'low miR-144' (n=9) and 'high miR-144' (n=9) groups and analyzed in comparison with HbAA. Clinical data is shown in Table 1.

To evaluate the clinical relevance of these HbSS subtypes, we compared the clinical profiles of the patients in these two groups. When compared with group II, group I patients had significantly lower hematocrit (Hct) (22% vs. 28%, p=0.04) and hemoglobin (Hb) level (7.77 vs. 9.92, p=0.04). Group I patients also had a higher reticulocyte percentage and absolute reticulocyte count (Table 2). Taken together, these results indicated that group I patients exhibited a more severely anemic phenotype than group II patients, with lower Hb and Hct levels and a higher degree of reticulocytosis. On the other hand, these two groups did not vary significantly in terms of their gender, age, HbF level or other clinical parameters (Table 2).

High serum lactate dehydrogenase (LDH) has been identified as a biomarker for a hemolytic subtype of SCD (Kato, McGowan et al. 2006). To determine if our HbSS groups, based purely on erythrocyte microRNA expression patterns, corresponded to this reported subtype, we compared serum LDH and bilirubin but found no significant differences between the two groups.

3.3 Materials and Methods

Procedures for blood collection, microRNA isolation and purification from mature human erythrocytes are as described in (Sangokoya, LaMonte et al. 2010).

3.4 Discussion

The heterogeneity of miR-144 expression among HbSS patients demonstrated one group of HbSS erythrocyte samples with significantly higher miR-144 expression levels and one group with miR-144 expression levels that were not significantly different from HbAA erythrocytes. This finding of a HbSS group with lower miR-144 levels similar to HbAA led us to consider the possibility that the functional connection between miR-144 and anemia severity among HbSS samples may be related to a phenotypic difference between HbSS and HbAA erythrocytes. Thus in order to understand the potential mechanistic consequences of miR-144 overexpression in association with anemia severity among HbSS samples, it would be necessary to determine the developmental stage at which the dysregulation of miR-144 expression might occur between HbSS and HbAA samples.

4. MiR-144 Expression in primary erythroid progenitors during erythroid maturation

4.1 Expression of MiR-144 and MiR-451 during maturation of normal and SCD primary erythroid progenitors

To determine the expression of miR-144 during erythroid maturation we performed ex-vivo differentiation studies with HbSS and HbAA CD34+ cells in identical culture conditions and cytokine milieu (Figure 4). During the maturation of purified erythroid precursor cells, we found that the degree of induction of miR-144 was higher HbSS precursors during early maturation (Figure 4, Figure 5). At the same time, miR-451 expression was not significantly different between HbSS and HbAA precursors.

4.2 Materials and Methods

HbAA CD34+ cells (ALLCells) and HbSS CD34+ cells were purified from blood collected from apheresis with donor consent according to Duke IRB protocol. Mononuclear cells were separated by Ficoll-Hypaque density gradient, and CD34+ cells were enriched by immunomagnetic selection (Miltenyi Biotec), and cultured in StemLine II (Sigma) medium with human serum albumin, insulin, and transferrin. For the first stage of erythroid differentiation (days 0-7), CD34+ cells were stimulated with 50ng/mL stem cell factor (SCF, Invitrogen), 20ng/mL IL-3 (Invitrogen), and 3 U/mL erythropoietin (EPO, Calbiochem). On day 7 the progenitor cells were further enriched for CD71+ using immunomagnetic selection (Miltenyi Biotec). During the second stage (days 8-12), IL-3 was removed and the cells were maintained in both EPO and SCF. In the final stage (days 12-14), the cells were cultured in the same media with the addition of EPO alone. May Grunwald Giemsa staining was used to assess cell morphology and differentiation status. On selected days, the cells were lysed for RNA and protein extraction. RNA expression was measured relative to RNU6 expression control.

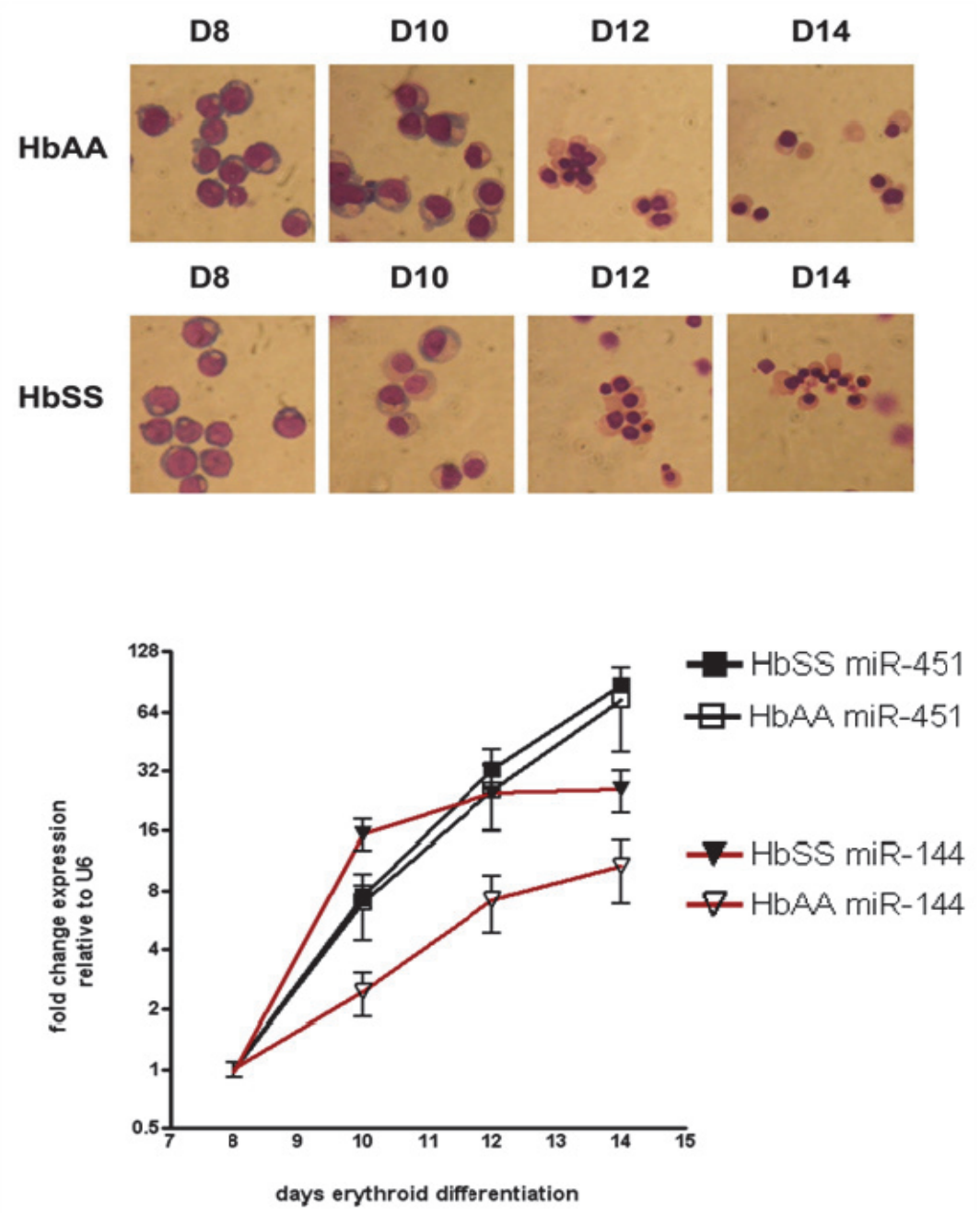


Figure 4: Erythroid maturation of HbSS and HbAA CD34+ progenitors

(top) May-Grunwald-Giemsa staining of cells in culture at day 8, 10, 12, and 14 of erythroid maturation in HbAA and HbSS progenitors. **(bottom)** Fold change in miR-144 and miR-451 expression at the indicated day of differentiation relative to Day 8 levels in HbAA (n=3) and HbSS (n=2) progenitors

Northern blotting of representative HbAA and HbSS erythroid progenitor RNA samples at different stages of erythroid maturation (D8, D10, D12) was also performed. Five micrograms of total RNA were separated in 15% TBE-urea polyacrylamide gel (Bio-Rad, CA), and electro-transferred onto Hybond-N+ (Amersham Biosciences, UK) membranes. The blot was then probed with end-labeled locked nucleotide acid-modified probes for miR-144 (Exiqon) in Express-Hyb buffer (Clontech, CA) at 50°C. The intensity of the 5.8S rRNA stained with ethidium bromide was used as a loading control.

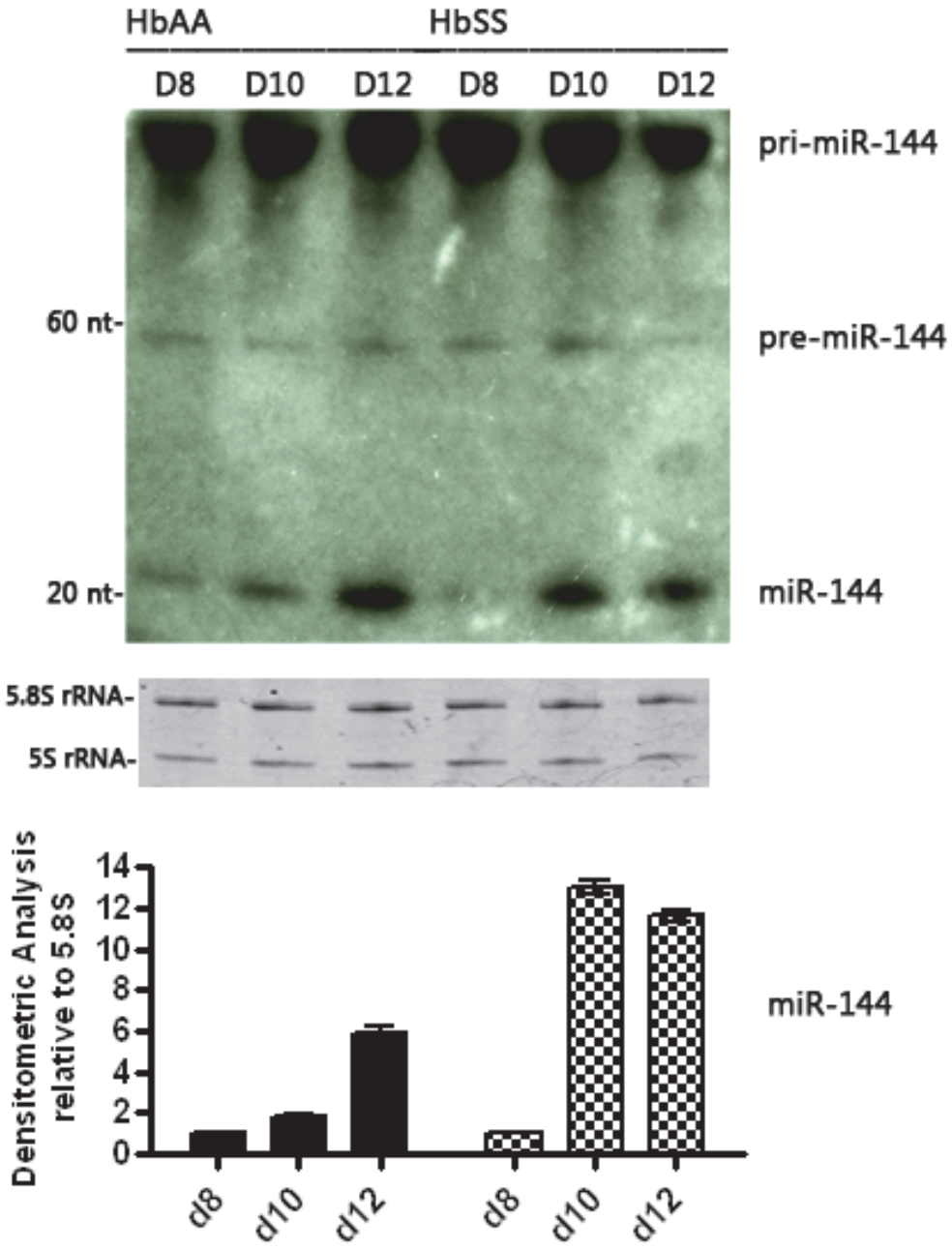


Figure 5: Northern blot analysis miR-144 expression during the maturation of HbAA and HbSS erythroid progenitors

(top) microRNA northern blot (middle) ethidium-bromide-stained gel as control for loadin, **(bottom)** densitometric analysis of mature miR-144 microRNA relative to loading control

4.3 Discussion

The overexpression of miR-144 during the ex-vivo maturation of HbSS and HbAA erythroid progenitor cells suggests that miR-144 overexpression may in fact be a cell-autonomous component of the HbSS cells. This expression difference can stem from the higher induction in the HbSS at early stage of maturation (D8 to D10), which is seen in both the real-time qPCR and northern analyses. Since the high miR-144 levels first seen in mature HbSS erythrocytes can also be seen during the erythroid precursor stages of development, we turned our focus to the potential functional relevance of miR-144 overexpression in erythroid cells.

5. Functional Role for miR-144 in SCD pathobiology

To understand the potential functional connection between miR-144 and anemia severity, we analyzed the predicted targets of miR-144 using TargetScanS and MiRbase (Lewis, Burge et al. 2005; Griffiths-Jones, Saini et al. 2008) and looked for targets related to known factors in the pathobiology of events that characterize Sickle Cell Disease on the cellular level, including erythrocyte dehydration, enhanced endothelial adhesion, oxidant perturbation, lipid bilayer dysfunction, and cytoskeletal abnormalities. Among the predicted targets of miR-144, we identified *NFE2L2* (NRF2) as a functionally relevant target due to its importance in mediating oxidative stress tolerance.

5.1 Antioxidant capacity and Oxidative Stress Response in SCD pathobiology

The normally effective balance between oxidative stress and antioxidant capacity of the erythrocyte is significantly altered in SCD (Rank, Carlsson et al. 1985; Amer, Ghoti et al. 2006). Compared to normal erythrocytes, SCD erythrocytes have an increased level of oxidative stress and ROS (Hebbel, Eaton et al. 1982). To prevent hemolysis under this environment, SCD erythrocytes require an even higher antioxidant capacity and enhanced repair mechanisms for oxidant damage. However, what is actually seen in SCD is a significantly lower antioxidant capacity in SCD erythrocytes, with reduced level of GSH (Tatum and Chow 1996; Morris, Suh et al. 2008) and decreased SOD, CAT, and GPX1 activities (Chiu and Lubin 1979; Schacter, Warth et al. 1988). This reduced antioxidant capacity makes SCD erythrocytes especially susceptible to oxidative insult and hemolysis (Tatum and Chow 1996; Steinberg and Brugnara 2003; Amer, Ghoti et al. 2006). The basis for such reduced capacity to defend against oxidative stress in SCD is currently unknown. Once the cells' glutathione levels are insufficient, normally protected sulfhydryl groups can become exposed and oxidized, alterations in globin conformation can

occur, heme can dissociate from globin, and oxidized membrane and cytoskeletal proteins can become sites for hemichrome binding (Tsantes, Bonovas et al. 2006) — all of which can lead to hemolysis and are often seen in SCD erythrocytes (Deneke and Fanburg 1989; Dumaswala, Zhuo et al. 2001; Morris, Suh et al. 2008).

In a set of preliminary studies, we characterized some of these known differences between HbSS and HbAA. As expected, mature HbSS erythrocytes were much more susceptible to H_2O_2 -induced oxidative stress compared to HbAA erythrocytes (Figure 6A, Figure A1). HbSS erythrocytes also demonstrated significantly decreased glutathione levels (-32.3%, $p<.0001$) and higher (+83%, $p<.0001$) levels of reactive oxygen species when compared to HbAA erythrocytes, consistent with previous observations (Figure 6B,6C). Since the SOD1 is the predominant SOD isoform present in mature erythrocytes, we used SOD enzymatic activity in erythrocytes as an alternative means of determining SOD1 levels in HbSS erythrocytes. We compared SOD1 activity in a cohort of 4 HbAA and 8 HbSS samples and found significantly decreased SOD activity (-40.9% $p<.05$) in HbSS samples (Figure 6D).

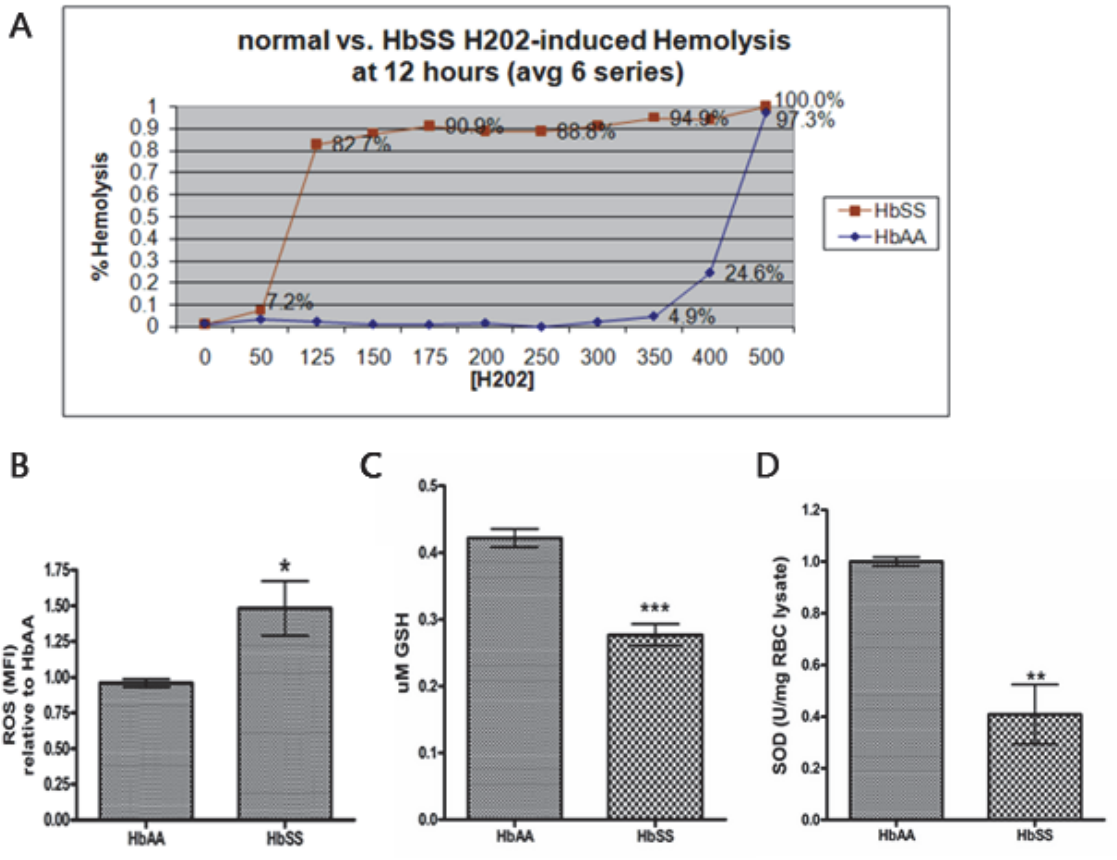


Figure 6: Oxidative Stress and Antioxidant Capacity in HbSS and HbAA Erythrocytes

HbSS erythrocytes (A) demonstrate increased susceptibility to oxidative stress-induced hemolysis, have (B) increased reactive oxygen species, (C) decreased glutathione levels, and (D) decreased superoxide dismutase activity levels compared to normal HbAA erythrocytes

5.1.1.1 Materials and Methods

5.1.1.1.1 Hemolysis assay

Procedure is modified from (Lee, Chan et al. 2004). Oxidative stress-induced hemolysis of the red blood cells was done using titrated concentrations of H_2O_2 and PBS with a 2% hematocrit solution of each sample. After incubation (37C, 5% CO2) for indicated time intervals, followed by brief centrifugation, determination of hemoglobin concentration of the cell supernatant was made using Drabkin's reagent and a spectrophotometer to measure the

absorbance at 540nm. Measurements were compared to '100% hemolysis' samples to determine percent hemolysis.

5.1.1.1.2 Reactive Oxygen Species Assay

For ROS measurement, erythrocytes were washed twice with PBS, resuspended to 2% packed cell volume, and incubated in 500uM H_2O_2 (37C, 5% CO2) for 1 hour. Cells were then resuspended in PBS and HEPES buffer and incubated with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) probe (Molecular Probes) for 30 minutes. Samples were immediately assayed by flow cytometry (FACScan, BD Biosciences) and analyzed by the CellQuest program (BD Biosciences). Results are reported as differences in mean fluorescence intensity. All samples were assayed in triplicate.

5.1.1.1.3 Superoxide Dismutase assay

Total superoxide dismutase was assessed using an assay that measures the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine (Cayman Chemical). One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. All samples were assayed in triplicate. Samples were immediately analyzed for absorbance at 450nm by microplate reader (FLUOstar OPTIMA, BMG LabTech).

5.2 Role of NRF2 and NRF2-regulated genes in cellular oxidative stress response of the erythrocyte

The erythrocyte is a chief oxidative sink and important mobile detoxifying system in the human body. The effective antioxidant capacity of the red blood cell allows it to serve as an antioxidant for itself as well as other cells and tissues. In doing so, it is susceptible to hemolysis due to various contributors of oxidative stress. One central regulator of antioxidant response is NRF2. Under oxidative stress, NRF2 binds to the antioxidant response element (ARE) found on

the promoters of key genes involved in oxidative stress response (Ishii, Itoh et al. 2000; Motohashi and Yamamoto 2004; Li, Johnson et al. 2005; Kensler, Wakabayashi et al. 2007). The binding of NRF2 to ARE is important for the coordinately inducible expression of antioxidant enzymes such as SOD (superoxide dismutase), catalase (CAT), GPX1, phase II detoxification enzymes such as NAD(P)H:quinone oxidoreductase (NQO1), and GSH synthesis and processing enzymes such as gamma-glutamylcysteine synthetase (GCS) and glutathione reductase. NRF2-regulated expression of ARE-driven genes, especially those involved GSH biosynthesis and recycling (Harvey, Thimmulappa et al. 2009), has been shown to be critical for cell survival during oxidative stress in various *in vivo* and *in vitro* models (Itoh, Chiba et al. 1997; Chan and Kwong 2000; Lee, Calkins et al. 2003).

The importance of NRF2 in the cellular defense mechanism of the erythrocyte is illustrated in mice with targeted deletions of NRF2 or NRF2-dependent genes. NRF2-deficient mice develop hemolytic anemia, increased sensitivity to oxidative stress, decreased glutathione, and decreased expression levels of NRF2-dependent genes (Lee, Chan et al. 2004). For example, targeted deletion of the peroxiredoxin PRDX1, a NRF2-dependent gene involved in preventing oxidative stress damage, also leads to severe hemolytic anemia characterized by an increase in erythrocyte reactive oxygen species (ROS), protein oxidation, hemoglobin instability, and decreased lifespan (Neumann, Krause et al. 2003).

5.3 The MiR-144-NRF2 regulatory axis in K562 erythroid cells

5.3.1 NRF2 is a direct target of miR-144

There are two evolutionarily conserved potential miR-144 target sites in the 3'UTR of NRF2 (positions 265-271 and 370-377) (Figure 7). To determine whether miR-144 directly regulates NRF2, we used a NRF2 3'UTR reporter construct consisting of firefly luciferase reporter followed by the full length 3'UTR of NRF2. Expression constructs for miR-144, miR-

320, or empty vector were cotransfected with the NRF2 3'UTR reporter into K562 cells to measure the effect of their respective overexpression on luciferase reporter activity. We found that miR-144 overexpression was able to repress luciferase activity by 70.3% (± 1.37 SEM $p<.0001$) relative to the empty vector control, while miR-320, which does not have a target site in the NRF2 3'UTR, did not have a significant effect on luciferase activity (Figure 7). Thus, NRF2 is a direct regulatory target of miR-144 through the specific interaction between miR-144 and the NRF2 3'UTR. Similar results were also found using HEK293 cells. To test which of the two predicted miR-144 target sites in the 3'UTR of NRF2 were relevant for miR-144-mediated repression, we mutated the two binding sites individually on the reporter construct. The mutation of the first target site led to reduced repression by miR-144 (from 70.3% to 39.3% (± 8.69 SEM $p=0.022$)), whereas the mutation of the second binding site completely abolished the repression by miR-144 (Figure 7). MiR-320 overexpression did not have a significant effect on luciferase activity after mutation of either of these binding sites.

We then sought to determine how these microRNAs affect endogenous NRF2 protein levels. MiR-144 overexpression led to a detectable change in NRF2 protein level under baseline conditions, compared to empty vector control and miR-142-5p. However, under H_2O_2-induced oxidative stress, miR-144 led to a significant and reproducible decrease in NRF2 protein level (Figure 8A). Taken together, these results indicate that NRF2 is a valid target gene of miR-144, especially under oxidative stress.

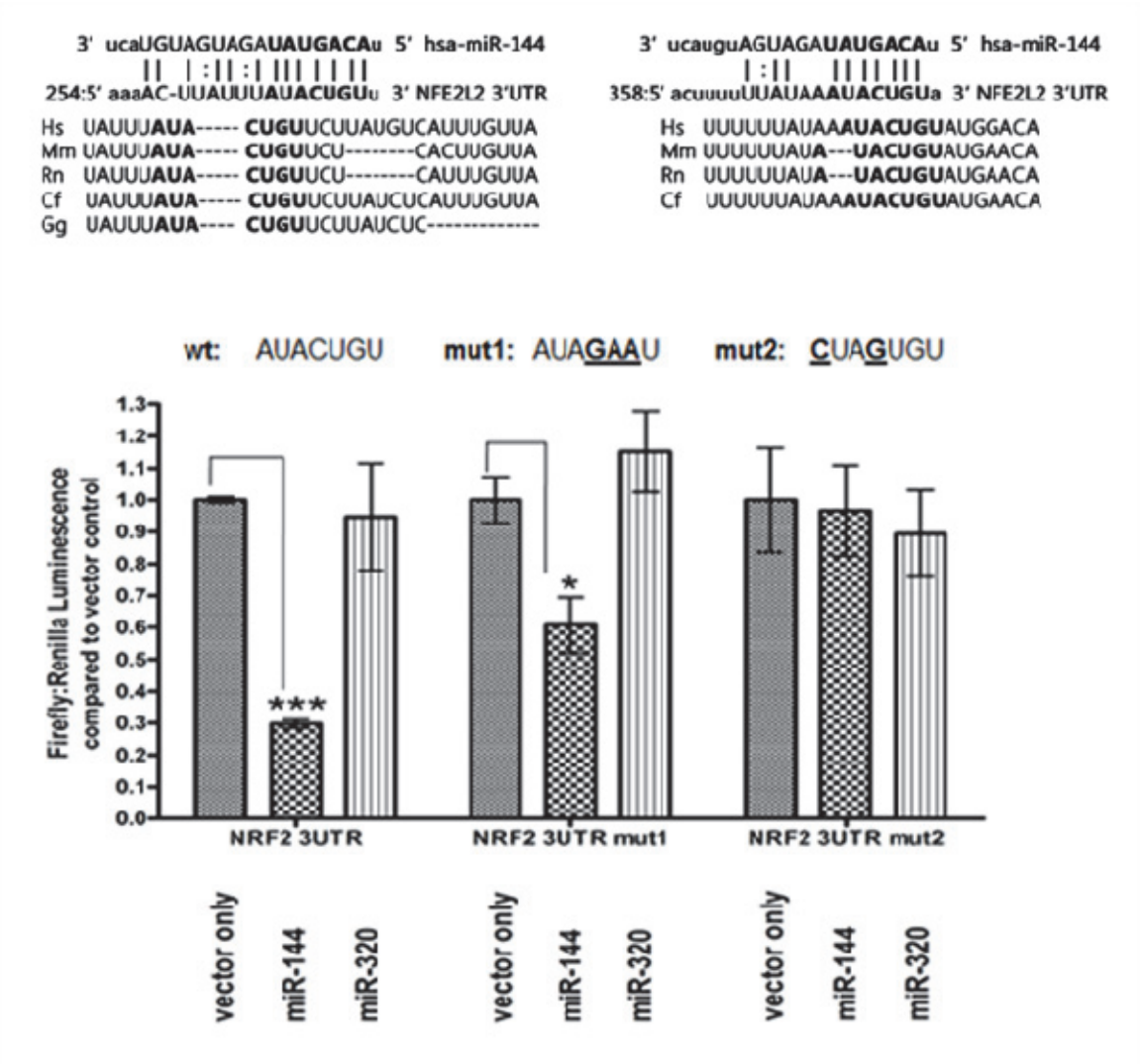


Figure 7: NRF2 is a direct target of miR-144

(top) sequence alignment and evolutionary conservation between miR-144 and its two putative binding sites in the NRF2 3'UTR

(bottom) NRF2 3'UTR luciferase reporter analysis shows wild-type repression by miR-144 and de-repression of mutant miR-144 binding sites. While mutation of the first miR-144 binding site (mut1) modestly decreased miR-144 mediated repression, mutation of the second site (mut2) abolished this effect.

5.3.1.1 Materials and Methods

5.3.1.1.1 Cell Culture, Cell Transfection, RNA and protein extraction

K562 cells (ATCC) were maintained in standard RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics. Transfection experiments with K562 cells were done using Amaxa nucleofection (Amaxa) or Lipofectamine LTX (Invitrogen) lipofection according to their respective protocols. Small RNAs were extracted 48 hours after transfection using the mirVana microRNA Extraction Kit (Ambion) and stored at -80C until thawed for assay. For protein analysis, cells were harvested 48 hours after transfection, washed twice with Dulbecco's Phosphate-Buffered Saline (PBS), pelleted by centrifugation, and stored at -80C until thawed for the indicated assays.

5.3.1.1.2 Construction and validation of microRNA expression vectors

Expression constructs encoding the precursor hairpin sequences of miR-144, miR-142-5p, and miR-320 were each created by insertion into a CMV-based pcDNA3 cloning vector (Invitrogen, CA) that coexpresses green fluorescent protein (GFP). Vector GFP expression allowed for simple assessment of transfection efficiency. Primers designed for the overexpression construct amplification were as follows: miR-144 (forward: gaagctgtgtgtgtccagcc; reverse: gcagcatctctcctgtcctc), miR-142-5p (forward: gcggccagccaggggttc; reverse: tccacgtaccatcccttcc), miR-320 (forward: caggaaccagacagggacgc; reverse: ccgactcttaagtccaggtc**).**

Additionally, a construct for a seed-mutated- miR-144 expression construct "miR-144-seedmut1" was made by taking the miR-144 pcDNA-based expression construct and performing site-directed mutagenesis with QuikChange II Site-Directed Mutagenesis (Strategene) using primers **(forward:** 5'-tgtaagtttgcgatgagacactattctatagatgatgtactagtccggg-3'**; reverse:** 5'-cccggactagtacatcatctatagaatagtgtctcatcgcaaacttaca-3'**)** to encode the following changes (underlined) in

the seed region (in bold) of miR-144: U**ACAGUAU**AGAUGAUGUACU => U***A<u>UUC</u>*****UAU**AGAUGAUGUACU . This construct is endogenously processed to produce mature miR-144 with a mutated seed region and thus serves as a control for specificity of the miR-144 sensor for measuring the mature form of miR-144.

These expression constructs were validated to confirm overexpression after transient transfection into K562 cells (Figure 9)**.** K562 cells were transiently transfected with expression constructs by nucleofection. After RNA extraction, mature microRNA levels were measured using Real-Time PCR. Relative to empty vector, cells transfected with the miR-144 construct overexpressed miR-144 by 5.7 fold (± 1.60 SEM p=0.0011). MiR-451, due to its close proximity to miR-144 precursor (92bp apart), was also measured as a control, and its expression was not significantly different (.891 fold ±.124 SEM, p=0.0010) from that of empty vector in the cells with enforced miR-144 expression. Thus we were able to significantly overexpress mature miR-144. Relative to empty vector, cells with the miR-142-5p construct overexpressed miR-142-5p by 1.96 fold (± .097 SEM, p=0.0011) (Figure 9C).

5.3.1.1.3 Construction of NRF2 luciferase reporters

The 3'UTR of the NRF2 was amplified using primers (forward: atttaggaggatttgacc; reverse: tttttgccagagctaaacaattt) and cloned into the XbaI site downstream of the firefly luciferase gene in the pGL3-control vector (Promega). The NRF2 3'UTR mutant reporters were constructed with QuikChange II Site-Directed Mutagenesis (Stratagene, CA), which introduced base pair changes in the miR-144 seed sequence-targeted regions.

The NRF2 3'UTR mutant reporters were constructed with QuikChange II Site-Directed Mutagenesis (Stratagene, CA), which created base pair changes in the miR-144 seed sequence-targeted regions **(**site 1: tttata**ctg**ttcttat to tttata**<u>gaa</u>**<u>t</u>tcttat and site 2: aaata**c**tgtatgga to aa**<u>c</u>**tact**g**tg<u>t</u>atgga**).** A plasmid encoding the full sequence of NRF2 cDNA was obtained from

Origene (Rockville, MD) and served as a template for the NRF2-without-3UTR expression plasmid.

5.3.1.1.4 Construction and validation of miR-144 sensor luciferase reporters

Two reverse complement 'target' sites for mature miR-144 were cloned in tandem between the XhoI and NotI sites of the si-CHECK-2 luciferase reporter plasmid (Promega), which encodes both the firefly luciferase gene as the internal control for transfection and renilla luciferase gene as the reporter. The sensor assay is performed by cotransfection of the sensor construct with indicated expression constructs into K562 cells using Lipofectamine LTX (Invitrogen) followed by Dual Luciferase assay (Promega) performed according to manufacturer's instructions. Each sensor assay is performed alongside a control assay cotransfecting the 'empty' no-insert siCHECK-2 luciferase reporter plasmid under the same conditions. The relative difference between the no-insert reporter and the sensor construct is reported.

MiR-144 expression was further validated using this miR-144 sensor construct (Figure 9B). The sensor was transfected alone (mock) or cotransfected with pcDNA (empty vector), miR-144, miR-320, or miR-144-seedmut1 expression vectors into K562 cells by Lipofectamine LTX lipofection method as described in text. Cotransfection with miR-144 expression vector showed significant repression (83.5%±.80 SEM $p<.0001$) of miR-144 sensor luciferase reporter activity (relative to empty vector). Expression of empty vector, miR-144-seedmut1, and miR-320 did not significantly repress luciferase activity of the miR-144 sensor construct compared to mock transfection.

5.3.1.1.5 Luciferase assay

K562 cells were cotransfected with 0.5 ug of indicated luciferase reporter, 20 ng Renilla luciferase construct and 2 ug of indicated microRNA expression constructs using Lipofectamine

LTX (Invitrogen). All samples were assayed in triplicate. After 24 hours, the transfected cells were washed and lysed with passive lysis buffer (Promega). Firefly and Renilla luciferase activity were measured using the Dual-Glo Luciferase assay (Promega) and a luminometer (Lumat, Berthold Technology).

5.3.2 MiR-144 modulates oxidative stress tolerance

Given the importance of NRF2 in the transcriptional response to oxidative stress, we hypothesized that miR-144 could directly influence the cellular tolerance to oxidative stress through modulation of NRF2 expression. Although microRNAs have been shown to be modulators of cellular stress (Bhattacharyya, Habermacher et al. 2006; Sunkar, Kapoor et al. 2006), this has not been reported in erythroid cells.

In K562 erythroid cells we observed a dose-dependent decrease in miR-144 expression in response to H_2O_2-induced oxidative stress (Figure 10). To test the functional effect of miR-144 overexpression on oxidative stress tolerance, we overexpressed miR-144 into K562 cells and measured their survival under different concentrations of H_2O_2. We found that miR-144 overexpression leads to a 19.78%, 36.41%, and 47.19% (±11.6 SEM, p=0.0049) increase in sensitivity to 2.5, 5, and 10uM H_2O_2-induced oxidative stress, respectively (Figure 8B). In contrast, overexpression of miR-142-5p does not lead to significant differences in oxidative stress sensitivity. To test whether this effect is mediated through NRF2, we cotransfected a NRF2 cDNA expression construct with miR-144 and found that this significantly rescued miR-144-mediated sensitivity to 10uM H_2O_2 oxidative stress (p=0.0037) (Figure 8C).

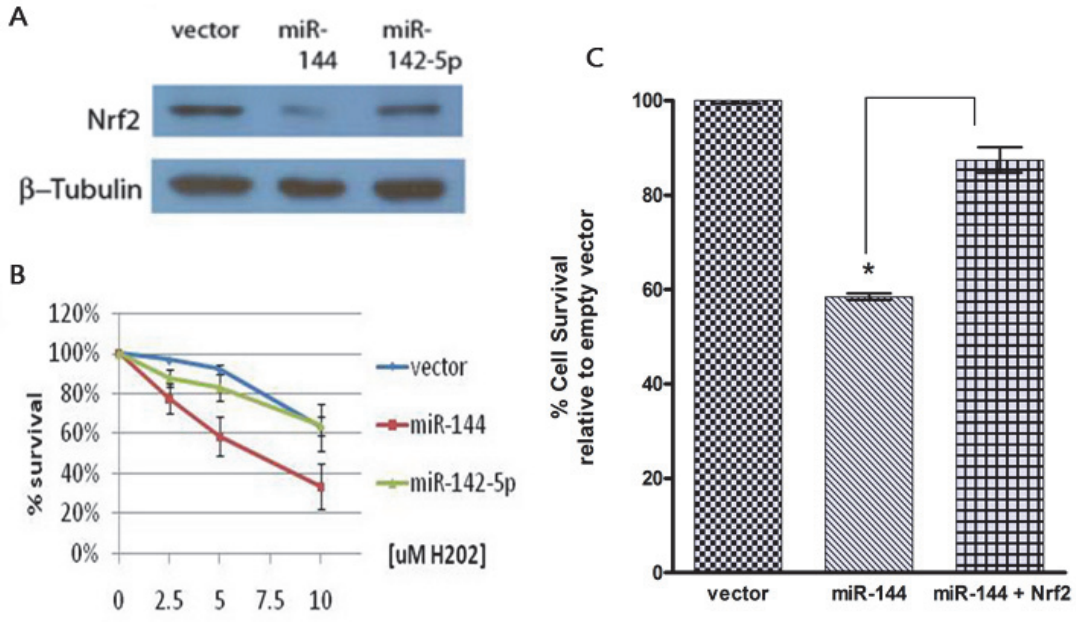


Figure 8: MiR-144 overexpression reduces cellular response to oxidative stress

(A) Western blot analysis of NRF2 protein levels after the overexpression of miR-144 and miR-142-5p in K562 cells and H_2O_2-induced oxidative stress. (B) MiR-144 overexpression leads to significantly increased sensitivity to oxidative stress at indicated concentrations of H_2O_2 as measured by MTS assay. (C) MiR-144-mediated sensitivity to H_2O_2 oxidative stress is partially rescued by NRF2 overexpression.

Furthermore, miR-144 overexpression also leads to a detectable reduction of NRF2 protein expression in primary erythroid cells (Figure 11A). The effect of miR-144 on NRF2 protein was more pronounced under oxidative stress (Figure 11B), as seen in K562 cells. Importantly, miR-144 overexpression in primary erythroid cells also leads to significantly increased sensitivity to H_2O_2-induced oxidative stress ($p=0.012$), and cotransfection of a NRF2 cDNA construct without its 3'UTR rescued this miR-144-mediated sensitivity (Figure 11C). These results indicate that miR-144-mediated NRF2 repression can play a direct and important role in the reduction of oxidative stress tolerance in erythroid cells.

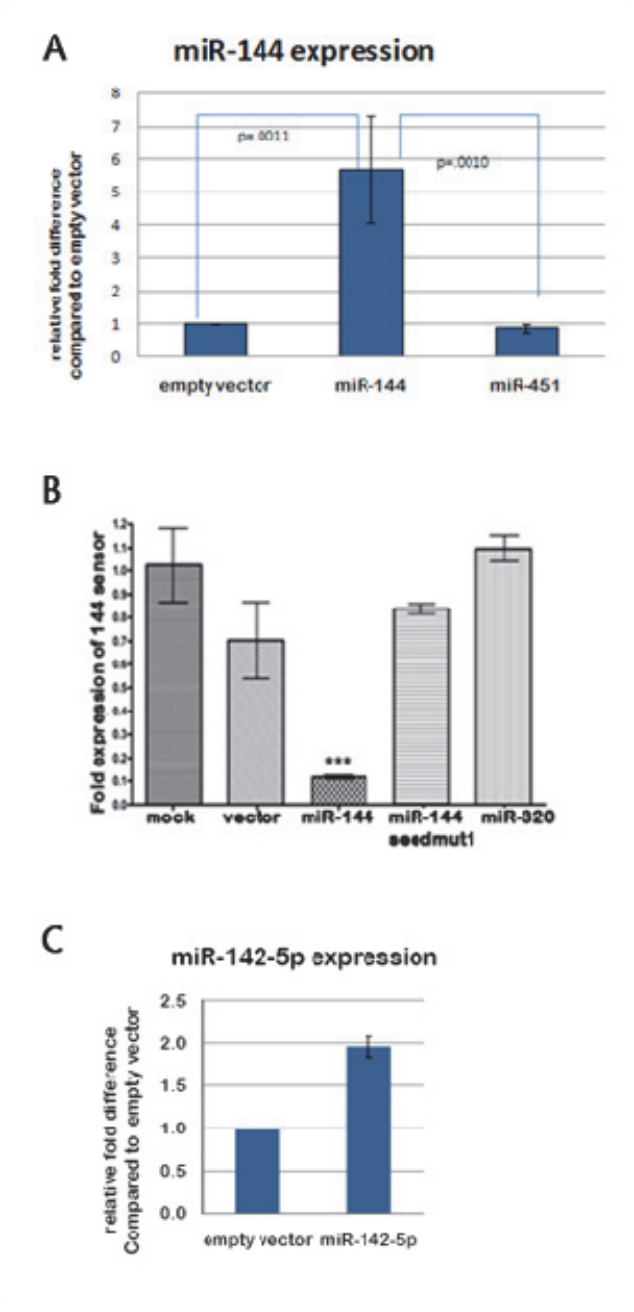


Figure 9: Validation of MicroRNA Expression Constructs and Luciferase Reporters

MiR-144 overexpression vector specifically upregulates miR-144 levels, shown by **(A)** microRNA qRT-PCR assay and **(B)** miR-144 sensor luciferase constructs. **(C)** validation of miR-142-5p overexpression using qRT-PCR assay.

5.3.2.1 Materials and Methods

5.3.2.1.1 Oxidative Stress assay

To determine the effects of H_2O_2 on cell viability, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay (Promega) was used. K562 cells were seeded at 2×10^4 cells well and treated with H_2O_2 for 6h. Primary erythroid cells were stressed for 2h. Samples were treated and analyzed in quadruplicate. Results are expressed as a percentage mean of metabolically active cells with untreated control cells set at 100%.

5.3.2.1.2 NRF2-ELISA assay

The NRF2-ELISA assay was done using the NRF2 TRANS-AM assay (Active-Motif) according to manufacturer's directions. All samples were assayed in triplicate.

5.3.2.1.3 Transfection of primary erythroid cells

Transfection of erythroid precursor cells was done by Amaxa nucleofection on day 8 of differentiation using the CD34 nucleofector kit.

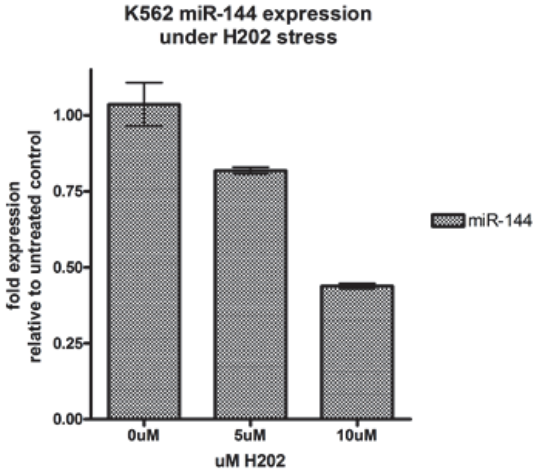


Figure 10: K562 miR-144 expression under oxidative stress

Expression of miR-144 in K562 cells under different concentrations of H202-induced oxidative stress, compared to untreated control.

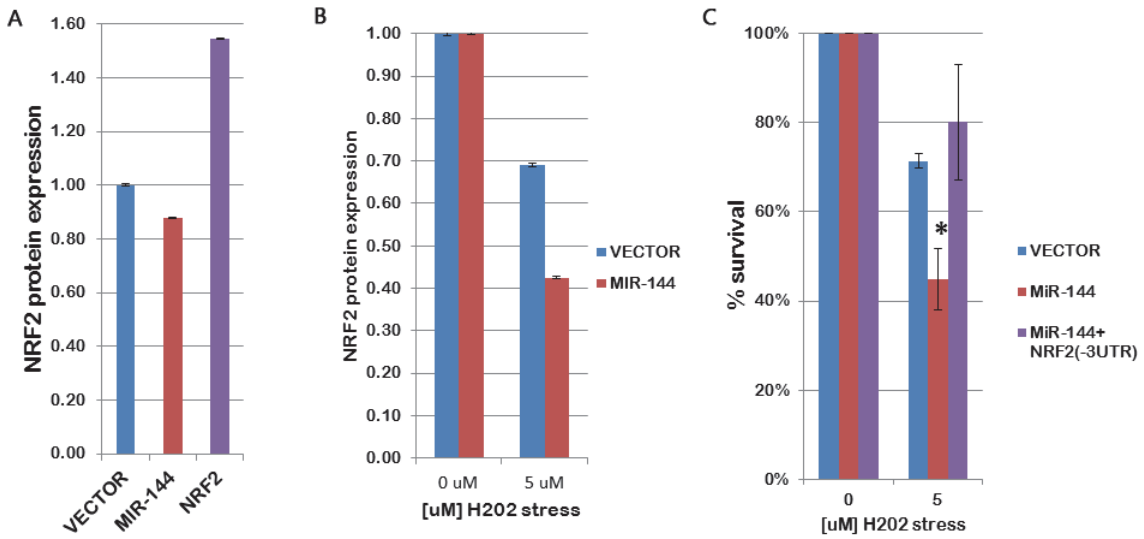


Figure 11: Effect of miR-144 overexpression on NRF2 protein and oxidative stress tolerance in primary erythroid cells

(A) miR-144 overexpression effect on NRF2 protein expression at baseline and **(B)** under oxidative stress. **(C)** miR-144 co-transfection with NRF2-without its 3'UTR rescues the miR-144-mediated sensitivity to oxidative stress

5.3.3 MiR-144 reduces antioxidant response element (ARE)-driven gene expression

The transcriptional activity of NRF2 in oxidative stress states is mediated by its binding to the antioxidant response elements (AREs) located in the promoter regions of many genes encoding antioxidants and detoxification enzymes. This promoter-enhancer element regulates the basal transcription and induction of these genes through the binding of NRF2 in response to ROS and other forms of oxidative stress. This process is vital to the cellular stress response and survival of many cell types, including erythrocyte precursors.

To determine whether miR-144 can have an inhibitory effect on the activation of genes that undergo ARE-mediated upregulation, we used a luciferase reporter consisting of an ARE promoter element upstream of luciferase cDNA (NQO1-ARE-Luc, a kind gift from Jeffrey A.

Johnson). This reporter construct was responsive to the level of NRF2 protein, since the reporter activity was shown to be increased by NRF2 overexpression and reduced with knockdown of endogenous NRF2 by si-NRF2 (Figure 12).

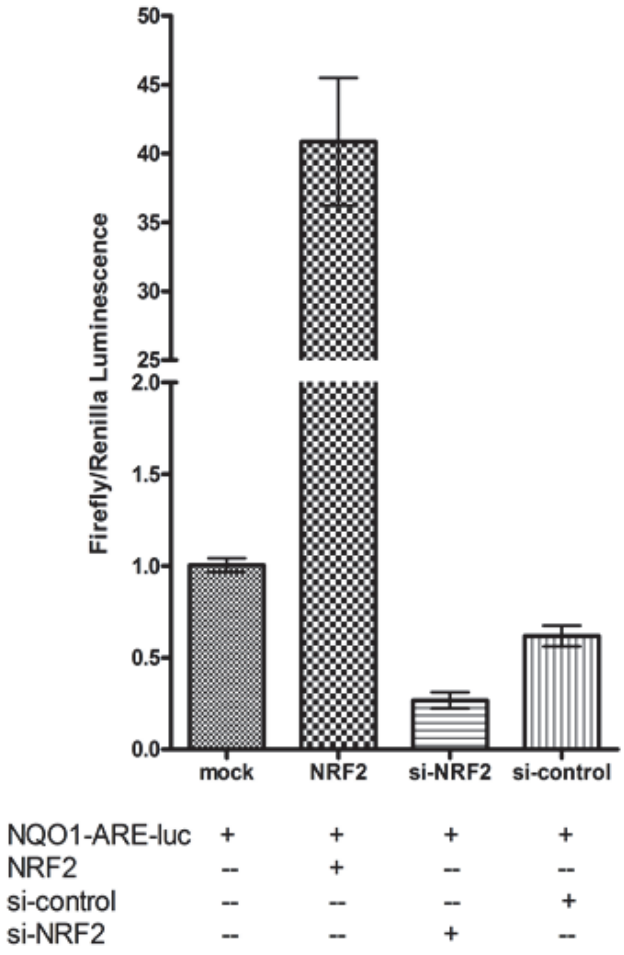


Figure 12: Validation of Antioxidant Response Element luciferase reporter
Overexpression of NRF2 drives expression of the ARE while knockdown of NRF2 reduces ARE reporter expression

Next the NQO1-ARE-Luc reporter was cotransfected into K562 cells with empty vector, miR-144, or miR-320 expression constructs to measure their respective effects on ARE-driven

gene expression. We found that miR-144 significantly inhibits activity of the ARE luciferase reporter by 62.1% (±2.64 SEM, $p<.0001$) relative to the empty vector control and miR-320 did not significantly inhibit reporter activity (Figure 13A). Taken together, this suggests that miR-144 overexpression leads to the subsequent inhibition of ARE-driven gene expression, presumably by its inhibitory effect on NRF2 expression. Similar results with this reporter were found in HEK293 cells (Figure 13B). The NQO1-ARE luciferase reporter construct was cotransfected with empty vector, miR-144, or NRF2 (as a positive control) into HEK293 cells to measure their respective effects on ARE-driven gene expression. We found that miR-144 (relative to vector control) significantly inhibited activity of the ARE luciferase reporter by 56.8% (±4.4 SEM, $p<.0001$), while NRF2 overexpression significantly increased reporter activity.

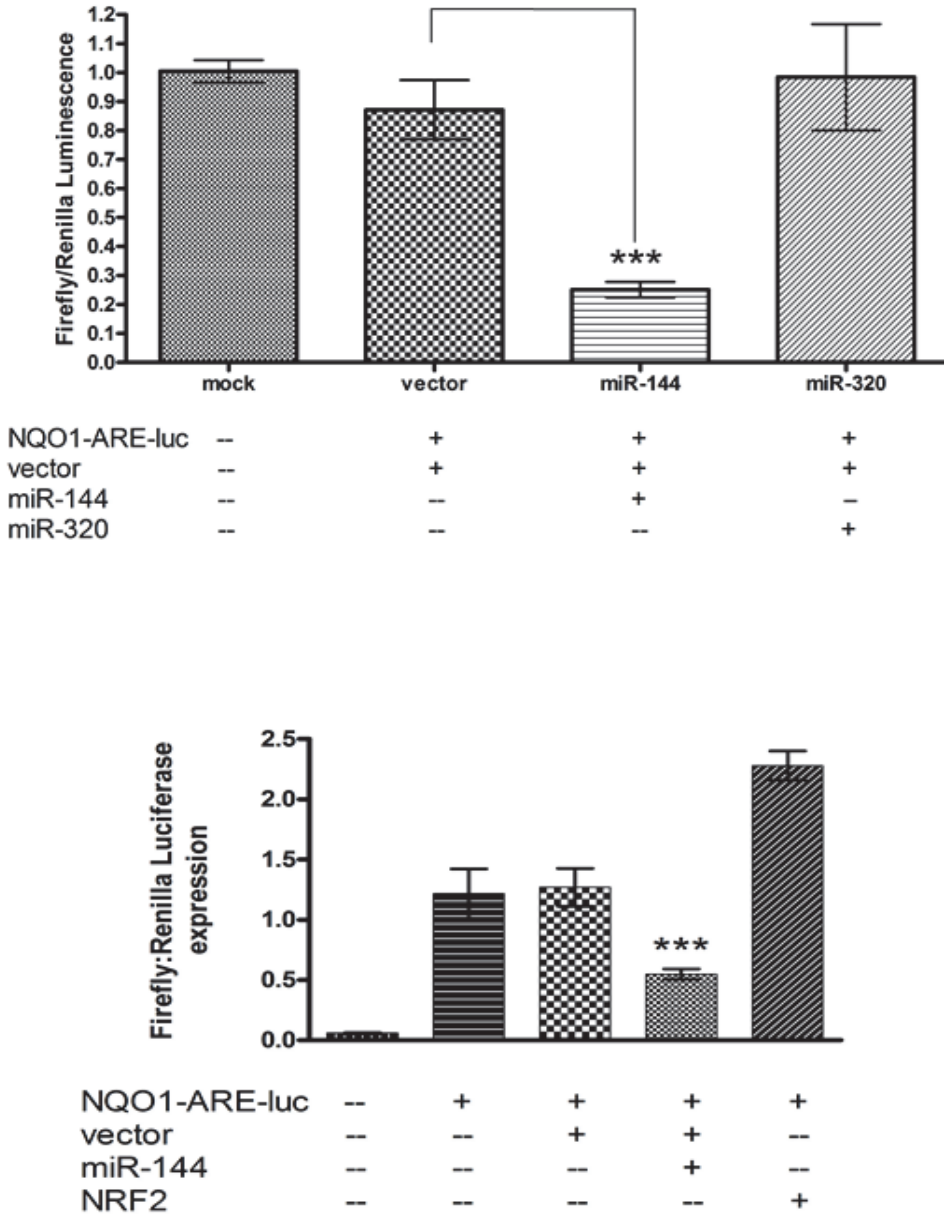


Figure 13: Enforced MiR-144 expression represses ARE-driven luciferase reporter

(top) miR-144 effect on ARE-reporter activity in K562 **(bottom)** miR-144 effect on ARE reporter activity in HEK293 with NRF2 overexpression shown as positive control

To further confirm the effect of miR-144 overexpression on the RNA expression of several endogenous ARE-driven genes under oxidative stress, we measured the gene expression of SOD1, CAT, and the catalytic (GCLC) and modulatory (GCLM) subunits of GCS in K562 cells at baseline (Figure 14A) and after treatment with H_2O_2-induced oxidative stress (Figure 14B). At baseline, miR-144 overexpression led to an 18.2% decrease in CAT expression (±2.3 SEM, p=0.001), and 10.0% increase in GCLM expression (±3.0 SEM, p=0.0021) in K562, and did not significantly affect the expression of SOD1 or GCLC. Under oxidative stress, miR-144 overexpression led to a 22.0% decrease (±2.3 SEM, p=0.0007) in SOD1 expression, 39.9% decrease (±8.4 SEM, p=0.009) in CAT expression, 59.1% decrease (±3.0 SEM, p=0.0001) in GCLC expression, and 15.2% decrease (±2.9 SEM, p=0.0083) in GCLM expression compared to expression of vector control.

5.3.3.1 Materials and Methods

5.3.3.1.1 Quantification of Antioxidant Gene Expression

RNA was reverse-transcribed with SuperScript II following manufacturer's protocol (Invitrogen) and assayed with primers specific for SOD1, CAT, glutamate-cysteine ligase, catalytic subunit (GCLC), and glutamate-cysteine ligase, modifier subunit (GCLM), with GAPDH as an internal control.

5.3.3.1.2 Use and validation of the ARE-luciferase reporter and si-NRF2 construct

The NQO1-ARE luciferase reporter construct was validated in K562 cells using NRF2 cDNA overexpression construct as a positive control, which led to a 40.85 fold increase in luciferase activity compared to mock-transfected cells (Figure 12). The downregulation endogenous NRF2 via gene silencing by siRNA against NRF2 leads to a 73.3% decrease in ARE-reporter activity compared to mock-transfected cells and 43.2% decrease compared to si-control

transfected cells (Figure 10). The si-NRF2 for knockdown of NRF2 was purchased from Dharmacon, along with 'si-control' for transfection control.

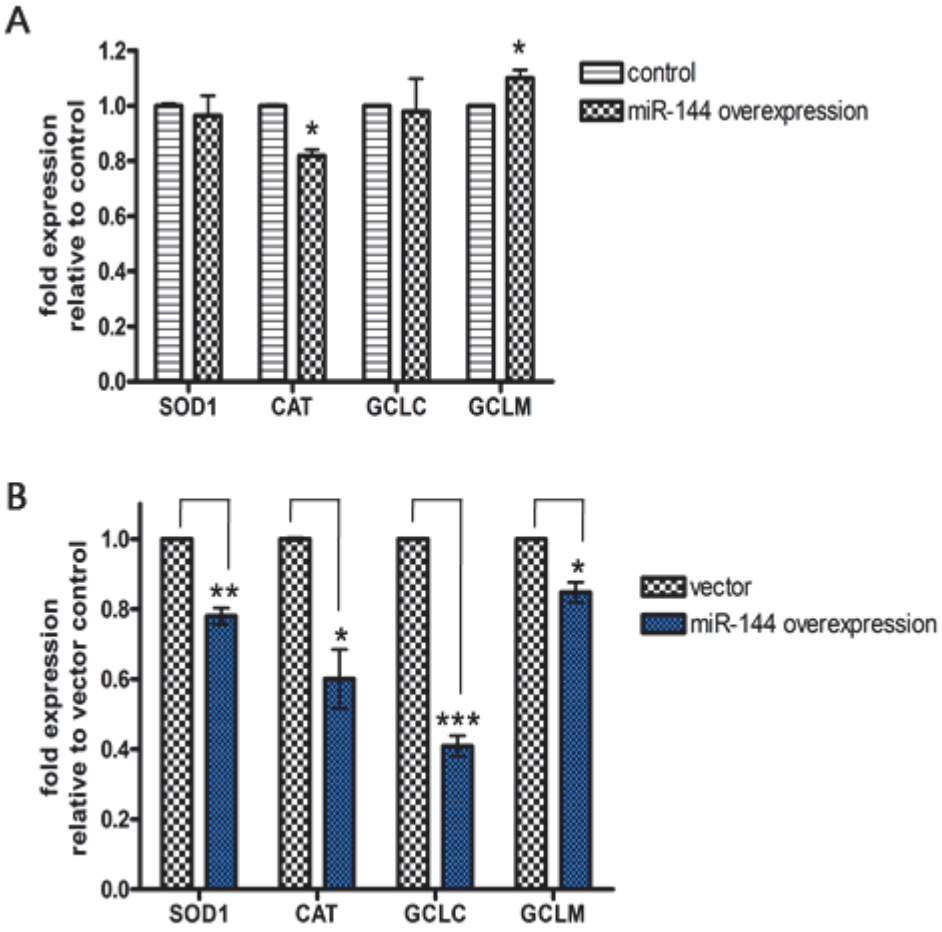


Figure 14: Enforced MiR-144 expression reduces ARE-driven gene expression
Effect of enforced miR-144 expression on the expression of select genes driven by the antioxidant response element (A) under baseline and (B) under oxidative stress condition in K562

5.3.4 MiR-144 decreases cellular glutathione concentration under oxidative stress

NRF2-regulated GSH regeneration is critical for the generation and maintenance of cellular response to oxidative stress and for cell survival (Harvey, Thimmulappa et al. 2009). Since mature erythrocytes lack regenerative capacity after oxidative damage, glutathione synthesis and recycling is even more vital to the mature erythrocyte. The role of glutathione is to maintain the redox state of protein sulfhydryl moieties and reduce hydrogen and lipid peroxides in erythrocytes before permanent oxidative damage occurs. Given the ability of miR-144 to reduce NRF2 protein and ARE-driven gene expression**,** we reasoned that miR-144 may also affect cellular glutathione concentration. To test this possibility, we overexpressed empty vector, miR-144, miR-144 cotransfected with NRF2, and NRF2 alone (as a positive control) in K562 cells. Total reduced GSH was measured after 2 hours under basal conditions or under 10uM of H_2O_2-induced oxidative stress. Under basal conditions, NRF2 overexpression led to an increase in glutathione concentration ([GSH]) of 73.5% while miR-144 overexpression did not lead to significant changes in [GSH] (Figure 15A). However, under oxidative stress, there was a significant decrease in [GSH] (34.6 ± 0.6%, $p<.0001$) in the cells with miR-144 overexpression compared to empty vector control (Figure 15B). This effect was partially rescued by the cotransfection of NRF2, which led to a 23.9% increase (± 3.67%, $p=0.0007$) in [GSH]. Therefore, high miR-144 expression can compromise GSH recycling and restoration during oxidative stress.

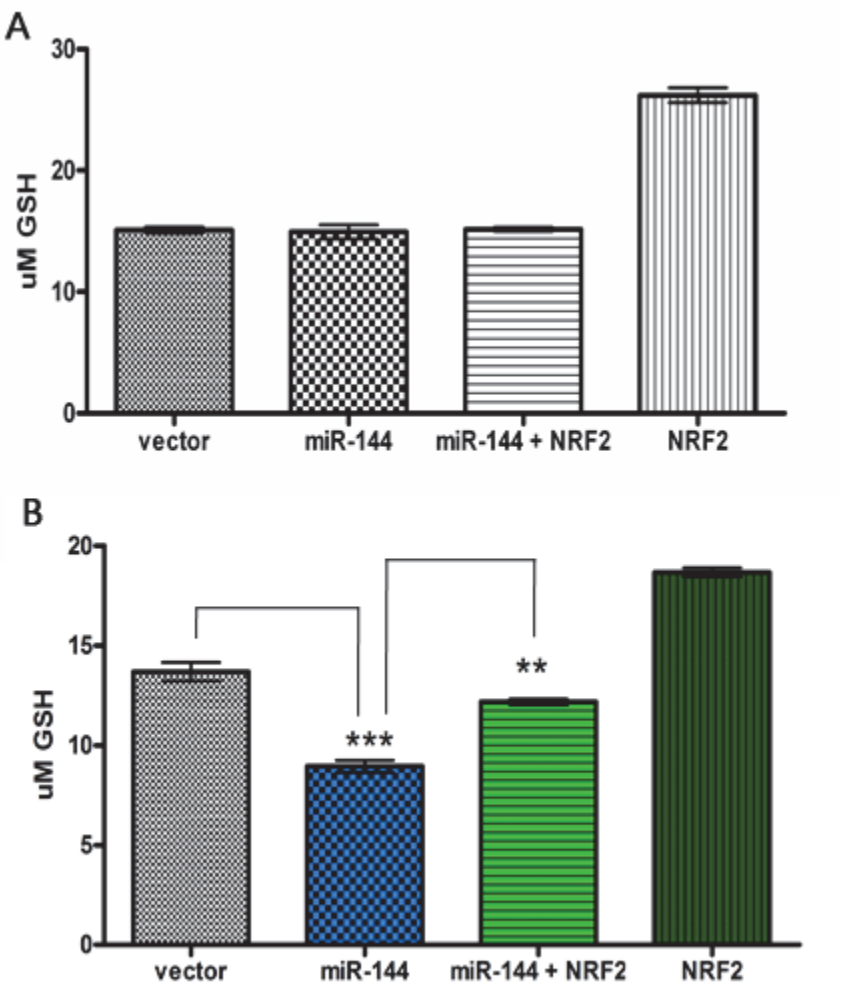


Figure 15: Enforced MiR-144 expression reduces cellular GSH levels

Effect of enforced miR-144 expression on glutathione levels (A) under baseline condition (B) under oxidative stress in K562

5.3.4.1 Materials and Methods

5.3.4.1.1 Glutathione assay

Intracellular glutathione concentration was measured using the GSH-GLO Glutathione assay (Promega) according to manufacturer's instructions. The assay is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione, catalyzed by glutathione S-transferase. For erythrocyte lysate experiments, the amount of glutathione is expressed as micromolars per cell. All samples were assayed in triplicate.

5.4 The MiR-144-NRF2 regulatory axis in erythrocytes, reticulocytes and primary erythroid progenitors

5.4.1 MiR-144 expression inversely correlates with antioxidant protein expression in HbSS erythrocytes

Since miR-144 was found to be overexpressed in all SCD erythrocytes, particularly among the group with more severe anemia, and was also found to repress the key regulator of oxidative stress response, we sought to determine the relationship between miR-144 expression and antioxidant capacity in SCD erythrocytes using an independent set of 18 HbSS patient samples (Table 1). Among this cohort, high miR-144 expression was also correlated with the severity of anemia as indicated by lower hemoglobin and hematocrit levels (Figure 16). We measured expression of several antioxidant proteins (GCLC, GCLM and SOD1) in these SCD erythrocytes using intracellular staining followed by FACS analysis to obtain quantitative single cell measurements. When we separated the samples into two groups (high vs. low miR-144) based on miR-144 expression, we noted that the intracellular expression of GCLC and GCLM was 1.74-fold and 2.37-fold higher respectively in 'low miR-144' group compared to the 'high miR-144' group (Figure 17). These results demonstrate that higher miR-144 in erythrocytes is

associated with lower levels of NRF2-driven antioxidant proteins among HbSS patients, supporting the role of miR-144 in the modulation of oxidative stress tolerance in sickle cell disease.

5.4.1.1 Materials and Methods

5.4.1.1.1 Intracellular Protein Expression assay

To detect the proteins SOD1, GCLM, and GCLC in mature erythrocytes, cells were fixed in 0.025% (v/v) glutaraldehyde in PBS. The cells were permeabilized with 0.01% saponin containing 1% (v/v) fetal bovine serum. Permeabilized cells were incubated with rabbit anti-human SOD1 (Abcam), rabbit anti-human GCLC (Santa Cruz Biotechnology), or rabbit anti-human GCLM (Santa Cruz Biotechnology), and Allophycocyanin (APC)-labeled goat anti-rabbit secondary antibody (Invitrogen), followed by flow cytometry analysis. Representative FACS results are shown in Figure 17.

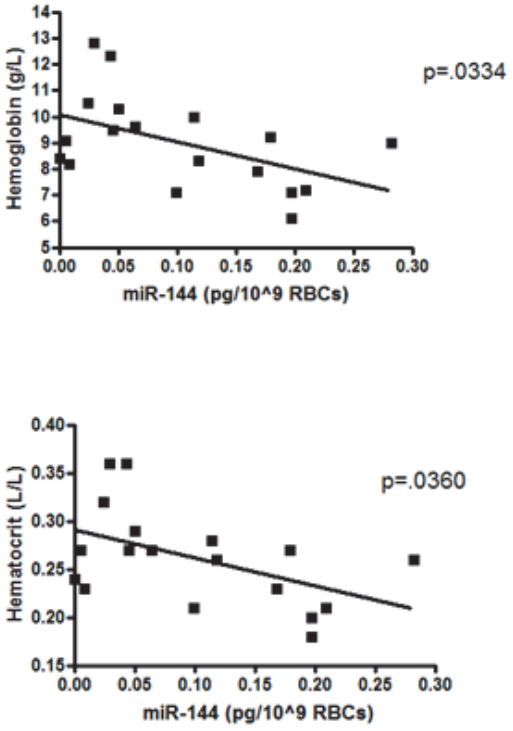


Figure 16: Correlation of miR-144 with low Hemoglobin, Hematocrit in HbSS erythrocytes

Negative correlation of erythrocyte miR-144 with hemoglobin **(upper panel)** and hematocrit **(lower panel)** in HbSS erythrocytes

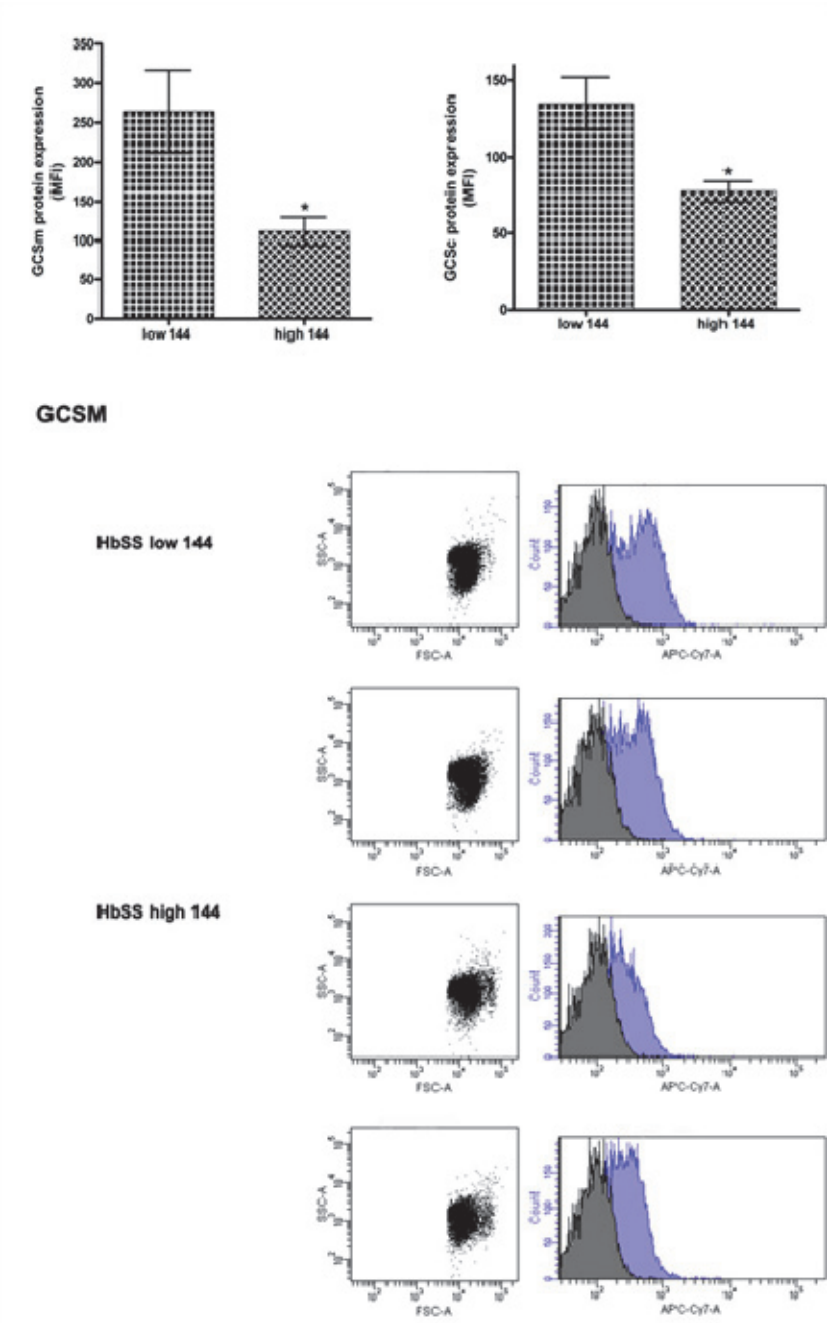


Figure 17: Intracellular Protein Levels in HbSS Erythrocytes with high vs low miR-144

(top panel) GCLM (left and GCLC (right) intracellular protein expression
(bottom panel) representative FACS plot analysis showing population of cells by size and expression of APC-control stained (gray,darker) versus APC-conjugated-GCLM-antibody-stained (purple, lighter) cells

5.4.2 MiR-144 expression inversely correlates with NRF2 protein levels in HbSS reticulocytes and primary erythroid progenitors

Since high miR-144 was correlated with poor antioxidant capacity in HbSS mature erythrocytes, we sought to determine the relationship between miR-144 expression and NRF2 protein levels in CD71+ HbSS reticulocytes, using an independent set of 25 HbSS patient samples (Table 3). Since reticulocytes have recently undergone the process of enucleation, there are still detectable levels of residual NRF2 protein. Among this cohort, high miR-144 expression in HbSS reticulocytes was also correlated with the severity of anemia as indicated by lower hemoglobin and hematocrit levels (Figure 18A, 18B). Analysis of the level of NRF2 proteins in a subset of this cohort (n=15) using quantitative ELISA revealed a significant inverse correlation of NRF2 protein levels with miR-144 expression (p=0.0383) in reticulocytes (Figure 18C).

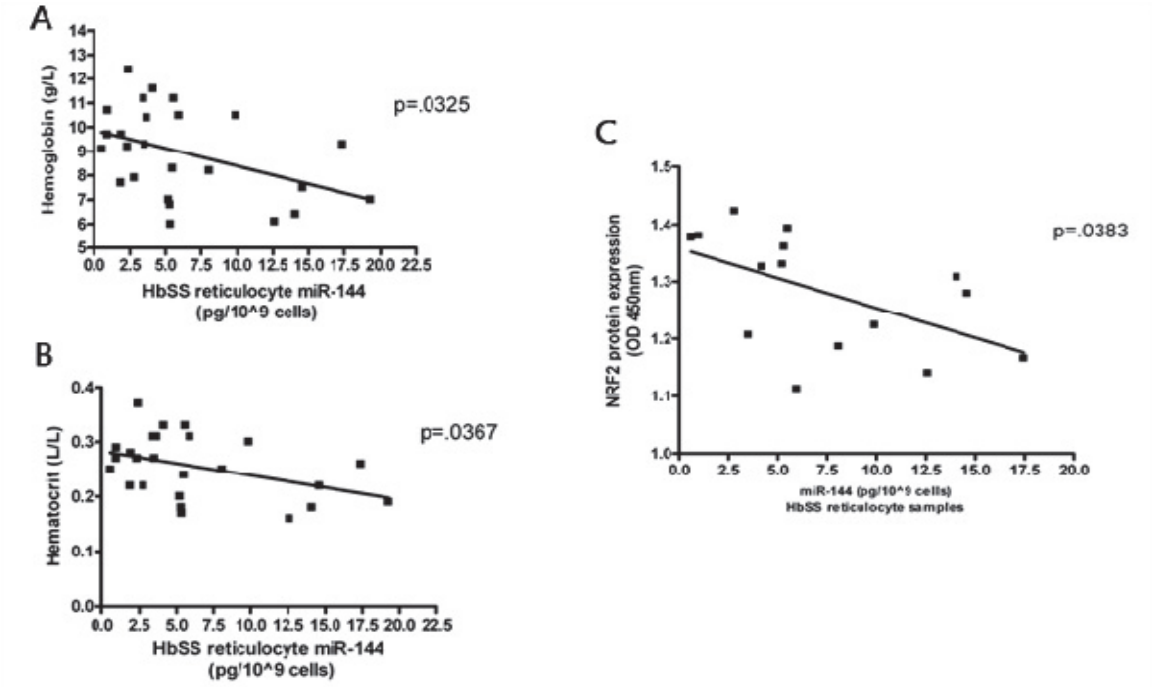


Figure 18: Correlation of HbSS reticulocyte miR-144 expression with Anemia indicators and NRF2 levels

Negative correlation of miR144 with **(A)** hemoglobin, **(B)** hematocrit, and **(C)** NRF2 protein in HbSS reticulocytes

5.4.3 MiR-144 overexpression decreases NRF2 protein levels and oxidative stress tolerance in primary HbSS erythroid progenitors

To determine the relationship between miR-144 and NRF2 during the erythroid maturation stages prior to the reticulocyte, we performed ex-vivo differentiation studies with HbSS CD34+ cells. In the purified erythroid precursor cells, we found significant upregulation of miR-144 expression and a corresponding decrease in NRF2 protein level during erythroid maturation (Figure 19A, 19B). Glycophorin A protein levels are shown as a control for successful erythroid maturation. Importantly, NRF2 protein levels were lower in HbSS compared with HbAA erythroid cells during differentiation. Both HbAA and HbSS samples respectively demonstrated significant decreases in NRF2 expression between D8 and D10 ($p=0.021$, $p<.0001$), D12 ($p<.0001$, $p=0.0002$), and D14 ($p=0.026$, $p=0.013$). In HbAA samples, expression change between D10 and D12 is statistically significant ($p=0.049$).

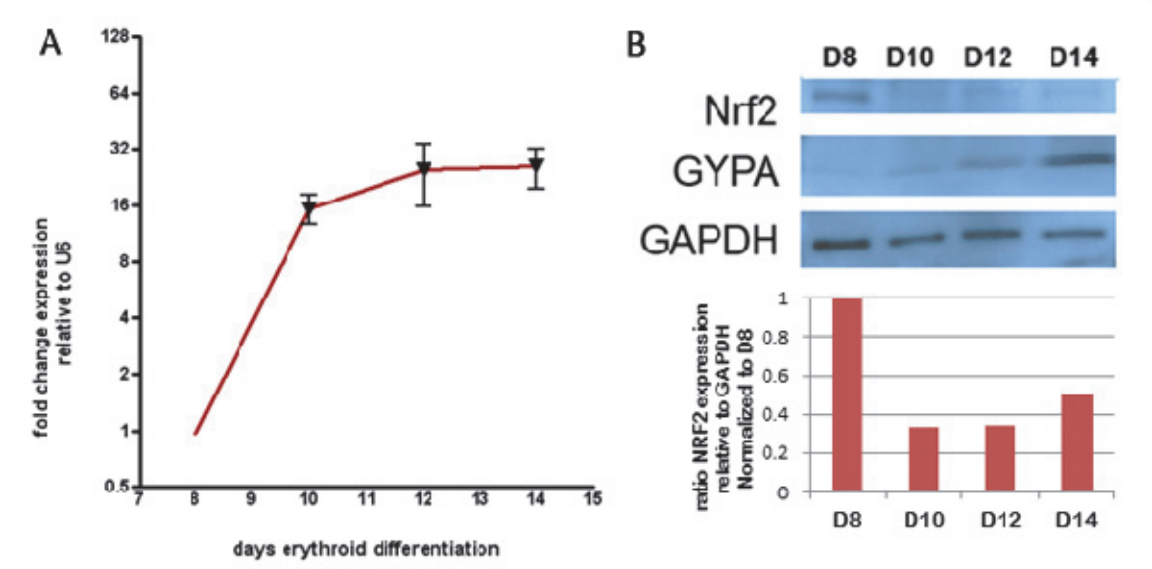


Figure 19: MiR-144 and NRF2 expression during ex-vivo HbSS erythroid maturation
MiR-144 expression by qRT-PCR **(A)**, Western blot **(B)** and densitometric analyses **(C)** of NRF2 expression during erythroid maturation

5.5 Model for the Functional Role of miR-144 in SCD pathobiology

Taken together, our study suggests a model in which erythroid precursor miR-144 expression modulates NRF2 expression, alters oxidative stress tolerance, and subsequently influences the reduced ability of mature RBCs to tolerate oxidative stress present in SCD, thus contributing to the clinical severity and erythrocyte properties seen in HbSS patients (Figure 20).

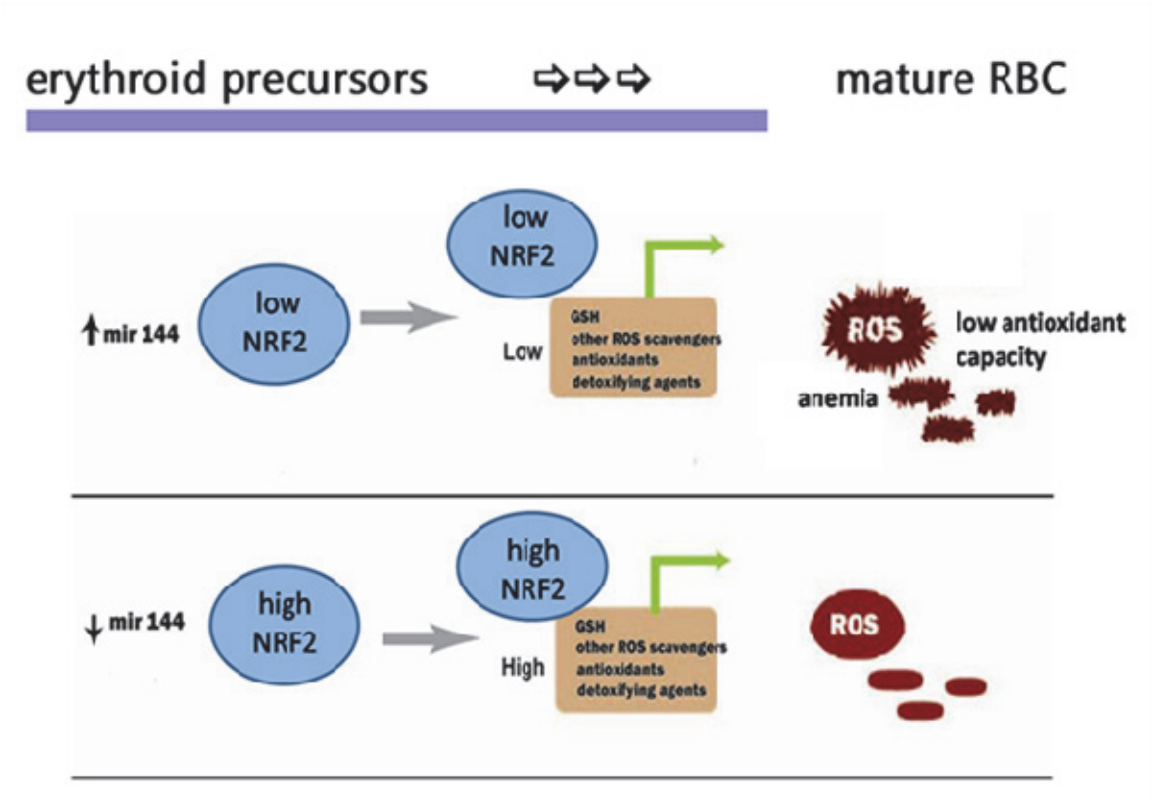


Figure 20: Model of the functional consequences of miR-144 overexpression in SCD

High miR-144 levels during erythroid precursor maturation leads to low NRF2 levels, low antioxidant capacity for the mature erythrocyte, greater susceptibility to oxidative stress, and an association with more severe anemia **(top)**, whereas normal/low miR-144 levels during erythroid precursor maturation leads to high NRF2 levels, high antioxidant capacity for the mature erythrocyte, and a greater oxidative stress response **(bottom)**.

6. Conclusions and Perspectives I

6.1 Conclusions

The microRNA profiles of erythrocytes are likely to reflect the complex, dynamic, and multi-dimensional processes between erythrocyte production (erythropoiesis) and destruction (turnover kinetics) that influence their phenotypes and clinical manifestations. In this set of studies, we have used global erythrocyte microRNA expression to uncover heterogeneity among SCD patients based on high miR-144 expression, and have found an association with increased anemia severity. We have discovered the miR-144-NRF2 regulatory axis and have found it to be present in erythroid cells. We have observed the downregulation of miR-144 expression during oxidative stress and the concurrent expression of NRF2, which coordinately regulates the oxidative stress response. Conversely, we have demonstrated that high miR-144 expression during oxidative stress leads to the miR-144-mediated direct repression of NRF2.

Since NRF2 is a transcription factor that functions in the nuclei of mammalian cells, it is unlikely that it is functional in the same role in mature erythrocytes. However, NRF2 can play an important role in oxidative stress tolerance during the erythroid developmental and survival until maturity. A hemolytic anemia phenotype with decreased antioxidant capacity has previously been seen in mice with disrupted NRF2 loci (Lee, Chan et al. 2004), thus supporting the functional relevance of NRF2 for the erythrocyte. Antioxidant capacity, as illustrated by glutathione concentration, is a feature that is of vital importance to survival and resistance to hemolysis in the mature cell, where the ability to regenerate machinery damaged by oxidative stress is absent. Therefore, the high level of miR-144 expression and reduced levels of NRF2 and ARE-driven gene products such as glutathione and superoxide dismutase in the mature erythrocyte together comprise a plausible mechanism for the higher anemia severity among a subset of SCD patients. Furthermore, we know that the higher levels of reactive oxygen species

and lower antioxidant proteins are a natural feature of HbSS compared to HbAA erythrocytes. Thus our finding of the cell autonomous high level of miR-144 expression and lower levels of NRF2 protein observed in SCD (compared to HbAA) erythroid progenitors is also consistent with a known but previously unexplained phenotype.

In SCD, it is important that effective and efficient commensurate erythropoiesis in the bone marrow can compensate for increased hemolysis. When erythrocyte loss in SCD becomes excessive, one compensatory mechanism is that of augmented erythropoiesis (termed 'stress erythropoiesis'), which occurs in order to quickly counteract this loss. One manifestation of stress erythropoiesis is an increased level of reticulocytes, such as is seen in patients with SCD. However, the increased reticulocyte levels seen in SCD patients do not always lead to improvement of hemoglobin and hematocrit levels. We wonder why stress erythropoiesis in SCD can better compensate for anemia in some patients than in others. There is evidence that this stress erythropoiesis response is distinct from normal erythropoiesis and involves different microenvironmental factors, progenitor cell characteristics, cytokine growth factors and signaling pathways (Longmore 2006; Socolovsky 2007). The observed heterogeneity among the SCD erythrocyte microRNA expression profile may additionally be illustrative of differences among SCD patients in this ability to compensate during stress erythropoiesis.

6.2 Future Directions

Our findings have several important clinical implications and areas for further study in the future. First, these observations indicate that erythroid microRNAs may contribute to the complex regulatory network of disease modifiers in SCD pathobiology and can aid in the identification and dissection of heterogeneity in clinical phenotypes. When combined with genetic polymorphisms associated with risk for certain SCD clinical manifestations such as stroke (Hoppe, Klitz et al. 2003; Hoppe, Klitz et al. 2004; Sebastiani, Ramoni et al. 2005), microRNA

expression profiles are likely to enhance our ability to further identify risk of specific complications and disease severity. Second, our data shows that miR-144 expression in erythrocytes may serve as useful molecular markers to identify patients who are likely to benefit from treatment with antioxidants or NRF2 inducers (Thimmulappa, Mai et al. 2002; Ahn, Hwang et al. 2010) given their relatively lower level of NRF2 and antioxidant response. Third, in addition to SCD, other anemia disorders (e.g. thalassemia and G6PD-deficiency) also manifest enhanced oxidative stress and impaired antioxidant status in their erythrocytes. It will be important to determine whether the dysregulation of miR-144 or other microRNAs may also play a role in their reduced ability to deal with oxidative stress and subsequent susceptibility to hemolysis. Lastly, these results indicate the potential of using microRNA expression profiles as an indicator for the quality of mature erythrocytes, either *in vivo* during physiological and pathological adaptations or *in vitro* during the storage of blood products. The unknown functions and the regulation of expression of other erythroid-specific microRNAs could provide further clues in order to elucidate the remaining mysteries of SCD and other disorders of erythroid biology.

6.3 Perspective: Function of the miR-144/451 locus

Although miR-144 is transcribed as part of a bicistronic transcript with miR-451, we show that only miR-144 is associated with anemia severity in SCD. This difference suggests that differential processing between the primary and mature forms of miR-144 may play a role in the observed elevation of miR-144 in a subset of HbSS patients. In our studies we found that while miR-451 was also expressed at a significantly higher level in the erythrocytes of the high miR-144, more severely anemic subset of HbSS patients, the ability of miR-451 as a predictor to

accurately separate the two HbSS groups was not as significant as using miR-144. Furthermore miR-451 was not found to have a predicted site on the NRF2 3' UTR.

Recent studies have found that during normal erythropoiesis, the *miR-144/451* locus is important in the erythroid differentiation, homeostasis, and fine-tuning of gene expression in erythroid cells in zebrafish (Dore, Amigo et al. 2008; Fu, Du et al. 2009; Pase, Layton et al. 2009). Recent reports have also shown that in mice, disruption of this locus leads to a mild anemia (Rasmussen, Simmini et al. 2010; Yu, dos Santos et al. 2010). While it is apparent that miR-451 is of greater importance in erythropoiesis, due to the fact that its expression alone has been reported to rescue the defective erythropoiesis caused by knockout of the complete locus (Dore, Amigo et al. 2008; Pase, Layton et al. 2009), this may not be the case for the other phenotypes for which this locus is important, such as for homeostasis under stress conditions in mice (Papapetrou, Korkola et al. 2010). Thus far, all of the current literature have reported investigations of either the complete disruption of the *144/451* locus or the selective disruption of miR-451, so there are no published studies yet using a loss-of-function approach to decipher the precise function of miR-144 in the erythroid cell.

Of interest, there have been two recent papers (published around the same time as our findings) which report that loss of the miR-144/451 locus (Yu, dos Santos et al. 2010) or selective disruption of miR-451 (Patrick, Zhang et al. 2010) exacerbates the effects of oxidative stress in erythroid cells. Both studies identify miR-451 as targeting a phospho-serine/threonine binding protein encoded by *ywhaz* (14-3-3zeta) that is partially important for the nuclear localization of the transcription factor FoxO3. FoxO3 has been reported to contribute to the regulation of oxidative stress, maturation, and lifespan of erythroid cells during erythropoiesis (Marinkovic, Zhang et al. 2007). Patrick et al. place emphasis on the finding that miR-451 loss of function leads to a reduction in hematocrit, an erythroid differentiation defect, and ineffective

erythropoiesis in response to oxidative stress, and claim based on their studies that the *ywhaz* product may play a role in hematopoiesis as a regulator of erythroid differentiation. Yu et al. focus on the relationship between 14-3-3zeta and FoxO3 nuclear accumulation and claim with more emphasis that miR-451 protects against erythroid oxidant stress, yet the majority of their studies are done in mice with knockout of the complete miR-*144/451* locus. Thus the individual contributions of miR-451 and miR-144 to the oxidative stress response phenotype are yet to be determined. In our studies, the enforced expression of miR-144 in K562 cells did not alter significantly alter miR-451 levels at baseline (Figure 9), but a more rigorous look at the relationship between the two microRNAs under different conditions relevant to the erythroid cell (erythropoiesis, stress conditions) is warranted.

Interestingly, although the miR-*144/451* locus is co-transcribed, there are 7332 predicted sites for miR-144 in the human genome compared to 1605 predicted sites for miR-451 (Betel, Wilson et al. 2008). Only 821 sites are predicted to be shared between these two microRNAs. Thus a majority of the predicted miR-451 sites are shared by miR-144, yet only a fraction of miR-144 predicted sites are shared by miR-451. Given the stability and long half-life of microRNAs, the processes of differential regulation are just beginning to be understood in other model organisms (Ramachandran and Chen 2008; Chatterjee and Grosshans 2009). Therefore the differential regulation of miR-144 and miR-451 presents an attractive model to dissect microRNA-specific processing and decay in mammalian cells.

6.4 Perspective: MiR-144, NRF2, and Fetal Hemoglobin in SCD

Two known influences on the severity of anemia in SCD are expression of fetal hemoglobin (hemoglobin F or HbF), which is associated with protection from sickling (Dover, Chang et al. 1987; Platt, Brambilla et al. 1994; Franco, Lohmann et al. 1998), and the coinheritance of alpha-thalessemia, which is associated with improved erythrocyte hydration,

increased hemoglobin concentration, and increased cell survival (Embury 1994; Hoffman 2005). HbF does not incorporate with sickle hemoglobin polymers, thus inhibiting the major mechanism of erythrocyte damage and clinical sequellae in SCD. This fact, in addition to its inverse association with mortality (Castro, Brambilla et al. 1994; Platt, Brambilla et al. 1994) is one of the reasons that the HbF inducer hydroxyurea is the sole FDA-approved agent for treating SCD. However, hydroxyurea is only effective in approximately half of SCD patients (Steinberg, Lu et al. 1997). Many other pharmacological inducers of HbF expression are cytotoxic or can suppress erythropoiesis.

In a recent publication, Macari et al. have discovered that gamma-globin, one of the components of HbF, has a functional antioxidant response element that is responsive to NRF2 (Macari and Lowrey 2011). They have shown that inducers of the NRF2 signalling pathway can increase gamma-globin mRNA levels in human erythroid progenitors, leading to increased levels of HbF . This new information fits our model and emphasizes the role of NRF2 for the prevention of oxidative stress and the additional amelioration of sickling by a new mechanism—HbF production--that has been closely studied in SCD for decades. The pharmalogical induction of HbF by a NRF2-inducing mechanism can potentially be the next standard-of-care therapeutic for SCD.

6.5 Perspective: microRNAs in cellular stress response

Cells are able to adapt their gene expression programs by selectively translating certain messenger RNAs while repressing translation of others, and microRNAs have been implicated as mediators of this selective translation during states of stress. There is an emerging concept that microRNAs may be more essential for responding to changing environments and stress conditions or fine-tuning physiological functions than for viability or development (reviewed in (Leung and Sharp 2010).

In this set of studies examining the consequence of enforced miR-144 expression, we often observed detectable changes in target protein, antioxidant gene expression and glutathione levels at baseline, but a much more salient phenotype for all of these were seen only under the oxidative stress condition. Because stress conditions can alter the biogenesis of microRNAs (at the levels of transcription, processing, or stability), the expression and actions of mRNA targets, and the activities of miRNA-protein complexes, it is of great interest to investigate the roles for microRNAs not only under baseline states, but particularly in the stress condition when the cells are under some phenotypic crisis. The roles for microRNAs under stress can thus vary significantly from their roles at baseline. The discovery of the integrated networks between microRNAs, transcription factors, RNA-binding proteins, and their collective targets will allow a fuller description of the stress response program.

The strategic location of microRNAs within these regulatory networks can determine their functions and functional relevance even if their direct contributions are subtle. In our studies we focus on the actions of miR-144 on the transcription factor NRF2. Because the effect of miR-144 on NRF2 is then amplified due to the array of targets that it activates, much more salient downstream phenotypes of decreased oxidative stress tolerance and glutathione levels (Figure 14, Figure 15) under the oxidative stress condition.

The timing of dysregulation is also an important aspect of microRNA-mediated regulation. In our studies we see a much more amplified phenotype precisely under the condition when NRF2 is needed for the oxidative stress response. Furthermore, miR-144 is necessary for the development of the erythroid cell, its expression is required in the erythroid cell, and its enforced expression during erythropoiesis is not pathologic under normal erythropoiesis, yet we show that the timing of dysregulation (early erythroid maturation) in HbSS erythroid progenitors can impact the antioxidant capacity of the mature erythrocyte.

Our first study demonstrates a role for microRNA in the regulation of oxidative stress response in erythroid cells and the functional consequences of dysregulated microRNA expression in Sickle Cell Disease pathobiology. The second study examines a functional role for microRNA in the cellular response to changes in cellular iron concentration. Together these studies illustrate the scope of importance of microRNAs at a very basic level of biology: in the coordination of cellular responses to diverse stresses.

7. Post-transcriptional Regulation of Intracellular Iron Concentration

Iron is an essential nutrient that is necessary for all cells. The maintenance of cellular iron homeostasis involves RNA-binding proteins (RBPs) in the post-transcriptional coordination of iron uptake, utilization, and storage in order to prevent toxicity to the cell while ensuring availability when appropriate (Wallander, Leibold et al. 2006; Andrews 2008). This exquisite and well-characterized regulatory system uses two RBPs, iron regulatory proteins (IRP) 1 and 2, to bind sequence specific iron response/regulatory elements (IREs) in the 5' UTRs of target mRNAs to inhibit translation and in the 3'UTR to increase mRNA stability (Muckenthaler, Galy et al. 2008).

7.1 The IRP/IRE system

The IRE was first identified as a conserved cis-acting sequence element in the 5'UTR of one of the most well-known iron-regulated mRNAs, ferritin, and was found to be necessary and sufficient for the translational regulation of ferritin expression in response to iron (Aziz and Munro 1987; Hentze, Caughman et al. 1987). These IREs form a characteristic stem-loop secondary structure, shown to be prevalent across iron-regulated targets (Hentze, Caughman et al. 1987; Casey, Hentze et al. 1988). Shortly after the discovery of IREs, iron regulatory proteins were identified for their ability to recognize and bind to these IREs (Caughman, Hentze et al. 1988; Leibold and Munro 1988; Rouault, Hentze et al. 1988). IRP1 and IRP2 both have the ability to bind to IREs, but differ with respect to target RNA sequence recognition and binding specificity (Butt, Kim et al. 1996).

The functionality of the IRPs as RNA-binding proteins is itself regulated by iron. IRP1 is regulated in terms of its bi-functionality as an aconitase enzyme or RNA-binding protein, and IRP2 is regulated at the level of protein stability. IRP1 functions as a cytosolic aconitase (ACO1)

when cellular iron levels are appropriate and as an RNA-binding protein when iron levels are deficient. These activities are mutually exclusive, and the regulatory switch is based on the incorporation of an iron-sulfur cluster when iron is prevalent and the disassembly of this cluster when iron is scarce (Haile, Rouault et al. 1992). In contrast to IRP1, IRP2 is regulated at the level of protein stability. Under cellular iron deficiency, IRP2 accumulates and is available as an RNA-binding protein. When iron is abundant, the F-box substrate adaptor protein FBXL5 associates as part of the SKP1-CUL1-FBXL5 ubiquitin-ligase protein complex and promotes the iron-dependent ubiquitination and degradation of IRP2. FBXL5 is itself degraded during iron deficiency, thus allowing for IRP2 accumulation (Salahudeen, Thompson et al. 2009; Vashisht, Zumbrennen et al. 2009).

7.2 The IRP/IRE system and regulation of cellular iron homeostasis

In iron-deficient cells, IRPs target single IREs located in the 5'UTRs of target mRNAs involved in iron storage (ferritin heavy and light subunits, FtH1 and FtL), utilization (ALAS2, for heme synthesis), and export (ferroportin, FPN). At the same time, the multiple IREs present in the 3'UTR of transferrin receptor 1 (TFRC for cellular iron uptake) are bound and stabilized by IRP1 and IRP2 (Figure 21). Thus, iron-deplete cells experience increased iron uptake by TFRC and decreased export by FPN, storage by ferritin, and utilization by ALAS2 for heme synthesis (in erythroid cells). In iron-replete cells, the cellular expression program is reversed: iron no longer needs to be imported by TFRC, iron is appropriately exported by FPN, stored by ferritin, and used for heme synthesis by ALAS2 (Hentze and Kuhn 1996; Hentze, Muckenthaler et al. 2004) (Figure 21).

While IRP/IRE interactions are crucial for many iron-responsive mRNA targets, there are still unknown actors and mechanisms involved in control of the expression and activity of IRPs and members of the IRP regulon. The continuous state of destabilization and degradation of

TFRC mRNA during iron repletion is performed by an as- yet-unknown iron-controlled endonuclease (Binder, Horowitz et al. 1994). The iron-regulated ubiquitin-ligase FBXL5 regulates IRP2 protein stability during iron repletion, yet is itself also degraded during iron deficiency by an unknown actor. Each newly discovered iron sensor has subsequently been found to be indirectly regulated by iron. Whether microRNAs are involved in regulation of cellular iron homeostasis is unknown. Since microRNAs have been shown to mediate cellular responses to nutrient, oxidative, and hypoxic stresses, we wonder whether microRNAs might mediate the intracellular response to iron by regulating targets with roles in cellular iron homeostasis.

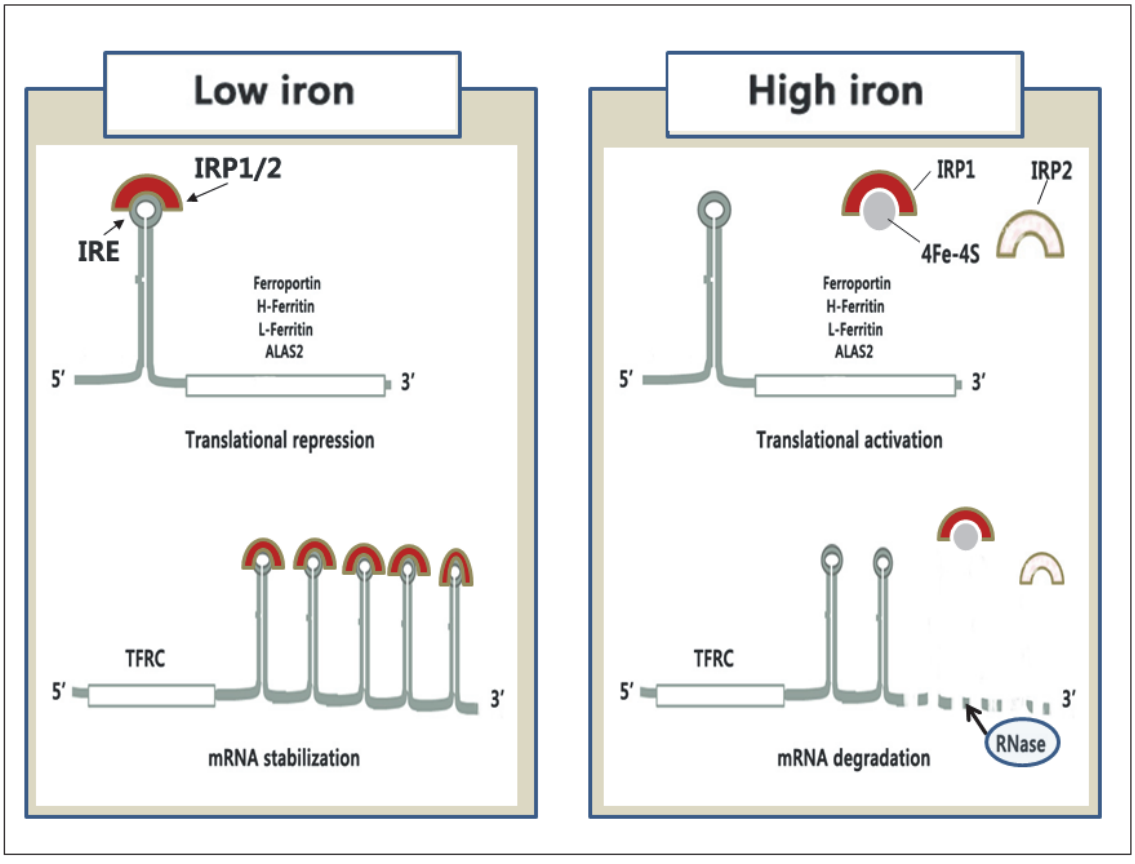


Figure 21: Cellular regulation of mammalian iron homeostasis by the IRP/IRE system

7.3 Identification of iron-regulated microRNAs

To identify microRNAs that are regulated by iron levels, we used the quantitative Real-Time PCR Taqman Low Density Array (TLDA) to measure the expression of microRNAs from K562 cells treated with the iron chelator desferrioxamine (DFE) to induce iron deficiency, treated with ferric ammonium citrate (FAC) to induce an iron-rich condition, or mock-treated to represent the baseline state (Figure 22). Of the 300 microRNAs expressed in these conditions, the expressions of 44 microRNAs were found to correlate with iron concentration (Figure 22, Table 4). Among this subset of microRNAs, we focused on microRNAs with predicted targets important in cellular iron metabolism. We confirmed the TLDA expression results for miR-30a*, miR-194, miR-149, miR-485-3p, and miR-502-3p in additional independent studies with K562 cells (Figure 23, Table 5). We were not able to confirm the results for miR-744* and miR-103 (not shown). To determine if this response could be seen in other cell types, the iron stress conditions were induced in biological replicates from K562 (human erythroid), HEL (human erythroid), and HEK293 (human embryonic kidney) cell lines and microRNA expression was measured using TaqMan mature microRNA Real-time assays. We found that the most uniform and significant change across these cell lines was in the increased expression of miR-485-3p during iron deficiency (Figure 23, Table 5). In addition, we identified miR-485-3p to be among 30 microRNAs with at least 1.5 fold expression difference under DFE-induced iron-deficiency in global analysis of microRNA expression in HEK293 (Figure A2).

MiR-485-3p was first isolated and cloned from human fetal liver, and has also been found to be expressed in the adult liver (Fu, Tie et al. 2005). Since the liver is one of the most important organs of the body for the production and storage of nutrients as well for the detoxification and the removal of waste products from the blood, the expression of miR-485-3p within this organ system suggests its potential to play a role in dealing with environmental stress

responses. Using gene ontology and literature resources, we found that among the 5155 predicted targets for miR-485-3p, there are four that are involved in cellular iron metabolism: SLC40A1, ISCU, HEPH, and NDFIP1.

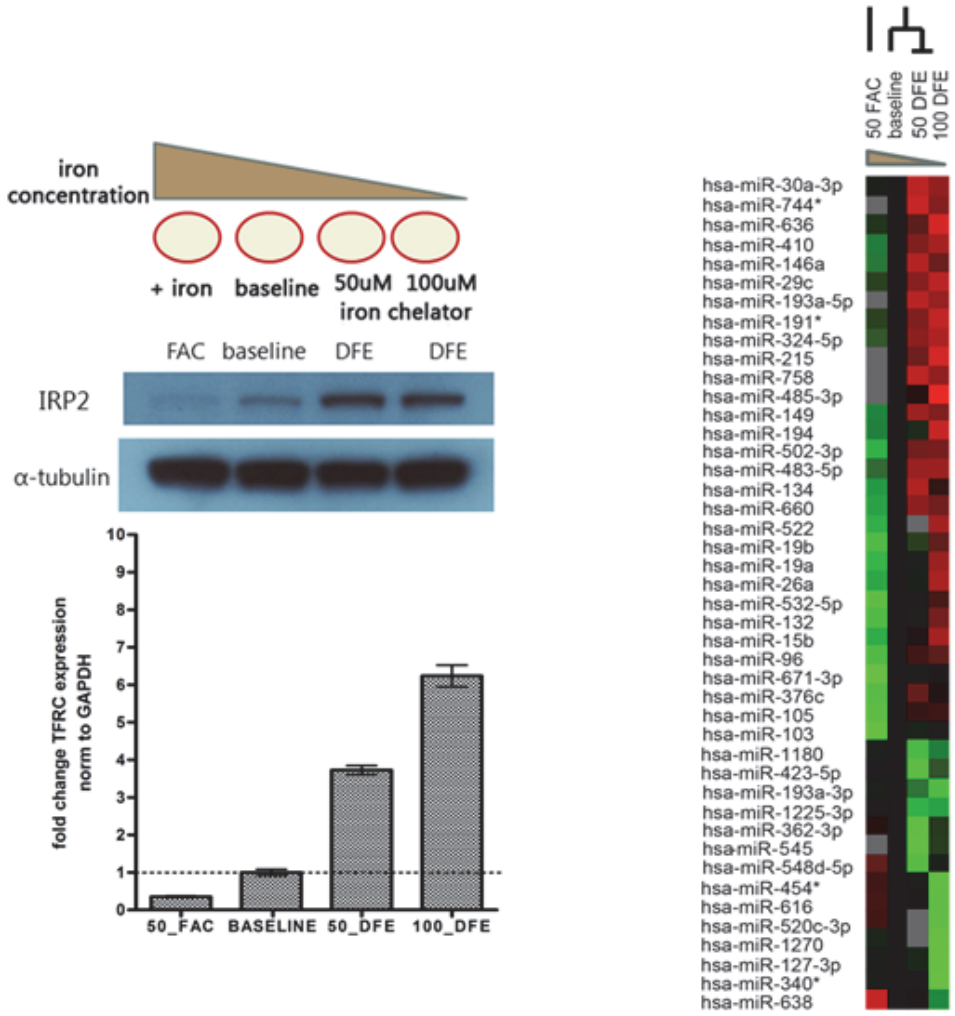


Figure 22: K562 model for cellular iron regulation and microRNA expression analysis

(left side) Schematic of iron treatments to achieve iron-rich (FAC) and iron-deficient conditions (DFE) (top), western blot analysis of IRP2 expression, with tubulin control, in iron conditions (middle), TFRC mRNA expression in indicated iron conditions (bottom)
(right side) Heatmap representation of expression of iron-regulated microRNA by TLDA anlysis

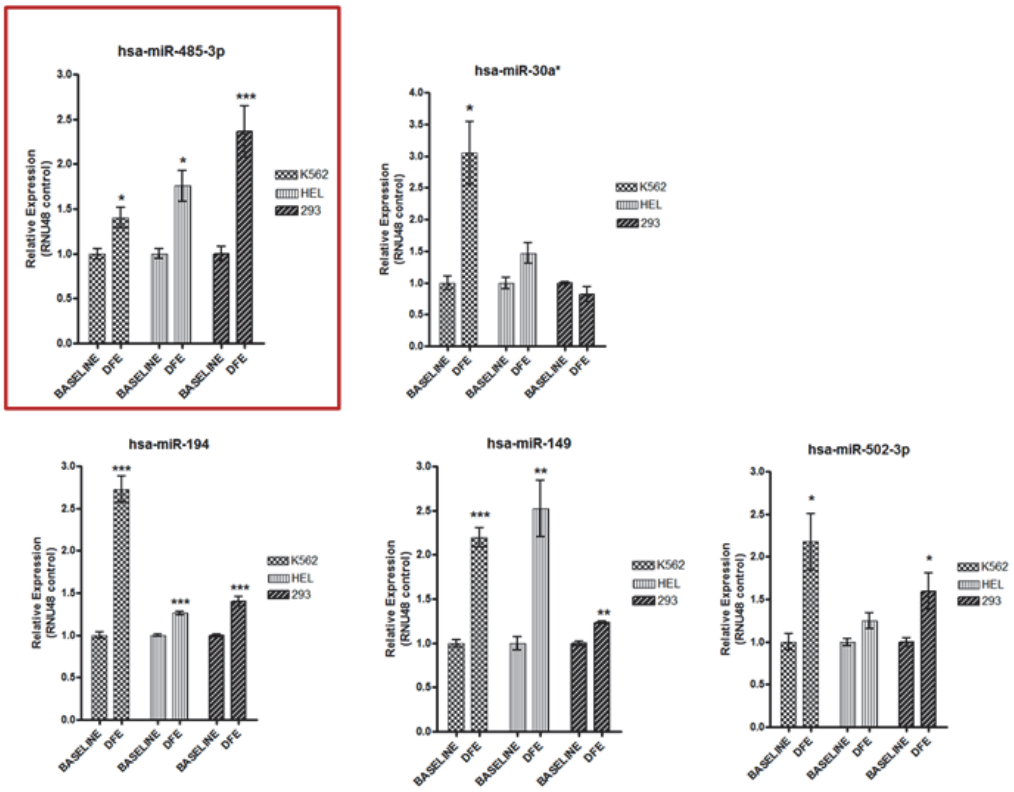


Figure 23: Validation of selected iron-regulated microRNA expression across cell lines

Expression of indicated microRNAs under baseline or iron-deficient condition in K562, HEL, and HEK293

7.3.1.1 Materials and Methods

7.3.1.1.1 In-vitro regulation of Cellular Iron Condition

For iron depletion, cells were treated with DFE (50uM or 100uM) diluted in PBS and added to the media for indicated time intervals (16-24 hours). For iron supplementation, cells were treated with FAC (500nM) diluted in PBS and added to the media for indicated time intervals (16-24 hours).

7.3.1.1.2 TLDA and MicroRNA expression qRT-PCR assays

We performed a quantitative real-time RT-PCR analysis using TaqMan Low Density Arrays (TLDA) Human microRNA Panel (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. For validation of results we used Taqman mature microRNA assays (Applied Biosystems).

7.4 Ferroportin: a predicted target for miR-485-3p

Ferroportin (FPN) is the product of the *SLC40A1* gene (aliases: FPN1, IREG1, MTP1) and functions as the sole known cellular iron exporter (Abboud and Haile 2000; McKie, Marciani et al. 2000; Donovan, Lima et al. 2005). Within the cell, the expression of FPN is post-transcriptionally regulated by the IRP/IRE system through a 5'UTR iron response element (Lymboussaki, Pignatti et al. 2003). FPN plays a critical role in systemic mammalian iron homeostasis and the general transfer of iron from the cells where it is processed and stored to the plasma for distribution to the tissues of the body. Its expression is abundant in cells known to export iron: duodenal enterocytes (iron uptake and export to the circulation), hepatocytes (site of iron storage), syncytiotrophoblasts (maternal transfer of iron to the embryo), and reticuloendothelial macrophages (iron recycling from senescent erythrocytes). Ferroportin is also present in erythroid precursors, lungs, kidneys, nervous system, and muscles (Abboud and Haile 2000; Yang, Haile et al. 2005; Ganz and Nemeth 2011).

Genetic iron disorders have provided insight into the mechanism of FPN in both maintenance of both cellular and systemic iron balance. Loss-of-function mutations have been found to lead to problems with protein localization to the cell membrane for iron export, thus manifesting chiefly as cellular iron overload, predominantly in organs containing abundant macrophages such as the liver, spleen, and bone marrow (Montosi, Donovan et al. 2001; Njajou,

Vaessen et al. 2001). Gain of function mutations in FPN cause the cell to become insensitive to the iron regulatory hormone, hepcidin (De Domenico, Ward et al. 2005; Drakesmith, Schimanski et al. 2005; Schimanski, Drakesmith et al. 2005). Hepcidin binds to ferroportin on the cell-surface and triggers its phosporylation, internalization, and ubiquitin-mediated degradation in the lysosomes (Nemeth, Tuttle et al. 2004; De Domenico, Ward et al. 2007). Therefore extracellular regulation by hepcidin leads to the removal of ferroportin from the cell membrane and a shut-down of cellular iron export. Insensitivity to this 'off-switch' thus results in continued export of iron to the plasma circulation and eventually leads to systemic iron overload in the form of hemochromatosis.

7.5 Evidence of IRP-independent regulation of Ferroportin mRNA

For the study of the potential role of miR-485-3p in targeting FPN for repression, we continued to use K562 and HEK293 cells as models for cellular iron regulation and added a third cell line—HepG2 (human liver epithelial cell/ hepatocellular carcinoma)---that could lend functional relevance for FPN regulation. We demonstrate the iron-dependent translational regulation of IRP2 and FTL by western blot (Figure 24A, top) and the accumulation of TFRC mRNA due to iron-dependent stabilization in HepG2 (Figure 24A, bottom). In models of iron regulation in 293 and K562, we confirm appropriate modulation cellular iron status by TFRC mRNA expression (Figure 24B) and find that FPN mRNA levels are not altered significantly during changes in iron concentration (Figure 24C).

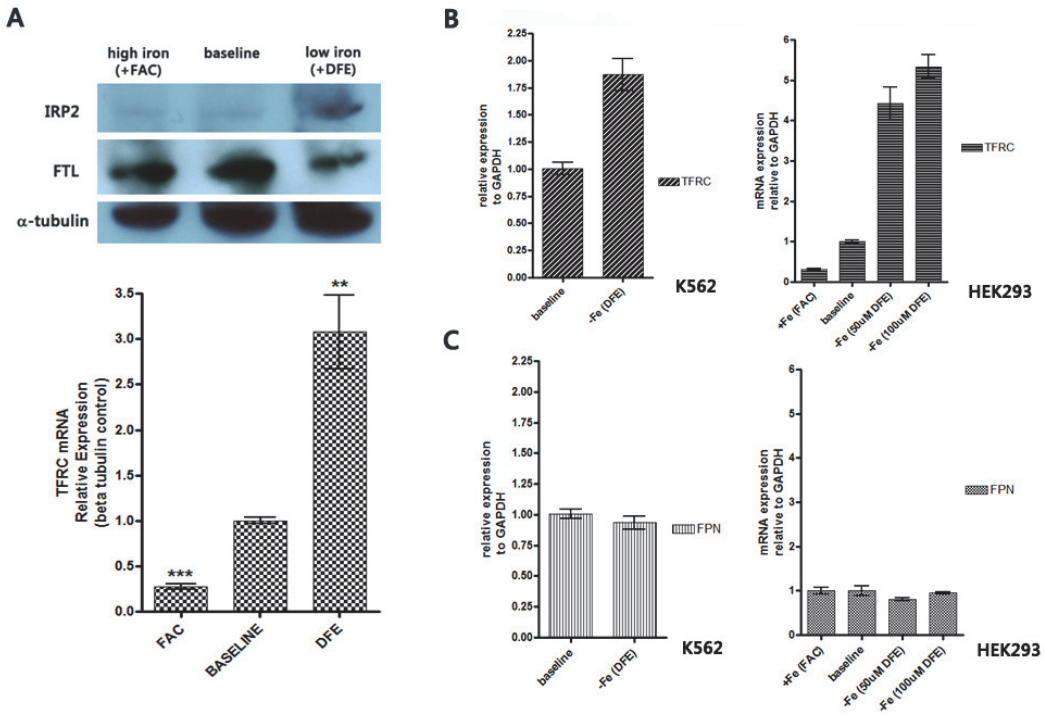


Figure 24: HepG2 model and FPN mRNA expression in cellular iron regulation

(A) Western blot analysis of iron-regulated protein expression in HepG2. **(B)** TFRC mRNA expression in baseline or iron-deficient condition in K562 (left) and HEK293 (right). **(C)** FPN mRNA expression in K562 (left) and HEK293 (right)

To demonstrate the known cellular regulation of the FPN 5'UTR and the TFRC 3'UTR by endogenous IRPs in iron replete and deficient conditions, luciferase reporters were designed based on the psiCHECK-2 (Promega) reporter system in which the expression of *Renilla* luciferase is regulated by sequences cloned into the 5'UTR or 3'UTR sites and the constituitive expression of firefly luciferase serves as an internal control. These reporters were transfected into K562, HEK293, or HepG2 cells followed by induction of the iron replete or deficient condition for 24 hours, lysis, and assay of the lysate for luciferase activity. Results were normalized to those of an empty reporter. In iron repletion, the expression level of the FPN 5'UTR reporter was significantly higher (1.88 ± 0.142, p=0.0002) than in the baseline condition,

indicating a release of repression, and the TFRC 3'UTR reporter expression was significantly lower (0.552 ± .075, p=0.0002) than baseline, demonstrating the effects of known destabilization of TFRC mRNA by endogenous endonucleolytic factors. In iron depletion, the expression level of the FPN 5'UTR reporter was significantly lower (0.437 ± 0.026, p<.0001) than in the baseline condition, demonstrating the repression of the reporter by the IRP/IRE system, and the TFRC 3'UTR reporter expression was significantly higher (2.078 ± .057, p<.0001) than baseline, demonstrating the known stabilization by IRPs. Similar results were seen in HEK293 and HepG2 (Figure 25).

To determine whether the FPN 3'UTR is a target for post-transcriptional regulation under iron replete or deficient conditions, we used a luciferase reporter with the full FPN 3'UTR. In the iron replete condition in HepG2, the expression of the FPN 3'UTR reporter was significantly higher (1.463 ± .012, p<.0025) than baseline, indicating a release of repression. During iron depletion, expression of the FPN 3'UTR reporter was significantly lower (0.437 ± .018, p<.041) than baseline, indicating that the 3'UTR is targeted for repression in this condition. Similar results were seen in K562 (Figure 26).

Thus the expression of FPN 3'UTR by itself (outside the regulation by the IRP/IRE system in the 5'UTR) is shown to be regulated by iron levels. The FPN 3'UTR is also shown to be de-repressed during iron abundance. There is the potential that microRNAs mediate these effects.

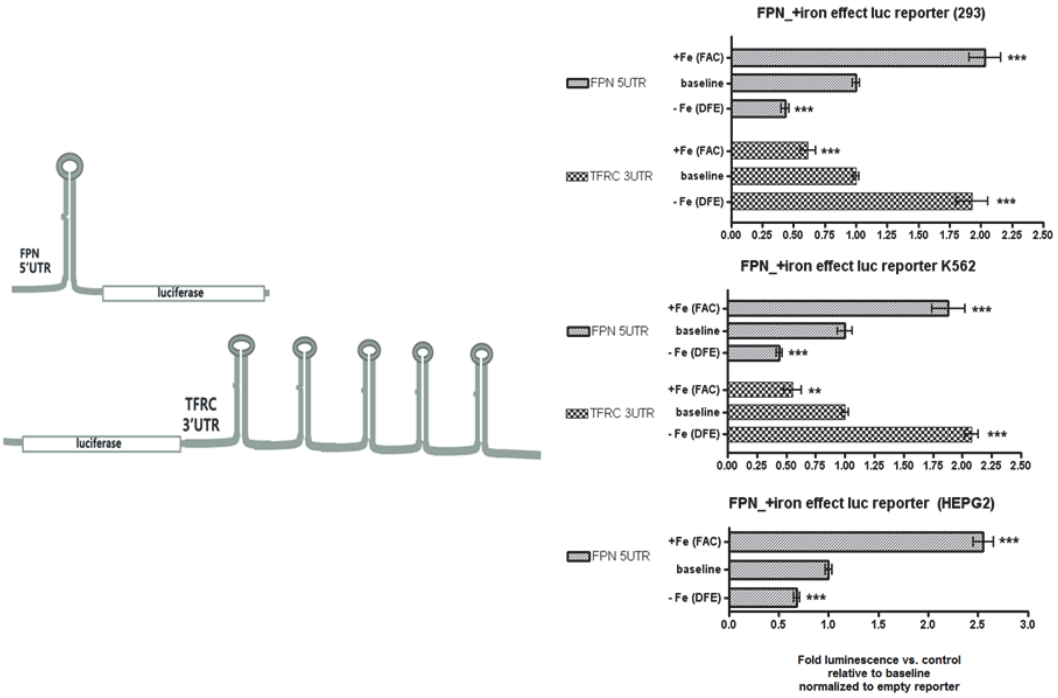


Figure 25: Iron-Dependent Regulation FPN 5'UTR and TFRC 3'UTR

(left side) schematic of FPN 5'UTR and TFRC 3'UTR luciferase reporters,
(right side) reporter assay for iron-dependent regulation of reporters in HEK293 (top), K562 (middle) and HepG2 (bottom)

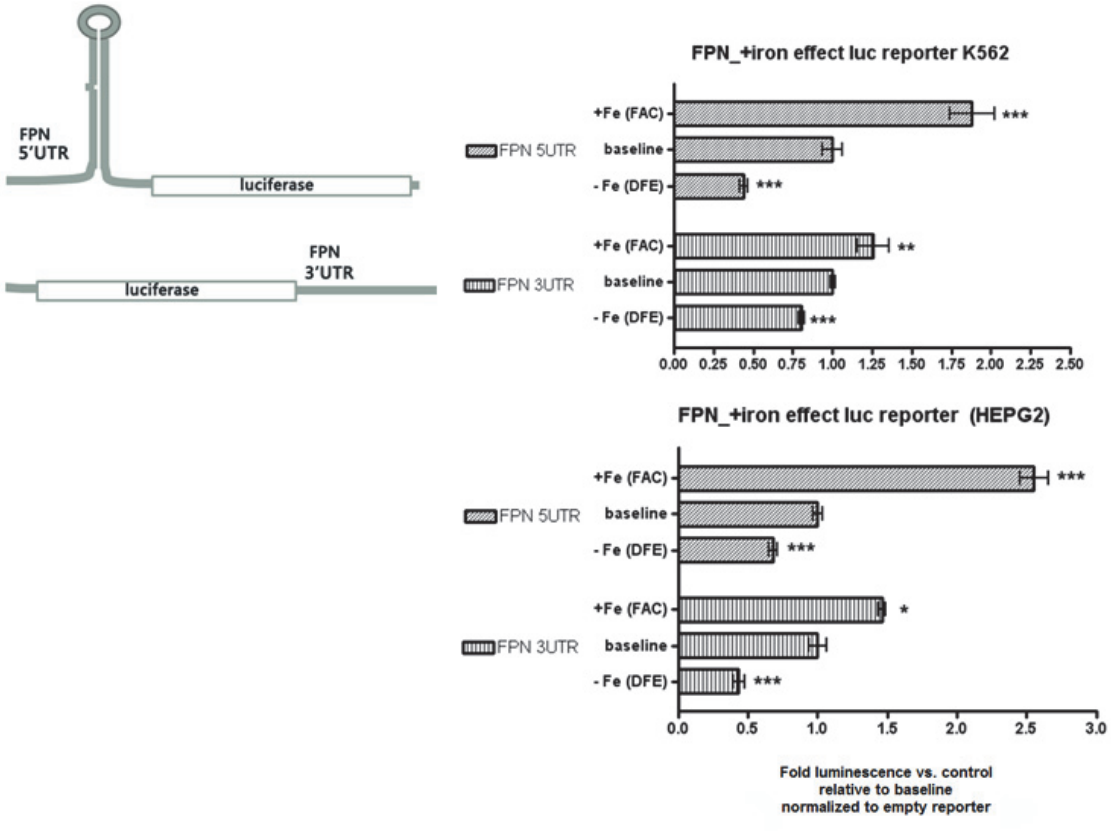


Figure 26: Iron-Dependent Regulation of the FPN 3'UTR
(**left side**) schematic of FPN 5'UTR and 3'UTR luciferase reporters
(**right side**) reporter assay for iron-dependent regulation of FPN 5'UTR and FPN 3'UTR in K562 (top) and HepG2 (bottom)

7.5.1.1 Materials and Methods

7.5.1.1.1 Western blot analysis, qRT-PCR mRNA Expression

Western blots were performed as described in (Sangokoya, Telen et al. 2010) with the following antibodies: IRP2 and FTL (Santa Cruz Biotechnology), alpha tubulin (Sigma).

7.5.1.1.2 Construction of Luciferase Reporters

A luciferase reporter was constructed with the 5'UTR of FPN cloned upstream of the sequence encoding Renilla luciferase (psi-CHECK, Promega). Luciferase reporters with the iron-regulated

region of the TFRC 3'UTR, and the full 3'UTR of FPN were also constructed using this vector, cloning into the MCS downstream of Renilla luciferase. Mutant reporters were constructed using primer-based overlapping PCR method for introducing mutations. All reporters were lipofected into K562, HEK293, and HepG2 cells using Lipofectamine 2000 or LTX (Invitrogen).

7.6 Ferroportin is a direct target of miR-485-3p

To identify FPN as a direct target of miR-485-3p, luciferase assays were performed using the FPN 3'UTR reporter and co-expression of an empty vector, miR-485-3p, or miR-744* expression construct. Results were normalized to those of an empty reporter. Overexpression of miR-485-3p significantly reduced FPN 3'UTR reporter expression to 0.501 ± .013 fold ($p<.0001$) compared to vector control, while there were no significant changes in expression (.978 ± .041, $p=0.89$) with the overexpression of miR-744* (Figure 27A).

Mutation of the FPN 3'UTR reporter at a predicted target site for miR-485-3p (FPN MT-485) led to significantly increased reporter expression (1.525 ± .093, $p=0.0009$) in comparison with the wild-type FPN 3'UTR (1.142 ± .074) and a control mutant reporter (0.998 ± .022) for an unrelated target site miR-103 (FPN MT-103) in HepG2. Similar results were seen in HEK293 (Figure 27B). Knockdown of miR-485-3p-mediated RISC activity by the transfection of 2'O-methyl-modified antisense-mediated oligonucleotides(Hutvagner, Simard et al. 2004) (AMO) complementary to the miR-485-3p sequence also led to a significant increase in FPN 3'UTR reporter expression (1.745 ± .142, $p=0.043$) compared to a control AMO (1.121 ± .210) or an AMO against miR-141 (1.102± .153, $p=0.94$), which lacks a target site on the FPN 3'UTR (Figure 27C).

The effective de-repression of FPN 3'UTR by knockdown of 485-3p-mediated RISC activity is exacerbated during iron deficiency. Compared to the baseline condition, AMO-485-

treated K562 cells demonstrated a significant increase (2.078 ± .280, p=0.0005) in FPN 3'UTR reporter expression under iron deficiency while AMO-CNTL (0.977 ± .093, p=0.82) and AMO-141-treated (0.913 ± .054, p=0.45) samples led to no significant changes. Similar results were seen in HEK293 (Figure 27D).

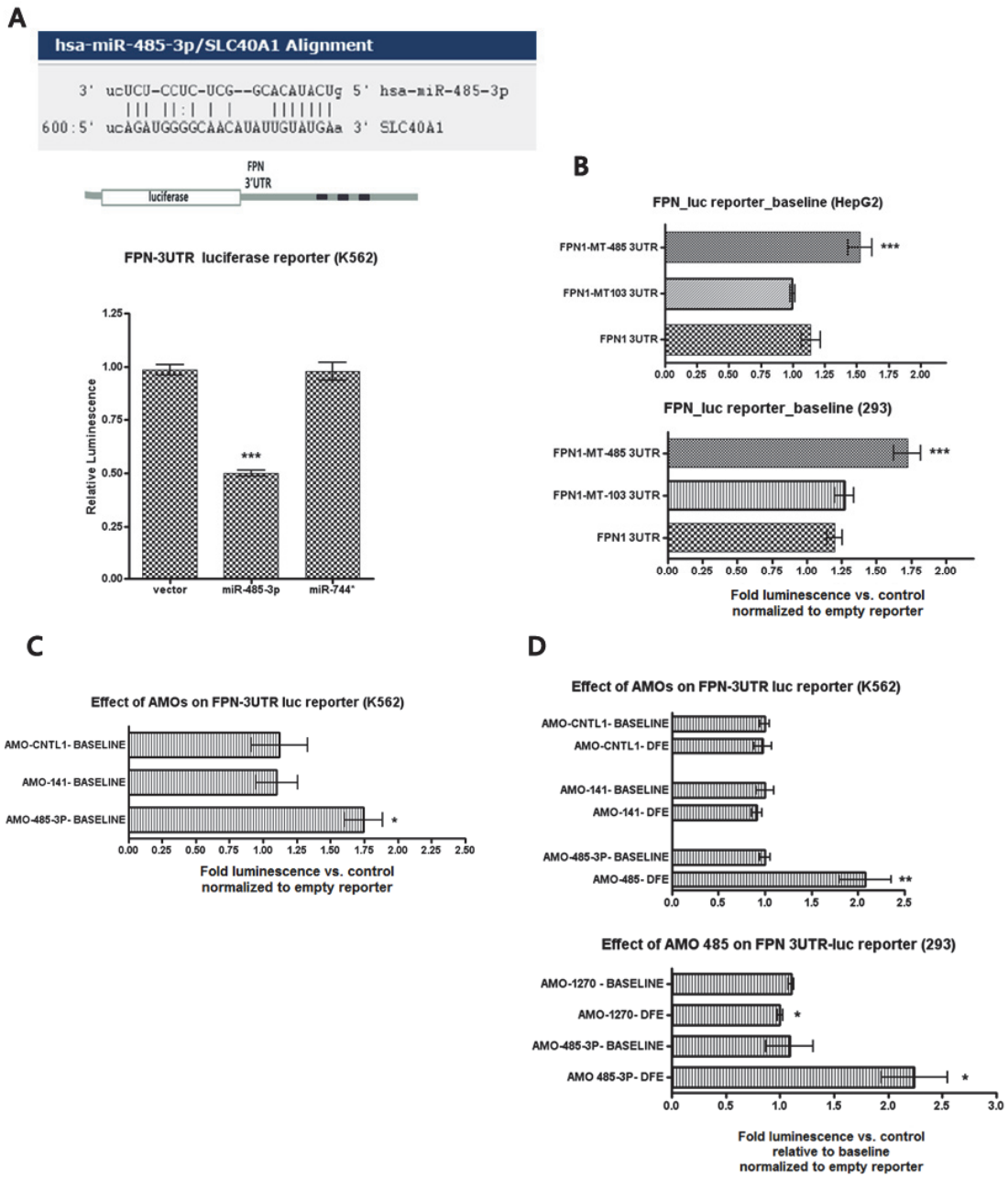


Figure 27: FPN is a direct target of miR-485-3p

A) Effect of miR-485-3p overexpression on the FPN 3'UTR reporter. **(B)** mutant versus wild-type FPN 3'UTR reporter assay in HepG2 (top) and HEK293 (bottom) . **(C)** Effect of AMOs on FPN 3'UTR reporter at baseline in K562. **(D)** Effect of AMOs on FPN 3'UTR reporter between the baseline and iron-deficient condition in K562 (top) and HEK293 (bottom)

Finally, the mutation of the miR-485-3p binding site on the FPN 3'UTR reporter to form the FPN MT-485 3'UTR reporter abolishes iron-dependent regulation of the reporter in HepG2. There is a significant decrease in wild type FPN 3'UTR (.753 ± .082, p=0.0002) and MT-103 FPN 3'UTR (.117 ± .018, p=0.015) reporter expression between the iron-replete and iron-deficient conditions, but there is no significant difference in expression of the FPN MT-485 3'UTR reporter (.021 ± .002, p=0.78) between these conditions (Figure 28).

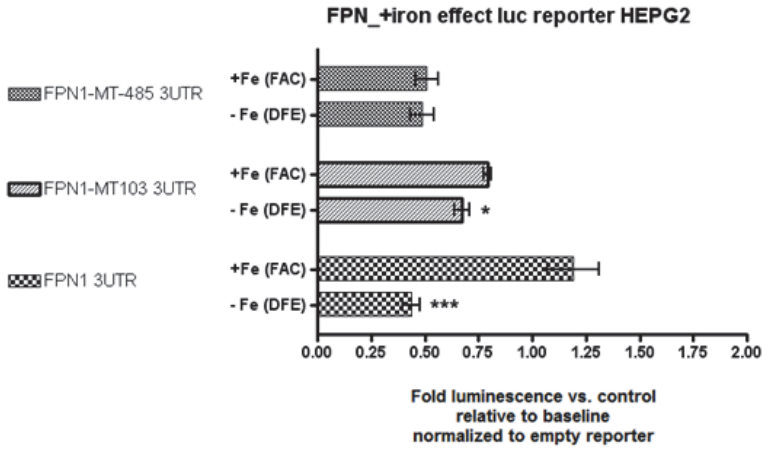


Figure 28: Loss of Iron Regulation of FPN 3'UTR with mutation of miR-485-3p target site

Expression of the FPN 3'UTR luciferase reporter demonstrates iron-dependent regulation. Loss of this regulation occurs after mutation of this reporter at the binding site for miR-485-3p (MT-485), but not with mutation at the binding site for miR-103 (MT-103).

Thus the de-repression of the FPN 3'UTR by the loss of the miR-485-3p binding site or by loss of function of miR-485-3p-mediated RISC confirms that FPN is a direct target of miR-485-3p. The loss of iron-dependent regulation associated with loss of the miR-485-3p binding site confirms the importance of this site to the FPN 3'UTR.

To determine the effect of miR-485-3p overexpression on endogenous FPN protein levels, expression constructs for miR-485-3p or vector were nucleofected into HepG2 and K562. Compared to vector control, miR-485-3p overexpression led to a reduction in the level of FPN

protein (Figure 29), and confirms the importance of miR-485-3p-mediated repression to the overall regulation of FPN.

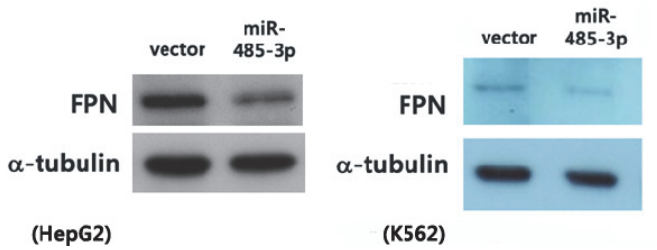


Figure 29: MiR-485-3p overexpression reduces endogenous FPN protein level

7.6.1.1 Materials and Methods

7.6.1.1.1 microRNA expression constructs, western blot

Expression constructs encoding the precursor hairpin sequences of miR-485-3p and miR-744* were each created by insertion into a CMV-based pcDNA3 cloning vector (Invitrogen, CA) that coexpresses green fluorescent protein (GFP). Western blot analysis: ferroportin antibody and antiserum were kindly provided by Tomasa Barrientos de Renshaw of the Andrews Lab.

7.7 Ago2-RNA-immunoprecipitation identifies enriched Ferroportin during cellular iron deficiency

To isolate and identify microRNA target mRNAs bound to Ago2 and the RISC, we used the direct biochemical approach of Ago2-RNA-immunoprecipitation (RIP). This method has been used successfully as a tool for microRNA target identification (Beitzinger, Peters et al. 2007; Karginov, Conaco et al. 2007; Hendrickson, Hogan et al. 2009). We induced an iron-deficient or mock-treated baseline condition in K562 cells and then performed a RIP. We found that the Ago2 precipitates from iron-deficient cells were significantly more enriched for FPN compared to those from the baseline condition (Figure 30). Beta-tubulin transcripts, which were not significantly enriched between the baseline and iron-deficient conditions, served as a control.

Since the FPN mRNA levels for the input fractions did not change between the baseline and iron-deficient state (Figure 24), we find that the enrichment of FPN within the Ago2 fraction identifies FPN is a target of the Ago2-associated RISC during the iron-deficient state.

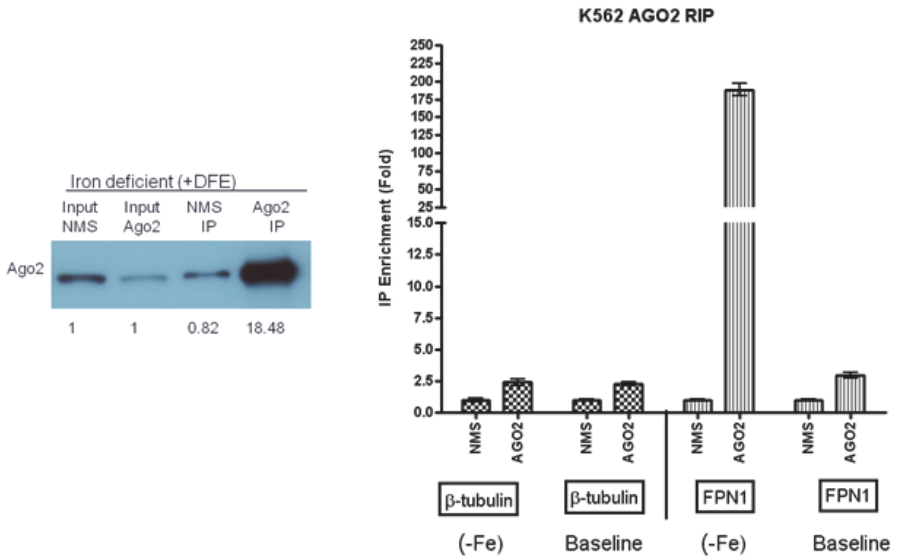


Figure 30: Ago2-RNA Immunoprecipitation shows enriched FPN in the RISC complex of iron-deficient K562 cells

Western blot (left) shows input and immunoprecipitate with normal mouse serum (NMS) control or Ago2. (right) qRT-PCR analysis of FPN and beta-tubulin mRNA expression in NMS or AGO2 IP fractions from RIP performed in baseline or iron-deficient condition

7.7.1.1 Materials and Methods

7.7.1.1.1 Ago2 RNA-Immunoprecipitation

For Ago2 RIP, a modified version of the method described in (Keene, Komisarow et al. 2006) followed by Real-time qPCR using primers specific for FPN and beta tubulin

7.8 Model for the Functional Role of miR-485-3p in Iron-Dependent Regulation of Ferroportin

Taken together, our study suggests a model in which ferroportin is regulated in an iron-dependent manner by both the IRP/IRE system and by the microRNA-mediated RISC (Figure 30). In iron deficiency, miR-485-mediated RISC binds to its target site on the 3'UTR of ferroportin and IRP1/2 binds to the IRE site in the 5'UTR. In iron abundance, expression of the 5'UTR is de-repressed by the IRP/IRE system, and concurrently, expression of the 3'UTR is de-repressed by miR-485-3p microRNA-mediated RISC, allowing full expression of ferroportin.

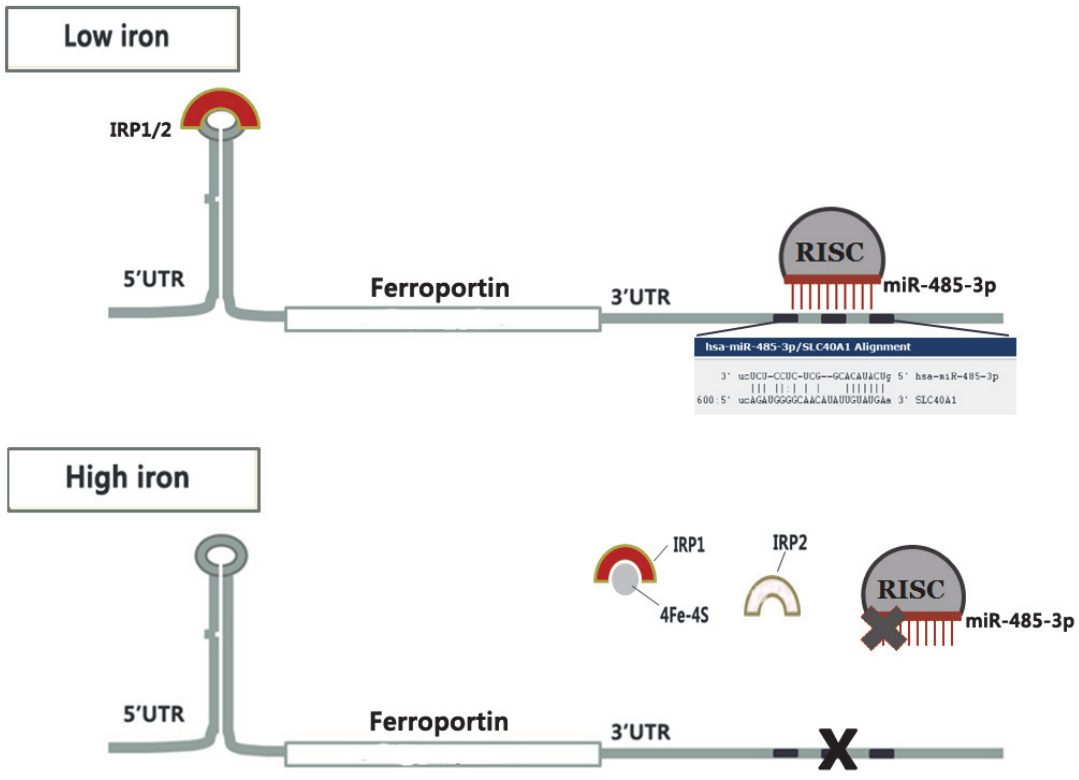


Figure 31: Model for the role of miR-485-3p and the IRP/IRE system in iron-responsive regulation of ferroportin

(top) Translational repression of FPN by the IRP/IRE system and miR-485-3p microRNA-mediated regulation of FPN transcript in the iron-deficient condition
(bottom) De-repression of FPN transcript in the iron-rich condition by both the IRP/IRE system and miR-485-mediated RISC allows for FPN expression

8. Conclusions and Perspectives II

8.1 Conclusions

In the present study, we identify iron-regulated microRNA expression and demonstrate the direct targeting of the cellular iron exporter ferroportin by one of these microRNAs---miR-485-3p. This finding of microRNA-mediated regulation of ferroportin, which is also known to be targeted by the IRP/IRE system under the same iron-dependent conditions, is of great interest as we have thus far known these processes to be mutually exclusive. We have shown that miR-485-3p overexpression is sufficient to regulate FPN on the protein level, but have not shown that it is essential. To do this, we will knockdown the activity of endogenous miR-485-3p and test if this affects iron efflux by looking for decreased iron storage by ferritin or other indices of cellular iron deficiency that would be present with increased levels of FPN. Along the same lines, since miR-485-3p usually increases during iron deficiency, then if we knockdown miR-485-3p activity during iron deficiency we would expect to see that instead of repressed levels of FPN as expected in this condition, we see normal or increased FPN levels and cell phenotype of a muted response to iron deficiency. Thus the actions of the microRNA would be shown to be an essential part of the normal response. It is possible that the IRP/IRE 5'UTR interaction can simply be influenced and specifically tuned by the actions of the microRNA-mediated RISC on a shared target. If this is the case, it can help to explain how specific RNA recognition and binding requirements for IRP1 and IRP2 have thus far remained elusive. MicroRNA-mediated RISC regulation in conjunction with IRP/IRE regulation can expand the range and versatility of the post-transcriptional regulatory system and lead to more fine-tuned protein expression over a wide range of conditions.

We find that different sets of microRNAs are identified as iron-regulated between K562 and HEK293 cell global microRNA expression analyses, and that the two sets of data share only

two microRNAs—miR-485-3p and miR-758. However, we then looked at the datasets (Table 4, Figure A2) within programs for microRNA target prediction and found that four microRNAs in the K562 set and three in the HEK293 set have significant predicted targets for ferroportin: miR-744*, miR-485-3p, miR-103, and miR-616 in K562 and miR-539, miR-485-3p, and miR-675 in HEK293. Of note is the fact that miR-103 is transcribed from a sequence within the intron of PANK2, and that patients with mutations or loss-of-function of PANK2 experience a pathological accumulation of iron in the brain (Zhou, Westaway et al. 2001). Furthermore, the silencing of PANK2 in cellular studies has been shown to increase ferroportin mRNA levels (Poli, Derosas et al. 2010). However, we were not able to confirm that the expression of miR-103 is iron-regulated (not shown), and mutation of the predicted target site for miR-103 on the FPN 3'UTR luciferase reporter showed no significant effect on expression. Thus a focus on identification of microRNA targets important within the cellular response could be a way to overcome the differences in cell-specific microRNA expression.

8.2 MicroRNA in cellular stress response II

There is evidence for combinatorial control of IRPs by multiple signaling pathways for cellular stresses such as iron, heme, oxidative stress, and hypoxia (Theil and Eisenstein 2000; Sanchez, Galy et al. 2007). For example, it is apparent that the effect of heme stress on the IRP/IRE system is distinct from the response to iron and the oxidative stress response even though the same IRP/IRE targets are involved (Hintze and Theil 2005; Theil 2006; Hintze, Katoh et al. 2007) . Also, although both ferritin and mitochondrial aconitase have 5'UTR IREs, the same amount of iron induces the production of ferritin in the liver up to 100-fold (Theil 1990) but mitochondrial aconitase is only induced 2-3 fold (Eisenstein and Blemings 1998). Finally, the NRF2/antioxidant response element pathway for the coordination of the antioxidant response has been identified as a transcriptional regulator of the expression of ferritin (Orino, Lehman et al.

2001; Pietsch, Chan et al. 2003) and ferroportin (Marro, Chiabrando et al. 2010; Harada, Kanayama et al. 2011) in response to oxidative stress and heme, respectively. Interestingly, Yanagawa et al. have reported a disorder in iron-transport in *Nrf2*$^{-/-}$ mice in which they found that the iron content of the enamel surface of their teeth was significantly decreased compared to wild-type mice. In addition, the general iron status of the mice were normal except for significantly (almost 2-fold) higher liver iron content (Yanagawa, Itoh et al. 2004). The precise mechanisms for this are yet to be reported, but these observations parallel what would be seen with a loss-of function disorder of ferroportin.

The mechanisms by which these stresses can induce a unique and selective cellular response by the coordination of both transcriptional and post-transcriptional regulation of the same pool of targets remains elusive. Based on the narrow tolerance of cells to specific stresses, this combinatorial control can be a function of the specific binding of the IRPs, a function of the potential microRNA-mediated RISC actions on the 3'UTR of these transcripts, a function of other RBP interactions with the transcripts, or combination of all three of these forms of regulation in a stress-dependent manner. Thus the study of the cellular stress responses to the actions of RBPs in post-transcriptional regulation such as the IRP/IRE system and can intersect with evidence for microRNA-mediated stress responses to yield a more complete understanding of the molecular circuitry and dynamics of these responses.

There is still the possibility that microRNAs may indeed be a primary sensor of stress responses. The mechanism by which these iron-regulated microRNAs are induced is still not known, but as we begin to identify the actors (targets, other RBPs) present in this cellular response regulatory network, we will be able to better describe how microRNAs can function within that network. Also, because different cell types express different subsets of microRNAs, it is difficult to use microRNA profiling alone as a tool to identify global stress changes among

different cell types if the characteristics or known roles of those microRNAs are not *a priori* known. Thus in the study of the roles of microRNAs in cellular stress responses it will prove useful to first determine the microRNA targets of the RISC between dynamic stages of a particular stress response, using methods available for identifying sequences bound by RBPs (particularly Ago2) such as RIP (RNA immunoprecipitation)-Chip , or the CLIP (cross-linking and immunoprecipitation)-based methods (Ule, Jensen et al. 2003; Ule, Jensen et al. 2005; Keene, Komisarow et al. 2006; Chi, Zang et al. 2009; Hafner, Landthaler et al. 2010; Hafner, Landthaler et al. 2010), as the targets involved within a given global stress response pathway are more likely to be more conserved among cell types than the microRNAs that target them.

8.3 Future Directions

It is of interest to determine whether and to what extent the IRP/IRE regulatory system interacts with microRNA-mediated RISC to modulate the target ferroportin, and whether this concurrent regulation also occurs with other target genes involved in cellular iron response. Recently novel biochemical methods such as HITS (high throughput sequencing)-CLIP (Licatalosi, Mele et al. 2008) and PAR (photoactivatable ribonucleoside-enhanced)-CLIP have enabled the identification of microRNA target sites on a transcriptome-wide scale. These methods identify RBP binding sites by cross-linking the RNA and proteins, immunoprecipitating the RBP of interest, and using next-generation sequencing to identify and characterize the bound RNAs. The use of Ago2 as the RBP with these methods have been successful and the overlap of Ago2 binding sites contained by CLIP with predicted microRNA target sites have been shown to be highly significant (Chi, Zang et al. 2009; Hafner, Landthaler et al. 2010). In a static state, the CLIP sequence data represents “snapshots” of RNA-protein interactions and many of these interactions could indeed be without any functional effect (Rajewsky 2011). The use of these

methods to follow a dynamic change such as a stress response has not been reported, but would strengthen the potential power to delineate functionally important binding sites.

Since ferroportin is a target important to both intracellular iron homeostasis and systemic iron homeostasis, it would be of interest to determine if miR-485-3p overexpression can parallel the inhibitory role of the iron regulatory hormone hepcidin in the regulation of cellular iron export. An alternative to miR-485-3p overexpression in this effort could be the use of inhibitors of miR-485-3p-associated RISC activity or oligonucleotides that can target the miR-485-3p binding site on endogenous intracellular ferroportin transcripts. This could modulate the iron-dependent miR-485-3p microRNA-mediated mechanism and could thus have the ability to regulate ferroportin from within the cell, potentially ameliorating disorders of ferroportin insensitivity to hepcidin by knockdown of ferroportin before it has reached the cell membrane.

8.4 Perspective: Comprehensive Regulation of Ferroportin

The regulation of FPN is comprehensive in that it can occur at the post-transcriptional (IRP/IRE system, microRNAs), post-translational (hepcidin), and transcriptional (Marro, Chiabrando et al. 2010; Harada, Kanayama et al. 2011) levels. Transcriptional variants govern the function of FPN during erythroid maturation. IRE-containing FPN transcripts are mainly expressed in undifferentiated erythroid progenitors and mature, hemoglobin-producing, erythroblasts, but during erythroid proliferation and differentiation, alternative transcripts without an IRE are expressed in an attempt to evade translational control (Figure 32). Once erythroid precursors undergo maturation and begin to produce hemoglobin, these alternative transcripts diminish and the IRE-containing form allows the cells to limit iron export during heme synthesis (Cianetti, Segnalini et al. 2005; Zhang, Hughes et al. 2009). The discovery of microRNA-mediated regulation of ferroportin further illustrates the diversity of methods within the mammalian system for the careful regulation of iron.

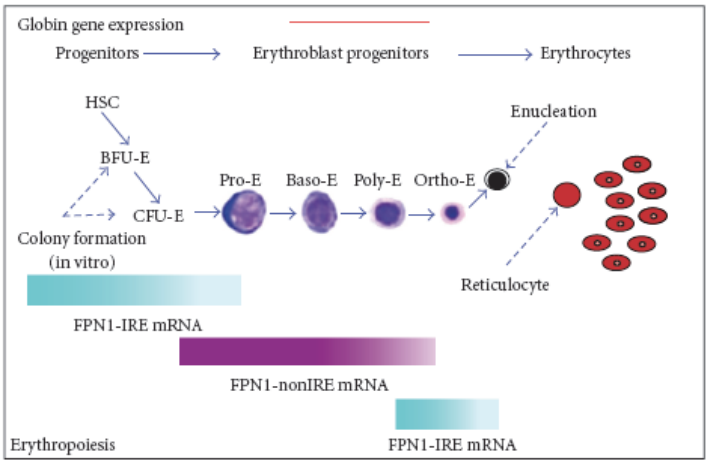


Figure 32: FPN in the pathway of erythropoiesis from progenitors to erythrocytes

Reproduced under Creative Commons Attribution License from (Cianetti, Gabbianelli et al. 2010)

Appendix A

Table 1: Clinical Parameters for HbSS dataset low-miR-144 (n=9), high-miR-144 (n=9)

Table 1

	HbSS sample	miR-144 pg/10^9 cells	RBC /uL	Hb (g/dL)	Hct (L/L)	Ret count (x 10^9) /L	Retic %	LDH (U/L)
	low 144	1.95E-05	2.63E+06	8.4	0.24	343.5	13.06	246
	low 144	4.92E-03	2.92E+06	9.1	0.27	228.6	7.67	191
	low 144	8.07E-03	2.25E+06	8.2	0.23	531.7	23.32	355
	low 144	2.40E-02	3.39E+06	10.5	0.32	107.5	3.21	416
	low 144	2.92E-02	4.45E+06	12.8	0.36	198.5	4.46	250
	low 144	4.27E-02	4.03E+06	12.3	0.36	159.2	3.98	181
	low 144	4.52E-02	2.24E+06	9.5	0.27	299.6	13.14	352
	low 144	4.97E-02	3.03E+06	10.3	0.29	349.8	11.47	318
	low 144	6.43E-02	2.31E+06	9.6	0.27	102.3	4.39	147
	high 144	9.89E-02	2.39E+06	7.1	0.21	61.7	2.52	208
	high 144	1.14E-01	3.13E+06	10	0.28	317	10	412
	high 144	1.18E-01	2.81E+06	8.3	0.26	73.4	2.53	299
	high 144	1.68E-01	2.18E+06	7.9	0.23	579.4	26.58	452
	high 144	1.79E-01	2.83E+06	9.2	0.27	399	13.76	275
	high 144	1.97E-01	1.95E+06	6.1	0.18	203	10.2	392
	high 144	1.97E-01	1.95E+06	7.1	0.2	156.6	8.03	559
	high 144	2.09E-01	2.31E+06	7.2	0.21	87.6	3.84	158
	high 144	2.82E-01	2.55E+06	9.00	0.26	157.10	6.31	147.00
p value		<.0001	0.073	0.007	0.011	0.674	0.976	0.391

Table 2: Clinical Parameters For SCD Subtype I (n=4) and Subtype II (n=8)

HbSS subtype	Gender	Age	RBC count x10^12	Retic Count x10^9	Retic %	Hemoglobin	Hematocrit	MCH	MCHC
I	M	34	2	393.6	17.57	7.1	0.19	35.5	37.6
I	M	22	2.88	604.9	17.35	9.4	0.26	32.6	36
I	F	28	1.74	204.3	11.88	7.6	0.22	43.7	34.7
I	F	35	2.57	421.8	16.16	7	0.22	27.2	32.3
II	M	32	2.82	370.3	13.13	8.4	0.24	29.8	34.7
II	F	29	2.88	274.5	9.4	9	0.25	31.3	36.6
II	F	45	4.34	243.1	5.76	12.5	0.36	28.8	35.1
II	M	40	3.15	336.9	10.56	10.7	0.29	34	37.2
II	M	40	2.06	101.2	5.01	8.6	0.25	41.7	35.1
II	M	18	2.56	155	6.08	9.7	0.29	37.9	33.6
II	M	25	3.01	169.8	5.68	12.3	0.33	40.9	36.9
II	F	44	3.01	190.5	6.35	8.2	0.24	27.1	33.9
Pvalue		0.43	0.098	0.04	0.001	0.04	0.04	0.83	0.82

HbSS subtype	Gender	Age	Hydroxyurea	Bilirubin, total	LDH	AST
I	M	34	no	3.9	1369	76
I	M	22	no	2.1	1029	33
I	F	28	y	6	672	40
I	F	35	y	4.7	708	55
II	M	32	no	3.5	1282	60
II	F	29	y	1.5	1029	35
II	F	45	no	1.4	983	74
II	M	40	no	4.5	1324	43
II	M	40	no	1	989	34
II	M	18	no	4.9	525	23
II	M	25	no	2.6	827	43
II	F	44	no	1.5	1239	46
Pvalue		0.43		0.13	0.54	0.56

HbSS subtype	Gender	Age	RDW	MCV	Nucleated RBC%	Nucleated RBC	platelet count	white blood cell	Fetal Hb
I	M	34	26.8	95	3.7	0.52	369	14.2	1.6
I	M	22	25.7	91	1.8	0.25	514	13.5	1.6
I	F	28	18.3	126	1.1	0.04	639	3.8	17.2
I	F	35	25.3	84	2.5	0.42	281	16.6	7.9
II	M	32	26.4	86	4.5	0.48	223	10.7	7.9
II	F	29	19.6	85	1.6	0.14	370	8.8	3.2
II	F	45	17	82	2.8	0.35	411	12.5	NA
II	M	40	20.9	91	1.2	0.17	473	13.8	4.3
II	M	40	18.6	119	8.3	0.57	397	6.9	4.4
II	M	18	16.2	113	14.6	0.66	261	4.5	9.1
II	M	25	15.5	111	0.7	0.06	195	8	18.1
II	F	44	23.2	80	2	0.17	415	8.7	NA
Pvalue		0.43	0.089	0.77	0.4	0.9	0.18	0.28	0.86

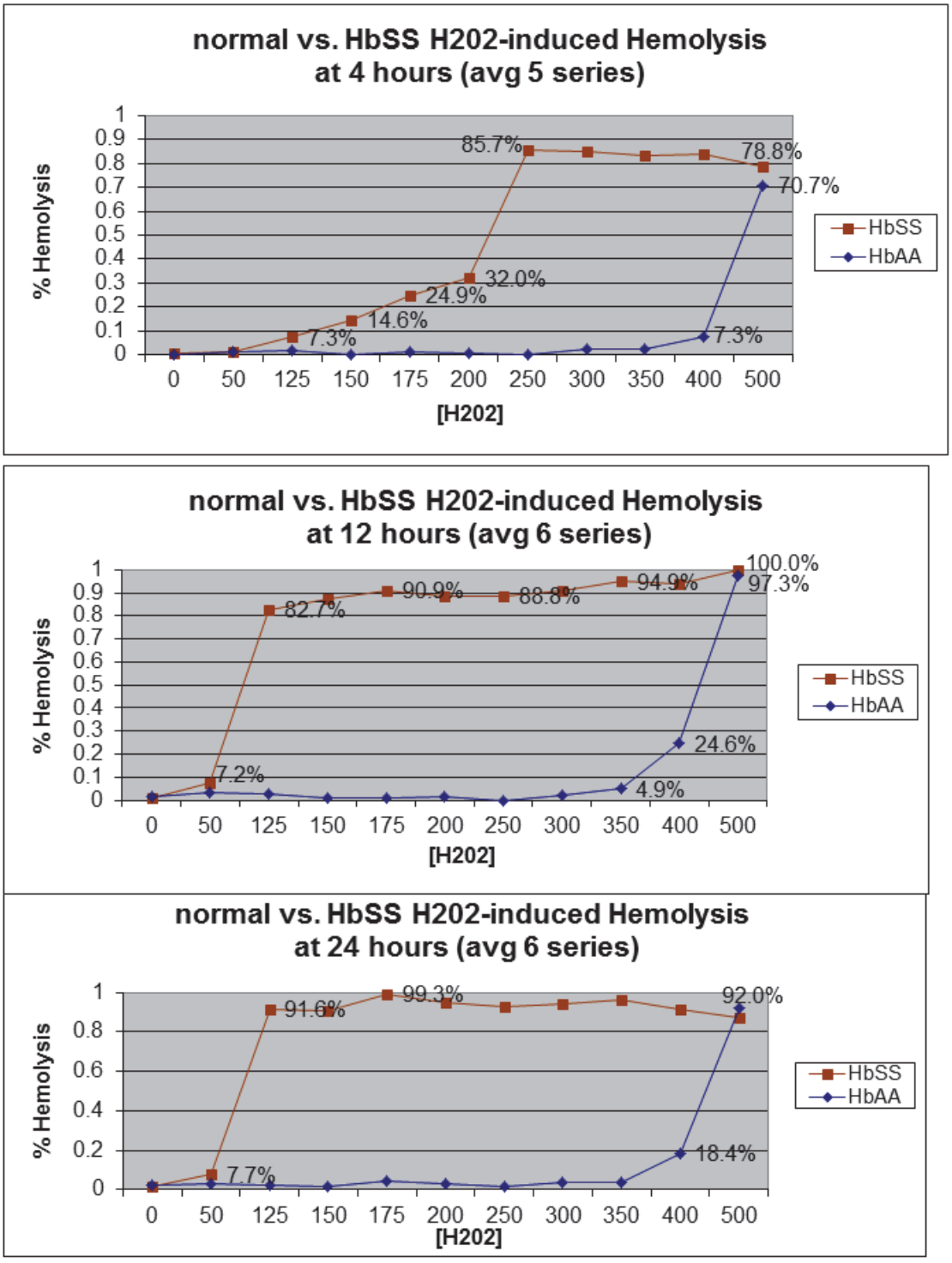


Figure A1: Oxidative Stress-Induced Hemolysis in HbAA and HbSS Erythrocytes

Table 3: Clinical Parameters for SCD reticulocyte dataset (n=25)

HbSS sample	miR-144 pg/10^9 cells	RBC /uL	Hb (g/dL)	Hct (L/L)	Retic %	LDH (U/L)
II-1	5.86E-01	2.49E+06	9.1	0.25	16.05	459
II-2	9.66E-01	3.13E+06	9.7	0.27	10.41	306
II-3	9.86E-01	2.84E+06	10.7	0.29	10.4	364
II-4	1.88E+00	2.72E+06	7.7	0.22	11.55	303
II-5	1.92E+00	3.86E+06	9.7	0.28	11.35	292
II-6	1.97E+00	2.32E+06	8.1	0.24	22.02	394
II-7	2.33E+00	2.44E+06	9.2	0.27	10.83	
II-8	2.41E+00	4.33E+06	12.4	0.37	6.66	201
II-9	2.79E+00	2.25E+06	7.9	0.22	15.74	452
II-10	3.48E+00	3.35E+06	11.2	0.31	3.69	318
II-11	3.54E+00	2.94E+06	9.3	0.27	8	244
II-12	3.68E+00	3.00E+06	10.4	0.31	9.46	
II-13	4.15E+00	2.84E+06	11.6	0.33	2.86	214
II-14	5.21E+00	2.17E+06	7	0.2	10.5	303
II-14	5.30E+00	2.01E+06	6.8	0.18	11.85	570
II-15	5.34E+00	1.87E+06	6	0.17	14.84	544
II-16	5.48E+00	3.28E+06	8.3	0.24	8.44	369
II-17	5.57E+00	3.71E+06	11.2	0.33	6.69	186
II-18	5.92E+00	4.30E+06	10.5	0.31	5.73	198
II-19	8.07E+00	2.62E+06	8.2	0.25	17.85	230
II-20	9.89E+00	3.38E+06	10.5	0.3	8.53	273
II-21	1.26E+01	1.73E+06	6.1	0.16	13.05	779
II-22	1.41E+01	1.86E+06	6.4	0.18	10.31	629
II-23	1.46E+01	2.02E+06	7.5	0.22	7.95	
II-24	1.74E+01	1.90E+06	9.3	0.26	3.56	321
II-25	1.93E+01	1.81E+06	7	0.19	8.83	540

Table 4: K562 Dataset- Iron-Dependent microRNA expression

K562 TLDA array

microRNA	baseline	50F	50D	100D	Predicted target important in cellular iron metabolism
hsa-miR-30a-3p	1	0.769117	10.42955	6.089224	*
hsa-miR-744*	1		5.909827	3.186908	*
hsa-miR-146a	1	0.370771	4.810414	2.441128	
hsa-miR-193a-5p	1		2.727646	2.241308	
hsa-miR-758	1		2.524518	2.02265	*
hsa-miR-410	1	0.499315	2.063459	2.620211	*
hsa-miR-324-5p	1	0.688204	1.8472	2.182758	
hsa-miR-134	1	0.59933	1.822471	1.197523	*
hsa-miR-29c	1	0.750824	1.818418	2.331601	*
hsa-miR-483-5p	1	0.700281	1.681186	1.71703	*
hsa-miR-191*	1	0.774558	1.643718	2.161767	
hsa-miR-502-3p	1	0.457069	1.620235	1.670195	*
hsa-miR-149	1	0.679044	1.593861	1.452254	*
hsa-miR-376c	1	0.355987	1.589732	1.115427	
hsa-miR-636	1	0.778126	1.572586	2.872032	
hsa-miR-215	1		1.42141	2.035362	
hsa-miR-96	1	0.342583	1.414417	1.59668	
hsa-miR-660	1	0.71914	1.332993	1.25106	
hsa-miR-105	1	0.617441	1.130471	1.151036	
hsa-miR-485-3p	1		1.08386	1.736544	*
hsa-miR-15b	1	0.631939	1.05369	1.556257	
hsa-miR-638	1	2.246984	1.035596	0.576702	
hsa-miR-616	1	1.571809	1.026745	0.247036	
hsa-miR-532-5p	1	0.573215	0.998862	1.204479	
hsa-miR-340*	1	0.954144	0.996266	0.296427	
hsa-miR-132	1	0.595128	0.993426	1.323865	
hsa-miR-671-3p	1	0.648682	0.982925	1.014039	
hsa-miR-19a	1	0.705393	0.979156	1.335628	
hsa-miR-454*	1	1.17042	0.972927	0.581094	
hsa-miR-26a	1	0.730861	0.957354	1.378519	
hsa-miR-194	1	0.770864	0.925655	1.506742	*
hsa-miR-103	1	0.496446	0.920084	0.9854	
hsa-miR-19b	1	0.668284	0.889903	1.199837	
hsa-miR-193a-3p	1	0.952846	0.756835	0.59062	
hsa-miR-127-3p	1	0.927393	0.756425	0.162702	
hsa-miR-1225-3p	1	1.019021	0.539505	0.574597	
hsa-miR-1180	1	0.926933	0.460168	0.624361	
hsa-miR-362-3p	1	1.130432	0.439222	0.81746	
hsa-miR-423-5p	1	0.998317	0.388004	0.708309	
hsa-miR-545	1		0.32382	0.748857	
hsa-miR-548d-5p	1	1.787621	0.262782	0.871561	
hsa-miR-522	1	0.611708		1.58067	*
hsa-miR-1270	1	0.837281		0.27157	
hsa-miR-520c-3p	1	1.568147		0.230891	

Table 5: Validation of Select Iron-Regulated MicroRNA expression across cell lines

K562

microRNA	baseline	DFE	p value vs baseline
hsa-miR-30a-3p	1.0 ± 0.11	3.05 ±0.50	.016
hsa-miR-502-3p	1.0 ±0.10	2.18 ±0.33	.027
hsa-miR-744*	1.0 ±.10	1.28 ±.09	.10
hsa-miR-194	1.0 ±0.04	2.73 ±0.16	.0004
hsa-miR-149	1.0 ±.04	2.20 ±.11	<.0001
hsa-miR-485-3p	1.0 ±.06	1.47 ±0.13	.039

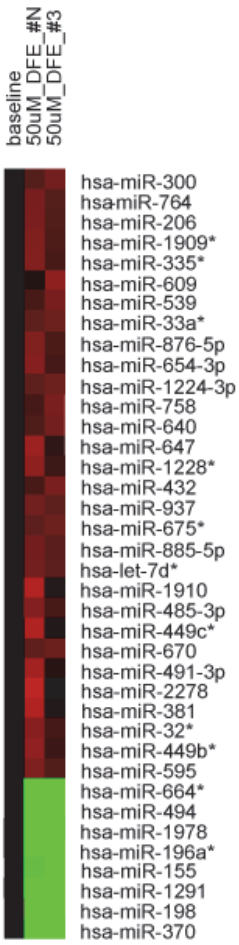
HEL

microRNA	baseline	DFE	p value vs baseline
hsa-miR-30a-3p	1.0 ± 0.09	1.47 ±0.16	.063
hsa-miR-502-3p	1.0 ±0.04	1.25 ± .09	.068
hsa-miR-744*	1.0 ±0.26	1.33 ±0.37	.50
hsa-miR-194	1.0 ±0.01	1.26 ±0.02	.0003
hsa-miR-149	1.0 ±0.08	2.53 ±0.32	.0097
hsa-miR-485-3p	1.0 ±0.06	1.76 ±0.17	.014

293

microRNA	baseline	DFE	p value vs baseline
hsa-miR-30a-3p	1.0 ±0.02	0.82 ±0.12	.20
hsa-miR-502-3p	1.0 ±0.05	1.60 ±0.21	.02
hsa-miR-744*	1.0 ±0.03	1.27 ±0.14	.13
hsa-miR-194	1.0 ±0.02	1.40 ±0.06	<.0001
hsa-miR-149	1.0 ±0.02	1.24 ±0.01	.0010
hsa-miR-485-3p	1.0 ±0.04	2.03 ±0.15	<.0001

293 microRNA array filtered for at least one >/= 1.4 fold change, n=2 biological replicates



MICRORNA	DFE #1	DFE #2	Predicted target important in cellular iron metabolism
hsa-miR-300	3.948	2.671	*
hsa-miR-764	3.715	3.195	
hsa-miR-206	3.304	2.919	*
hsa-miR-1909*	3.193	2.923	*
hsa-miR-335*	3.114	2.93	*
hsa-miR-609	2.941	1.2	
hsa-miR-539	2.91	1.805	*
hsa-miR-33a*	2.895	2.064	*
hsa-miR-876-5p	2.826	2.606	
hsa-miR-654-3p	2.65	2.549	
hsa-miR-1224-3p	2.607	1.861	
hsa-miR-758	2.573	1.567	
hsa-miR-640	2.429	1.587	*
hsa-miR-647	2.277	2.707	*
hsa-miR-1228*	2.025	2.162	*
hsa-miR-432	1.981	1.22	*
hsa-miR-937	1.944	1.574	
hsa-miR-675*	1.837	1.299	*
hsa-miR-885-5p	1.758	1.45	*
hsa-let-7d*	1.664	1.362	
hsa-miR-1910	1.63	2.586	
hsa-miR-485-3p	1.498	1.422	*
hsa-miR-449c*	1.483	2.276	
hsa-miR-670	1.455	1.048	*
hsa-miR-491-3p	1.446	1.869	*
hsa-miR-2278	1.429	2.596	*
hsa-miR-381	1.339	2.082	*
hsa-miR-664*	-0.9272	-2.191	
hsa-miR-494	-0.9493	-2.314	
hsa-miR-1978	-1.195	-2.327	
hsa-miR-196a*	-1.199	-1.603	
hsa-miR-155	-1.823	-0.8156	
hsa-miR-1291	-1.854	-2.874	
hsa-miR-198	-1.874	-2.556	
hsa-miR-370	-1.923	-2.794	

Figure A2: HEK293 Dataset- Iron-Dependent microRNA expression

References

Abboud, S. and D. J. Haile (2000). "A novel mammalian iron-regulated protein involved in intracellular iron metabolism." Journal of Biological Chemistry 275(26): 19906-19912.

Ahn, Y. H., Y. Hwang, et al. (2010). "Electrophilic tuning of the chemoprotective natural product sulforaphane." Proceedings of the National Academy of Sciences of the United States of America 107(21): 9590-9595.

Alizadeh, A. A., M. B. Eisen, et al. (2000). "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling." Nature 403(6769): 503-511.

Altuvia, Y., P. Landgraf, et al. (2005). "Clustering and conservation patterns of human microRNAs." Nucleic Acids Research 33(8): 2697-2706.

Ambros, V. (2004). "The functions of animal microRNAs." Nature 431(7006): 350-355.

Amer, J., H. Ghoti, et al. (2006). "Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants." British Journal of Haematology 132(1): 108-113.

Andrews, N. C. (2008). "Forging a field: the golden age of iron biology." Blood 112(2): 219-230.

Aziz, N. and H. N. Munro (1987). "Iron regulates ferritin mRNA translation through a segment of its 5' untranslated region." Proceedings of the National Academy of Sciences of the United States of America 84(23): 8478-8482.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell 116(2): 281-297.

Beitzinger, M., L. Peters, et al. (2007). "Identification of human microRNA targets from isolated argonaute protein complexes." RNA Biology 4(2): 76-84.

Betel, D., M. Wilson, et al. (2008). "The microRNA.org resource: targets and expression." Nucleic Acids Research 36(Database issue): D149-153.

Bhattacharyya, S. N., R. Habermacher, et al. (2006). "Relief of microRNA-mediated translational repression in human cells subjected to stress." Cell 125(6): 1111-1124.

Binder, R., J. A. Horowitz, et al. (1994). "Evidence that the pathway of transferrin receptor mRNA degradation involves an endonucleolytic cleavage within the 3' UTR and does not involve poly(A) tail shortening." The EMBO Journal 13(8): 1969-1980.

Bousquet, M., C. Quelen, et al. (2008). "Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the t(2;11)(p21;q23) translocation." Journal of Experimental Medicine 205(11): 2499-2506.

Bruchova, H., M. Merkerova, et al. (2008). "Aberrant expression of microRNA in polycythemia vera." Haematologica 93(7): 1009-1016.

Bruchova, H., D. Yoon, et al. (2007). "Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis." Experimental Hematology 35(11): 1657-1667.

Bushati, N. and S. M. Cohen (2008). "MicroRNAs in neurodegeneration." Current Opinion in Neurobiology 18(3): 292-296.

Butt, J., H. Y. Kim, et al. (1996). "Differences in the RNA binding sites of iron regulatory proteins and potential target diversity." Proceedings of the National Academy of Sciences of the United States of America 93(9): 4345-4349.

Calin, G. A., C. D. Dumitru, et al. (2002). "Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia." Proceedings of the National Academy of Sciences of the United States of America 99(24): 15524-15529.

Calin, G. A., M. Ferracin, et al. (2005). "A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia." New England Journal of Medicine 353(17): 1793-1801.

Casey, J. L., M. W. Hentze, et al. (1988). "Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation." Science 240(4854): 924-928.

Castro, O., D. J. Brambilla, et al. (1994). "The acute chest syndrome in sickle cell disease: incidence and risk factors. The Cooperative Study of Sickle Cell Disease." Blood 84(2): 643-649.

Caughman, S. W., M. W. Hentze, et al. (1988). "The iron-responsive element is the single element responsible for iron-dependent translational regulation of ferritin biosynthesis. Evidence for function as the binding site for a translational repressor." Journal of Biological Chemistry 263(35): 19048-19052.

Chan, J. Y. and M. Kwong (2000). "Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein." Biochimica et Biophysica Acta 1517(1): 19-26.

Chatterjee, S. and H. Grosshans (2009). "Active turnover modulates mature microRNA activity in Caenorhabditis elegans." Nature 461(7263): 546-549.

Chen, S. Y., Y. Wang, et al. (2008). "The genomic analysis of erythrocyte microRNA expression in sickle cell diseases." PLoS One 3(6): e2360.

Chi, S. W., J. B. Zang, et al. (2009). "Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps." Nature 460(7254): 479-486.

Chiu, D. and B. Lubin (1979). "Abnormal vitamin E and glutathione peroxidase levels in sickle cell anemia: evidence for increased susceptibility to lipid peroxidation in vivo." Journal of Laboratory & Clinical Medicine 94(4): 542-548.

Cianetti, L., M. Gabbianelli, et al. (2010). "Ferroportin and erythroid cells: an update." Advances in Hematology 2010.

Cianetti, L., P. Segnalini, et al. (2005). "Expression of alternative transcripts of ferroportin-1 during human erythroid differentiation." Haematologica 90(12): 1595-1606.

Croce, C. M. (2009). "Causes and consequences of microRNA dysregulation in cancer." Nature Reviews Genetics 10(10): 704-714.

De Domenico, I., D. M. Ward, et al. (2007). "The molecular mechanism of hepcidin-mediated ferroportin down-regulation." Molecular Biology of the Cell 18(7): 2569-2578.

De Domenico, I., D. M. Ward, et al. (2005). "The molecular basis of ferroportin-linked hemochromatosis." Proceedings of the National Academy of Sciences of the United States of America 102(25): 8955-8960.

Deneke, S. M. and B. L. Fanburg (1989). "Regulation of cellular glutathione." American Journal of Physiology 257(4 Pt 1): L163-173.

Diederichs, S. and D. A. Haber (2007). "Dual Role for Argonautes in MicroRNA Processing and Posttranscriptional Regulation of MicroRNA Expression." Cell 131(6): 1097-1108.

Donovan, A., C. A. Lima, et al. (2005). "The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis." Cell Metabolism 1(3): 191-200.

Dore, L. C., J. D. Amigo, et al. (2008). "A GATA-1-regulated microRNA locus essential for erythropoiesis." Proceedings of the National Academy of Sciences of the United States of America 105(9): 3333-3338.

Dore, L. C., J. D. Amigo, et al. (2008). "A GATA-1-regulated microRNA locus essential for erythropoiesis." Proc Natl Acad Sci U S A 105(9): 3333-3338.

Dover, G. J., V. T. Chang, et al. (1987). "The cellular basis for different fetal hemoglobin levels among sickle cell individuals with two, three, and four alpha-globin genes." Blood 69(1): 341-344.

Drakesmith, H., L. M. Schimanski, et al. (2005). "Resistance to hepcidin is conferred by hemochromatosis-associated mutations of ferroportin." Blood 106(3): 1092-1097.

Dumaswala, U. J., L. Zhuo, et al. (2001). "Glutathione protects chemokine-scavenging and antioxidative defense functions in human RBCs." American Journal of Physiology - Cell Physiology 280(4): C867-873.

Eisenstein, R. S. and K. P. Blemings (1998). "Iron regulatory proteins, iron responsive elements and iron homeostasis." The Journal of Nutrition 128(12): 2295-2298.

Embury, S. H. (1994). Sickle cell disease : Basic Principles and Clinical Practice. New York, Raven Press.

Franco, R. S., J. Lohmann, et al. (1998). "Time-dependent changes in the density and hemoglobin F content of biotin-labeled sickle cells." Journal of Clinical Investigation 101(12): 2730-2740.

Fu, H., Y. Tie, et al. (2005). "Identification of human fetal liver miRNAs by a novel method." FEBS Letters 579(17): 3849-3854.

Fu, Y. F., T. T. Du, et al. (2009). "Mir-144 selectively regulates embryonic alpha-hemoglobin synthesis during primitive erythropoiesis." Blood 113(6): 1340-1349.

Ganz, T. and E. Nemeth (2011). "Hepcidin and disorders of iron metabolism." Annual Review of Medicine 62: 347-360.

Garzon, R., G. A. Calin, et al. (2009). "MicroRNAs in Cancer." Annual Review of Medicine 60: 167-179.

Garzon, R., M. Fabbri, et al. (2006). "MicroRNA expression and function in cancer." Trends in Molecular Medicine 12(12): 580-587.

Gill, F. M., L. A. Sleeper, et al. (1995). "Clinical events in the first decade in a cohort of infants with sickle cell disease. Cooperative Study of Sickle Cell Disease " Blood 86(2): 776-783.

Gregory, R. I., T. P. Chendrimada, et al. (2005). "Human RISC Couples MicroRNA Biogenesis and Posttranscriptional Gene Silencing." Cell 123(4): 631-640.

Griffiths-Jones, S., H. K. Saini, et al. (2008). "miRBase: tools for microRNA genomics." Nucleic Acids Research 36(Database issue): D154-158.

Hafner, M., M. Landthaler, et al. (2010). "Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP." Cell 141(1): 129-141.

Hafner, M., M. Landthaler, et al. (2010). "PAR-CliP--a method to identify transcriptome-wide the binding sites of RNA binding proteins." J Vis Exp(41).

Haile, D. J., T. A. Rouault, et al. (1992). "Reciprocal control of RNA-binding and aconitase activity in the regulation of the iron-responsive element binding protein: role of the iron-sulfur cluster." Proceedings of the National Academy of Sciences of the United States of America 89(16): 7536-7540.

Harada, N., M. Kanayama, et al. (2011). "Nrf2 regulates ferroportin 1-mediated iron efflux and counteracts lipopolysaccharide-induced ferroportin 1 mRNA suppression in macrophages." Archives of Biochemistry and Biophysics 508(1): 101-109.

Harvey, C. J., R. K. Thimmulappa, et al. (2009). "Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress." Free Radical Biology and Medicine 46(4): 443-453.

Hebbel, R. P., J. W. Eaton, et al. (1982). "Spontaneous oxygen radical generation by sickle erythrocytes." Journal of Clinical Investigation 70(6): 1253-1259.

Hendrickson, D. G., D. J. Hogan, et al. (2009). "Concordant regulation of translation and mRNA abundance for hundreds of targets of a human microRNA." PLoS Biology 7(11): e1000238.

Hentze, M. W., S. W. Caughman, et al. (1987). "Identification of the iron-responsive element for the translational regulation of human ferritin mRNA." Science 238(4833): 1570-1573.

Hentze, M. W. and L. C. Kuhn (1996). "Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress." Proceedings of the National Academy of Sciences of the United States of America 93(16): 8175-8182.

Hentze, M. W., M. U. Muckenthaler, et al. (2004). "Balancing acts: molecular control of mammalian iron metabolism." Cell 117(3): 285-297.

Hintze, K. J., Y. Katoh, et al. (2007). "Bach1 repression of ferritin and thioredoxin reductase1 is heme-sensitive in cells and in vitro and coordinates expression with heme oxygenase1, beta-globin, and NADP(H) quinone (oxido) reductase1." Journal of Biological Chemistry 282(47): 34365-34371.

Hintze, K. J. and E. C. Theil (2005). "DNA and mRNA elements with complementary responses to hemin, antioxidant inducers, and iron control ferritin-L expression." Proceedings of the National Academy of Sciences of the United States of America 102(42): 15048-15052.

Hoffman, R. (2005). Hematology: Basic Principles and Practice. Philadelphia, Pa. , Churchill Livingstone; MD Consult LLC.

Hoppe, C., W. Klitz, et al. (2004). "Gene interactions and stroke risk in children with sickle cell anemia." Blood 103(6): 2391-2396.

Hoppe, C., W. Klitz, et al. (2003). "Distinct HLA associations by stroke subtype in children with sickle cell anemia." Blood 101(7): 2865-2869.

Hutvagner, G., M. J. Simard, et al. (2004). "Sequence-specific inhibition of small RNA function." PLoS Biology 2(4): E98.

Ingram, V. M. (1959). "Abnormal human haemoglobins. III. The chemical difference between normal and sickle cell haemoglobins." Biochimica et Biophysica Acta 36: 402-411.

Ishii, T., K. Itoh, et al. (2000). "Transcription Factor Nrf2 Coordinately Regulates a Group of Oxidative Stress-inducible Genes in Macrophages." Journal of Biological Chemistry 275(21): 16023-16029.

Itoh, K., T. Chiba, et al. (1997). "An Nrf2/Small Maf Heterodimer Mediates the Induction of Phase II Detoxifying Enzyme Genes through Antioxidant Response Elements." Biochemical and Biophysical Research Communications 236(2): 313-322.

Ji, R., Y. Cheng, et al. (2007). "MicroRNA Expression Signature and Antisense-Mediated Depletion Reveal an Essential Role of MicroRNA in Vascular Neointimal Lesion Formation." Circulation Research 100(11): 1579-1588.

Karginov, F. V., C. Conaco, et al. (2007). "A biochemical approach to identifying microRNA targets." Proceedings of the National Academy of Sciences of the United States of America 104(49): 19291-19296.

Kato, G. J., V. McGowan, et al. (2006). "Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism, leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease." Blood 107(6): 2279-2285.

Kedde, M., M. J. Strasser, et al. (2007). "RNA-binding protein Dnd1 inhibits microRNA access to target mRNA." Cell 131(7): 1273-1286.

Keene, J. D. (2007). "RNA regulons: coordination of post-transcriptional events." Nature Reviews Genetics 8(7): 533-543.

Keene, J. D., J. M. Komisarow, et al. (2006). "RIP-Chip: the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts." Nature Protocols 1(1): 302-307.

Kensler, T. W., N. Wakabayashi, et al. (2007). "Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway." Annual Review of Pharmacology and Toxicology 47: 89-116.

Kolfschoten, I. G., E. Roggli, et al. (2009). "Role and therapeutic potential of microRNAs in diabetes." Diabetes, Obesity & Metabolism 11 Suppl 4: 118-129.

Lee, J. M., M. J. Calkins, et al. (2003). "Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis." Journal of Biological Chemistry 278(14): 12029-12038.

Lee, J. M., K. Chan, et al. (2004). "Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia." Proceedings of the National Academy of Sciences of the United States of America 101(26): 9751-9756.

Lee, Y., K. Jeon, et al. (2002). "MicroRNA maturation: stepwise processing and subcellular localization." The EMBO Journal 21(17): 4663-4670.

Lee, Y., M. Kim, et al. (2004). "MicroRNA genes are transcribed by RNA polymerase II." The EMBO Journal 23(20): 4051-4060.

Leibold, E. A. and H. N. Munro (1988). "Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5' untranslated region of ferritin heavy- and light-subunit mRNAs." Proceedings of the National Academy of Sciences of the United States of America 85(7): 2171-2175.

Leung, A. K. and P. A. Sharp (2010). "MicroRNA functions in stress responses." Molecular Cell 40(2): 205-215.

Lewis, B. P., C. B. Burge, et al. (2005). "Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets." Cell 120(1): 15-20.

Li, J., D. Johnson, et al. (2005). "Stabilization of Nrf2 by tBHQ confers protection against oxidative stress-induced cell death in human neural stem cells." Toxicological Sciences 83(2): 313-328.

Licatalosi, D. D., A. Mele, et al. (2008). "HITS-CLIP yields genome-wide insights into brain alternative RNA processing." Nature 456(7221): 464-469.

Longmore, G. D. (2006). "A unique role for Stat5 in recovery from acute anemia." Journal of Clinical Investigation 116(3): 626-628.

Lymboussaki, A., E. Pignatti, et al. (2003). "The role of the iron responsive element in the control of ferroportin1/IREG1/MTP1 gene expression." Journal of Hepatology 39(5): 710-715.

Macari, E. R. and C. H. Lowrey (2011). "Induction of human fetal hemoglobin via the NRF2 antioxidant response signaling pathway." Blood doi:10.1182/blood-2010-10-314096.

Marinkovic, D., X. Zhang, et al. (2007). "Foxo3 is required for the regulation of oxidative stress in erythropoiesis." Journal of Clinical Investigation 117(8): 2133-2144.

Marro, S., D. Chiabrando, et al. (2010). "Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position -7007 of the FPN1 promoter." Haematologica 95(8): 1261-1268.

Masaki, S., R. Ohtsuka, et al. (2007). "Expression patterns of microRNAs 155 and 451 during normal human erythropoiesis." Biochemical & Biophysical Research Communications 364(3): 509-514.

McKie, A. T., P. Marciani, et al. (2000). "A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation." Molecular Cell 5(2): 299-309.

Merkerova, M., M. Belickova, et al. (2008). "Differential expression of microRNAs in hematopoietic cell lineages." European Journal of Haematology 81(4): 304-310.

Montosi, G., A. Donovan, et al. (2001). "Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene." Journal of Clinical Investigation 108(4): 619-623.

Morris, C. R., J. H. Suh, et al. (2008). "Erythrocyte glutamine depletion, altered redox environment, and pulmonary hypertension in sickle cell disease." Blood 111(1): 402-410.

Motohashi, H. and M. Yamamoto (2004). "Nrf2-Keap1 defines a physiologically important stress response mechanism." Trends in Molecular Medicine 10(11): 549-557.

Muckenthaler, M. U., B. Galy, et al. (2008). "Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network." Annual Review of Nutrition 28: 197-213.

Naga Prasad, S. V., Z. H. Duan, et al. (2009). "Unique microRNA profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks." Journal of Biological Chemistry 284(40): 27487-27499.

Neel, J. V. (1949). "The Inheritance of Sickle Cell Anemia." Science 110(2846): 64-66.

Nelson, P. T., M. De Planell-Saguer, et al. (2007). "A novel monoclonal antibody against human Argonaute proteins reveals unexpected characteristics of miRNAs in human blood cells." RNA 13(10): 1787-1792.

Nemeth, E., M. S. Tuttle, et al. (2004). "Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization." Science 306(5704): 2090-2093.

Neumann, C. A., D. S. Krause, et al. (2003). "Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression." Nature 424(6948): 561-565.

Njajou, O. T., N. Vaessen, et al. (2001). "A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis." Nature Genetics 28(3): 213-214.

O'Carroll, D., I. Mecklenbrauker, et al. (2007). "A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway." Genes & Development 21(16): 1999-2004.

Orino, K., L. Lehman, et al. (2001). "Ferritin and the response to oxidative stress." The Biochemical Journal 357(Pt 1): 241-247.

Papapetrou, E. P., J. E. Korkola, et al. (2010). "A genetic strategy for single and combinatorial analysis of miRNA function in mammalian hematopoietic stem cells." Stem Cells 28(2): 287-296.

Pase, L., J. E. Layton, et al. (2009). "miR-451 regulates zebrafish erythroid maturation in vivo via its target gata2." Blood 113(8): 1794-1804.

Patrick, D. M., C. C. Zhang, et al. (2010). "Defective erythroid differentiation in miR-451 mutant mice mediated by 14-3-3zeta." Genes & Development 24(15): 1614-1619.

Pauling, L. and H. A. Itano (1949). "Sickle cell anemia a molecular disease." Science 110(2865): 543-548.

Perou, C. M., T. Sorlie, et al. (2000). "Molecular portraits of human breast tumours." Nature 406(6797): 747-752.

Pietsch, E. C., J. Y. Chan, et al. (2003). "Nrf2 mediates the induction of ferritin H in response to xenobiotics and cancer chemopreventive dithiolethiones." Journal of Biological Chemistry 278(4): 2361-2369.

Platt, O. S., D. J. Brambilla, et al. (1994). "Mortality in sickle cell disease. Life expectancy and risk factors for early death." New England Journal of Medicine 330(23): 1639-1644.

Poli, M., M. Derosas, et al. (2010). "Pantothenate kinase-2 (Pank2) silencing causes cell growth reduction, cell-specific ferroportin upregulation and iron deregulation." Neurobiology of Disease 39(2): 204-210.

Poy, M. N., L. Eliasson, et al. (2004). "A pancreatic islet-specific microRNA regulates insulin secretion." Nature 432(7014): 226-230.

Rajewsky, N. (2011). "microRNAs and the Operon Paper." Journal of Molecular Biology doi:10.1016/j.jmb.2011.03.021.

Ramachandran, V. and X. Chen (2008). "Degradation of microRNAs by a family of exoribonucleases in Arabidopsis." Science 321(5895): 1490-1492.

Rank, B. H., J. Carlsson, et al. (1985). "Abnormal redox status of membrane-protein thiols in sickle erythrocytes." Journal of Clinical Investigation 75(5): 1531-1537.

Rasmussen, K. D., S. Simmini, et al. (2010). "The miR-144/451 locus is required for erythroid homeostasis." Journal of Experimental Medicine 207(7): 1351-1358.

Rathjen, T., C. Nicol, et al. (2006). "Analysis of short RNAs in the malaria parasite and its red blood cell host." FEBS Letters 580(22): 5185-5188.

Regazzi, R. (2010). "Diabetes mellitus reveals its micro-signature." Circulation Research 107(6): 686-688.

Rouault, T. A., M. W. Hentze, et al. (1988). "Binding of a cytosolic protein to the iron-responsive element of human ferritin messenger RNA." Science 241(4870): 1207-1210.

Salahudeen, A. A., J. W. Thompson, et al. (2009). "An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis." Science 326(5953): 722-726.

Sanchez, M., B. Galy, et al. (2007). "Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency." Nature Structural & Molecular Biology 14(5): 420-426.

Sangokoya, C., G. LaMonte, et al. (2010). "Isolation and characterization of microRNAs of human mature erythrocytes." Methods in Molecular Biology 667: 193-203.

Sangokoya, C., M. J. Telen, et al. (2010). "microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease." Blood 116(20): 4338-4348.

Schacter, L., J. A. Warth, et al. (1988). "Altered amount and activity of superoxide dismutase in sickle cell anemia." The FASEB Journal 2(3): 237-243.

Schimanski, L. M., H. Drakesmith, et al. (2005). "In vitro functional analysis of human ferroportin (FPN) and hemochromatosis-associated FPN mutations." Blood 105(10): 4096-4102.

Schwarz, D. S., G. Hutvágner, et al. (2003). "Asymmetry in the Assembly of the RNAi Enzyme Complex." Cell 115(2): 199-208.

Sebastiani, P., M. F. Ramoni, et al. (2005). "Genetic dissection and prognostic modeling of overt stroke in sickle cell anemia." Nature Genetics 37(4): 435-440.

Sebastiani, P., N. Solovieff, et al. (2010). "Genetic modifiers of the severity of sickle cell anemia identified through a genome-wide association study." American Journal of Hematology 85(1): 29-35.

Small, E. M. and E. N. Olson (2011). "Pervasive roles of microRNAs in cardiovascular biology." Nature 469(7330): 336-342.

Socolovsky, M. (2007). "Molecular insights into stress erythropoiesis." Current Opinion in Hematology 14(3): 215-224.

Solovieff, N., J. N. Milton, et al. (2010). "Fetal hemoglobin in sickle cell anemia: genome-wide association studies suggest a regulatory region in the 5' olfactory receptor gene cluster." Blood 115(9): 1815-1822.

Srivastava, D. (2006). "Making or breaking the heart: from lineage determination to morphogenesis." Cell 126(6): 1037-1048.

Steinberg, M. H. and C. Brugnara (2003). "Pathophysiological-based approaches to treatment of sickle cell disease." Annual Review of Medicine 54: 89-112.

Steinberg, M. H., Z. H. Lu, et al. (1997). "Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea. Multicenter Study of Hydroxyurea." Blood 89(3): 1078-1088.

Sunkar, R., A. Kapoor, et al. (2006). "Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance." Plant Cell 18(8): 2051-2065.

Tatum, V. L. and C. K. Chow (1996). "Antioxidant status and susceptibility of sickle erythrocytes to oxidative and osmotic stress." Free Radical Research 25(2): 133-139.

Tenenbaum, S. A., J. Christiansen, et al. (2011). "The post-transcriptional operon." Methods in Molecular Biology 703: 237-245.

Theil, E. C. (1990). "Regulation of ferritin and transferrin receptor mRNAs." Journal of Biological Chemistry 265(9): 4771-4774.

Theil, E. C. (2006). "Integrating iron and oxygen/antioxidant signals via a combinatorial array of DNA - (antioxidant response elements) and mRNA (iron responsive elements) sequences." Journal of Inorganic Biochemistry 100(12): 2074-2078.

Theil, E. C. and R. S. Eisenstein (2000). "Combinatorial mRNA regulation: iron regulatory proteins and iso-iron-responsive elements (Iso-IREs)." Journal of Biological Chemistry 275(52): 40659-40662.

Thimmulappa, R. K., K. H. Mai, et al. (2002). "Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray." Cancer Research 62(18): 5196-5203.

Tibshirani, R., T. Hastie, et al. (2002). "Diagnosis of multiple cancer types by shrunken centroids of gene expression." Proceedings of the National Academy of Sciences of the United States of America 99(10): 6567-6572.

Tsantes, A. E., S. Bonovas, et al. (2006). "Redox imbalance, macrocytosis, and RBC homeostasis." Antioxidants & Redox Signaling 8(7-8): 1205-1216.

Ule, J., K. Jensen, et al. (2005). "CLIP: a method for identifying protein-RNA interaction sites in living cells." Methods 37(4): 376-386.

Ule, J., K. B. Jensen, et al. (2003). "CLIP identifies Nova-regulated RNA networks in the brain." Science 302(5648): 1212-1215.

Vashisht, A. A., K. B. Zumbrennen, et al. (2009). "Control of iron homeostasis by an iron-regulated ubiquitin ligase." Science 326(5953): 718-721.

Vasudevan, S. and J. A. Steitz (2007). "AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2." Cell 128(6): 1105-1118.

Vichinsky, E. P., L. A. Styles, et al. (1997). "Acute Chest Syndrome in Sickle Cell Disease: Clinical Presentation and Course." Blood 89(5): 1787-1792.

Wallander, M. L., E. A. Leibold, et al. (2006). "Molecular control of vertebrate iron homeostasis by iron regulatory proteins." Biochimica et Biophysica Acta 1763(7): 668-689.

Yanagawa, T., K. Itoh, et al. (2004). "Nrf2 deficiency causes tooth decolourization due to iron transport disorder in enamel organ." Genes to Cells 9(7): 641-651.

Yang, B., H. Lin, et al. (2007). "The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2." Nature Medicine 13(4): 486-491.

Yang, F., D. J. Haile, et al. (2005). "Apical location of ferroportin 1 in airway epithelia and its role in iron detoxification in the lung." Am J Physiol Lung Cell Mol Physiol 289(1): L14-23.

Yu, D., C. O. dos Santos, et al. (2010). "miR-451 protects against erythroid oxidant stress by repressing 14-3-3zeta." Genes & Development 24(15): 1620-1633.

Zeng, Y. and B. R. Cullen (2003). "Sequence requirements for micro RNA processing and function in human cells." RNA 9(1): 112-123.

Zhan, M., C. P. Miller, et al. (2007). "MicroRNA expression dynamics during murine and human erythroid differentiation." Experimental Hematology 35(7): 1015-1025.

Zhang, D. L., R. M. Hughes, et al. (2009). "A ferroportin transcript that lacks an iron-responsive element enables duodenal and erythroid precursor cells to evade translational repression." Cell Metabolism 9(5): 461-473.

Zhou, B., S. K. Westaway, et al. (2001). "A novel pantothenate kinase gene (PANK2) is defective in Hallervorden-Spatz syndrome." Nature Genetics 28(4): 345-349.

Biography

Carolyn Sangokoya was born in Washington, D.C. She has grown up in College Park, Maryland, Athens, Georgia, and Baton Rouge, Louisiana. In 1999 she graduated from Baton Rouge Magnet High School and began undergraduate studies at Stanford University in Palo Alto, California. As an undergraduate she studied Human Biology with a concentration in molecular genetics. In 2003, she received a Bachelor of Arts degree in Human Biology from Stanford University. In 2004, she entered the Medical Scientist Training Program at Duke University School of Medicine in Durham, North Carolina. In 2006, after completing the first two years of medical school, she began graduate studies in the University Program of Genetics and Genomics under the mentorship of Dr. Jen-Tsan Ashley Chi. Upon completion of graduate training, she will return for her final year of medical school.

Publications

Sangokoya C, Telen MJ, and JT Chi.
MicroRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. Blood. 2010 Nov 18;116(20):4338-48. PMID: 20709907

Sangokoya C, LaMonte G, and JT Chi.
Isolation and characterization of microRNAs of Human Mature Erythrocytes
MicroRNAs and the Immune System: Methods in Molecular Biology. 2010; vol. 667:193-203.
PMID: 20827535

Abstracts/Posters

Sangokoya C, Telen MJ, and JT Chi.
"MicroRNA miR-144, oxidative stress tolerance, and sickle cell disease."
Keystone Symposia "MicroRNAs in Human Disease" (Banff, Alberta, CA)

Sangokoya C, Wu J, Telen MJ, deCastro CM, and JT Chi
"Analysis of Erythrocyte MicroRNA Expression in Anemic Disorders."
National MD-PhD Student Annual Research Conference 2008 (Keystone, CO)

Chi JT, **Sangokoya C,** and CM deCastro
"MicroRNA expression in red blood cells from patients with PNH."
American Society of Hematologists Annual Conference 2007 (Atlanta, GA)