

ZINC TRANSPORTER EXPRESSION IN MATURE RED BLOOD CELLS AND
DIFFERENTIATING ERYTHROID PROGENITOR CELLS

By

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To my grandfather, Hong-Ryol Ryu (1911-1995). The lessons from him have always inspired me to pursue the delight to be wise with the right insights.

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Abstract of Thesis Presented to the Graduate School
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Animal and human studies have shown that the *in vitro* uptake rate of ^{65}Zn by red blood cells (RBCs) is inversely related to the subject's zinc status. The capability of RBCs to take up zinc may be a remnant of an earlier developmental stage of erythrocytes as zinc is essential for the activities of several proteins formed during the erythroid differentiation, e.g., carbonic anhydrase, $\text{Cu}^{2+}/\text{Zn}^{2+}$ -superoxide dismutase, and zinc finger transcription factors such as erythroid Krüppel-like factor (EKLF) and GATA-1. Conversely, excessive intracellular free zinc ions can interfere with incorporation of ferrous ions into heme at the final stage of erythroid differentiation. Thus, intracellular zinc homeostasis during erythroid differentiation must be tightly regulated.

To examine the hypothesis that the expression of zinc transporters would be involved in the strategic mechanism of erythroid zinc homeostasis, transporters in the membrane fraction of RBCs were screened by western analyses, and only Zip10 and ZnT1 were detectable among the zinc transporters tested. Erythroid progenitor cells were prepared from spleens of phenylhydrazine (PHZ)-treated anemic mice for the characterization of transporter gene expression during the terminal stage of erythropoiesis. Differentiation of cells into reticulocytes was induced by erythropoietin (EPO)-treatment *in vitro*. Hemoglobin (Hb) synthesis and

expression of erythroid δ -aminolevulinic acid synthase (ALAS-2) mRNA were measured for the confirmation of the ex vivo erythroid differentiation. Transcript levels of each transporter gene and other genes associated with zinc metabolism were quantified by quantitative real-time PCR (qRT-PCR). Temporal trends in expression of each gene were observed. Briefly, following addition of EPO, Zip10 mRNA levels peaked prior to the time-point when metal-responsive transcription factor-1 (MTF-1) transcripts reached its first peak level, and decreased dramatically afterwards. For ZnT1 mRNA, EPO-dependent expression was initiated later than Zip10 and was sustained until the experimental time-course was over. Metallothionein-1 (MT-1) transcript abundance decreased rapidly after addition of EPO and stayed lower than the 0 h basal levels until 48 h. Expression trends of Zip10 and ZnT1 were further confirmed by western blots utilizing total cell lysates and membrane fractions of these cells.

This is the first study to determine which zinc transporters are expressed in the erythroid system. The results presented here suggest that Zip10 and ZnT1 expression is induced in response to EPO. Furthermore, they could be the zinc transporters most directly involved in the regulation of intracellular zinc homeostasis in differentiating erythroid progenitor cells and circulating RBCs. The results may also be a route whereby RBCs accumulate excessive amounts of zinc during malaria.

CHAPTER 1 INTRODUCTION

Zinc is an essential element which is ubiquitously distributed in the human body, and its deficiency is known to cause reduced growth, hypogonadism, visceromegaly, and hematological abnormalities associated with iron deficiency anemia (1). The functional properties of zinc can be categorized by its catalytic, structural and regulatory roles in numerous metabolic processes of the biological system (2). Differential expression of zinc transporters is known to be involved in the regulatory mechanism of the intracellular zinc homeostasis in various tissues and cell types (3). There are two distinct gene families of zinc transporters composed of ten ZnT and fourteen Zip transporter genes, respectively. ZnT proteins facilitate the removal of cytosolic free zinc either by exporting through the plasma membrane or by sequestering zinc in vesicles, while the Zip transporters function in an opposite manner as a pathway for zinc influx from plasma or vesicles.

In mature red blood cells (RBCs), the zinc concentrations is about fifteen times larger than that in the plasma (4), and more than 90% of that is known to function as a component essential for the activity of zinc metalloenzymes, carbonic anhydrase and $\text{Cu}^{2+}/\text{Zn}^{2+}$ -superoxide dismutase (5). Various routes of circulating RBCs whereby zinc influx occurs have been reported by classical studies, of which suggested mechanisms involve the $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger activity, a neutral complex formation with thiocyanate, salicylate ions, and the chelation by amino acids (6,7). The calcium-dependent zinc efflux by a $\text{Ca}^{2+}/\text{Zn}^{2+}$ exchanger has been considered as the mechanism for the cellular zinc export from circulating RBCs (8). Additionally, there have been animal and human studies with zinc deficient subjects implying the expression of zinc-responsive intrinsic factors involved in the RBC zinc transport system (9-11). In these studies, RBCs from zinc deficient groups consistently revealed higher ^{65}Zn uptake rates than those

collected from normal subjects when cultured in conditions with identical zinc contents *in vitro*. Even though the modulation of zinc uptake rate is likely to be influenced by the zinc transporter expression, there have been no reports related to the determination of erythroid zinc transporters so far.

Proteins involved in the zinc metabolism of mature RBCs are likely to be remnants from earlier developmental stages as the capability for additional gene expression or protein production is deprived by enucleation at the final step of erythropoiesis. A study showing increased zinc uptake by the bone marrow during induced erythropoiesis in zinc deficiency supports the necessity of minimal amount of zinc during erythroid differentiation (12). One of the most well-studied features of zinc in erythroid differentiation is its incorporation into zinc finger transcription factors which are responsible for the expression of essential proteins involved in events of terminal erythroid maturation (13,14). Additionally, zinc metalloenzymes, such as carbonic anhydrase and $\text{Cu}^{2+}/\text{Zn}^{2+}$ -superoxide dismutase, are produced during erythropoiesis (15,16). Some clinical studies of zinc treatment for anemia have shown that it can reverse anemic symptoms by increasing the production of hemoglobin (Hb) and, consequently, facilitate the formation of normal RBCs (17,18). However, in converse, there have been reports of sideroblastic anemia caused by zinc intoxication as well (19,20). This may be due to the interference by excessive free zinc ions with incorporation of ferrous ions into protoporphyrin during the heme biosynthetic pathway (21). Based on these findings, it seems critical for the erythroid intracellular zinc level to be tightly regulated during late stage erythroid differentiation so that any adverse effects introduced by inadequate or excessive zinc supply can be avoided.

With consideration of these aspects related to the erythroid zinc metabolism, this study was designed upon the following hypotheses:

- **Hypothesis 1:** As in other tissues and cell types, certain ZnT and Zip proteins would be expressed in mature red blood cells for the maintenance of zinc homeostasis during circulation.
- **Hypothesis 2:** Zinc transporters detected in mature RBCs would be remnants from preceding developmental stages. Thus, the expression of respective transporters would occur during the EPO-mediated differentiation of late stage erythroid progenitor cells.

Consequently, the major aim of this study was to determine which zinc transporters may be involved in the erythroid zinc trafficking system, and characterize the transporter expression during RBC maturation. Initially, the zinc transporters expressed in circulating erythrocytes were identified at the protein level. Thereafter, temporal trends of each respective transporter expression in differentiating erythroid progenitor cells were determined. For a more comprehensive understanding of the zinc metabolism during terminal erythroid differentiation, mRNA levels of other zinc metabolism genes, *MT-1* and *MTF-1*, were also measured in differentiating cells.

CHAPTER 2 LITERATURE REVIEW

Erythropoiesis

Red blood cell (RBC) production initiates from a pluripotential hematopoietic stem cell and sequential differentiation of each intermediate cell type in the hematopoietic hierarchy depends on the activation by lineage-specific growth factors (22). Normally, the site of erythropoiesis is the bone marrow and in specific conditions, such as anemic subjects and embryos, the spleen and the liver becomes the major site of erythropoiesis, respectively (23). Once pluripotential hematopoietic stem cells become erythroid progenitor cells after carrying out several steps of erythropoiesis, they exclusively differentiate into RBCs as a response to a specific glycoprotein termed erythropoietin (EPO) (24). Erythroid progenitor cells have been classified into two types, the burst-forming unit-erythroid (BFU-E) and the colony-forming unit-erythroid (CFU-E). Both types require EPO for further differentiation; however, the BFU-E requires other growth factors such as interleukin-3 or granulocyte-macrophage colony stimulating factor (GM-CSF) in addition to EPO, while the CFU-E does not (24).

During the terminal stage of erythropoiesis, i.e., CFU-E differentiation, critical events for normal RBC formation occur. For instance, hemoglobin (Hb) biosynthesis and enucleation happen as a response to EPO-induction, and characterize the unique properties of erythrocytes (24). Most adverse effects of nutrient deficiencies or toxicities-related to anemia occur during this stage of RBC production. For example, in iron deficiency, an inadequate supply of ferrous ions yields hypochromic anemia by introducing an increased zinc/iron ratio in erythroblasts which leads the ferrochelatase-facilitated reaction to produce zinc protoporphyrin rather than the essential component of Hb, heme (25). Vitamin B6 deficiency results in sideroblastic anemia

because pyridoxal 5-phosphate is a cofactor for erythroid δ -aminolevulinic acid synthase (ALAS-2) activity (26).

***In Vitro* Models of Terminal Erythroid Differentiation**

As normal RBC formation depends on the sequential events during the terminal stage of erythropoiesis, several *ex vivo* models representing this step have been developed for hematological studies. For instance, splenocytes and bone marrow cells from phenylhydrazine (PHZ)-treated or Friend virus (FVA)-infected anemic animal models, and erythroleukemia (MEL) cell lines have been commonly used (23,27). Because of the absence of nucleus in mature RBCs, the use of these systems is necessary especially in approaches for the characterization of erythroid gene expression and the understanding functions of the respective proteins. Hodges et al. suggested that erythroblasts from PHZ-treated anemic mice show the highest homology to the *in vivo* erythroid system by evaluating the responsiveness of several erythroid specific genes in three different types of *in vitro* models after EPO-induction (27). In this cell model, sequential trends in expression of each gene were detected and the time-points of maximum mRNA abundance were shown to be dependent on the metabolic needs of the relevant protein activity during erythroid maturation.

Not only reliable *ex vivo* systems but also protocols for the confirmation of terminal erythroid differentiation are well-developed. Staining Hb, of which peroxidase activity yields a brownish-red product in staining solutions with o-dianisidine or benzidine, has been used as a classical method for erythroid differentiation assessment (27,28). Evaluating the expression of genes only induced in differentiating erythroids, such as ALAS-2 (27,29), is another reliable and also safer way to determine the differentiation status. This is especially relevant when the carcinogenic risks of the above reagents are concerned (US Department of Health and Human Services (DHHS), National Institute for Occupational Safety and Health (NIOSH) Publication

No. 81-106). Additionally, morphological changes of the cells, such as smaller size and nucleus extrusion, are markers of the terminal stage of differentiation (27).

Zinc Metabolism during Terminal Erythroid Differentiation

Zinc is required for the activities of several proteins related to erythroid differentiation. One of the most apparent biochemical properties of erythroid zinc is based on its role as a key structural component of the zinc finger proteins. Since the CFU-Es are committed to differentiate into mature erythrocytes by EPO-induction, an EPO-responsive zinc finger transcription factor, GATA-1, of which binding sites are located in all erythroid-specific genes, initiates its critical role as the coordinator of multiple events composing the differentiation process by regulating relevant gene expressions (14,15). Another red cell-specific zinc finger transcription factor, erythroid Krüppel-like factor (EKLF), is essential for the transcriptional induction of genes encoding β -globin, which with heme composes Hb, and ferrochelatase, which facilitates the incorporation of ferrous ions into protoporphyrin as the final step of heme synthesis (13). Additionally, both of these zinc finger proteins are known to be involved in the transcription of ALAS-2 and porphobilinogen deaminase (PBGD) genes (13,14). Their products are the rate-limiting enzymes of the heme biosynthetic pathway.

The production of the zinc metalloenzyme, carbonic anhydrase (CA), which contains around 87% of total RBC zinc content (5), occurs during the EPO-mediated terminal differentiation as well (15). This enzyme is the second most abundant protein in mature erythrocytes, after Hb, and requires zinc as an essential component for its catalytic activity. The induction of CA expression during the differentiation of erythroleukemia cells precedes hemoglobin synthesis (15), which may imply an increased requirement of zinc at the early stage of terminal erythroid differentiation. Supporting these functional properties of erythroid zinc, a

study by Huber et al. showed that zinc uptake by the bone marrow of zinc-restricted rats increased during induced-erythropoiesis (12).

Zinc Status and Anemia

Since a case of zinc deficiency associated-anemia was firstly reported by Prasad et al in 1961 (1), clinical studies on the value of zinc supplementation to anemic subjects have been performed. For instance, two consecutive studies by Nishiyama et al. were designed to determine effects of zinc supplementation on anemic middle-aged or pregnant women (17,18). Conclusive benefits were shown when adequate iron intake was ensured. Briefly, the concentrations of Hb, numbers of RBCs and reticulocytes in anemic patients were significantly increased by the zinc plus iron treatment, while the values from the other treatment groups, i.e., treated exclusively with zinc or iron, did not change. In addition, this research group also suggested that normocytic anemia with low total iron binding capacity (TIBC), which is inversely related to the transferrin saturation status and thus implicates the status of iron deficiency, may serve as an indicator of zinc deficiency (17).

While zinc supplementation has been reported to have beneficial effects on overcoming anemic symptoms, adverse effects leading hematological abnormalities introduced by excessive zinc treatment have been observed as well. Sideroblastic anemia associated with excessive and prolonged intake of oral zinc in human has been notified by several case reports (19,20). In addition, increased frequency of abnormal and/or immature erythrocytes in the blood stream has been observed in zinc intoxicated rats, mallards, and carps (30-32). Although secondary copper deficiency has been considered as the major reason of these symptoms, Bloomer et al. suggested that anemia introduced by excessive zinc may be attributed to the increased formation of a biologically inactive compound, zinc protoporphyrin (ZPP), instead of heme (21). Zinc strongly competes with iron for ferrochelatase and its product, ZPP, can exert the feedback inhibition of

ALAS-2 produced by heme. Even a slight decrease in iron availability by increased zinc can cause ZPP accumulation. Supporting this concept, increased formation of ZPP-globin by the decreased erythroid iron/zinc ratio in the maturing erythrocytes is known to be the reason of anemic symptoms during iron deficiency (25).

Erythroid Zinc Trafficking System

The intracellular zinc concentration in circulating RBCs is approximately fifteen times larger than the plasma levels (4). Over 90% of total erythrocyte zinc attributes to the enzyme activities of carbonic anhydrase and $\text{Cu}^{2+}/\text{Zn}^{2+}$ -superoxide dismutase as catalytic and structural elements, respectively (5). Accordingly, an adequate amount of zinc supply would be essential even after the erythroid maturation is accomplished. Supportively, impaired carbonic anhydrase activities, which catalyze the reversible hydration and dehydration of carbon dioxide, have been observed in subjects with low zinc diets (33).

The differential and tissue specific expression of zinc transporters have been strongly related to the homeostatic regulation of exchangeable systemic and cellular zinc pools in biological systems (3). Zinc transporters are composed of two distinct gene families, ZnT and Zip, of which proteins facilitate the decrease and increase of cytosolic zinc, respectively, by expression across plasma or vesicle membranes. The zinc removal by ZnT occurs either by exporting through the plasma membrane or by sequestering zinc in vesicles, while the influx by Zip is mediated in the opposite manner. Even though the zinc trafficking system in various tissues and cell types have been extensively studied during the past decade, little is known about the specific pathways for ionic zinc transport across RBC membranes. Some classical studies have suggested that the zinc uptake mechanisms would be mediated by interactions with other biological compounds. For instance, facilitated diffusion of zinc by chelation with amino acids, particularly L-histidine and L-cysteine, has been thought to be related the zinc influx in rat and

human RBCs (6). Additionally, two anion-dependent mechanisms related to the zinc influx system in human erythrocytes were suggested (7). One of these requires bicarbonate ions which induces the formation of a zinc-anion complex, $[\text{Zn}(\text{HCO}_3)_2\text{Cl}]^-$, that can be taken up via the $\text{Cl}^-/\text{HCO}_3^-$ anion exchanger. The other one, involving thiocyanate or salicylate ions, has been thought to be accomplished by forming a neutral zinc complex that migrates across the lipid bilayer. With regard of pathways whereby RBC zinc efflux may occur, a calcium-dependent mechanism through the $\text{Ca}^{2+}/\text{Zn}^{2+}$ exchanger is the only route speculated so far (8).

Despite absence of definitive evidences, it is likely that zinc transporters are involved in the homeostatic regulation of erythroid zinc metabolism. Animal and human studies have shown a modulation in the zinc trafficking rate of erythrocytes during zinc deficiency (9-11). Specifically, increased *in vitro* ^{65}Zn uptake rates of RBCs were observed in rat, sheep and human subjects when fed zinc-restricted diets. These results would imply the presence of a zinc-responsive intrinsic factor that enables the homeostatic regulation of erythroid intracellular zinc. In other words, the regulated activity of this factor would result in a higher zinc uptake rate which would correct the cellular zinc loss introduced by the zinc-deprived conditions *in vivo*. This aspect is quite consistent with the general properties of the homeostatic regulatory mechanism mediated by zinc transporter expressions in other tissues (3). Consequently, it was of interest to determine the transporter expressed in erythroid lineage cells. The purpose of the current study is to identify of zinc transporter thought to be involved in erythroid zinc trafficking system, and characterize the expression trends during the maturation of RBCs.

CHAPTER 3 MATERIALS AND METHODS

Preparation of RBC Membranes

Whole blood (~ 400 µl/subject) was collected from anesthetized CD-1 mice through cardiac puncture and aliquoted into EDTA-treated centrifuge tubes. Three volumes of wash buffer [5 mM Sodium Phosphate (pH 7.4~8), 0.15M NaCl] was added to the blood sample which was then centrifuged at 2,000 x g x 10 min, 4°C. After carefully aspirating the supernatant and buffy coat, the pellet was washed with 2 vol of wash buffer, extensively. Following the final wash, the presence of RBCs was confirmed by phase contrast microscopy. Isolated RBCs were then lysed by washing with 3 vol of a hypotonic lysis buffer [5 mM Sodium Phosphate (pH 7.4~8) with protease inhibitor cocktail (Sigma, St. Louis, MO)]. After centrifugation at 12,000 x g x 10 min, 4°C, the supernatant was aspirated. Then the tube was tilted and rotated for the aspiration of the red button, which is beneath the ghost pellet and contains proteases. The cell lysis step was repeated until the pellet lost its red color (3~7 times). Each final pellet was dissolved in 200 µl of storage buffer [5 mM Tris-HCl, 0.5% Triton X-100 with protease inhibitor cocktail (Sigma)] for solubilization. Finally, the protein concentration of the red cell membrane preparation was determined colorimetrically with the D_C Protein Assay kit (Bio-Rad, Hercules, CA). Standards developed with diluted bovine serum albumin solutions and the absorbance was read at 750 nm using a Beckman DU 640 spectrophotometer (Beckman, Fullerton, CA).

Production of Primary Erythroid Progenitor Cells

Primary erythroid progenitor cells were prepared from spleens of PHZ-treated anemic mice. CD-1 mice were injected with PHZ hydrochloride (Sigma) in 0.9% saline (60 mg/kg bw) intraperitoneally on day 1 and day 2. On day 5, the PHZ-treated mice were sacrificed and the spleen was collected. Monocellular splenocyte suspensions were prepared by cutting the spleen

into small fragments in AMEM (Mediatech, Herndon, VA) containing 10% FBS and antibiotic antimycotic solution (Sigma), and by passing the disrupted spleen through nylon mesh. The viability and concentration of cells were determined by staining an aliquot of the cell suspension with Trypan Blue solution (0.4%; Sigma).

Cell Culture

To induce further differentiation of the late stage erythroid progenitors, cells were incubated with recombinant human EPO (ProSpec-Tany, Rehovot, Israel). The single spleen cell suspension was diluted into 1.0×10^7 cells/ml with AMEM containing 10% FBS and antibiotics/antimycotics for treatment. The treatment was conducted by a further 1:2 dilution with an equal volume of fresh medium containing EPO as 10 IU/ml. With the final concentration of cells and EPO as 5.0×10^6 cells/ml and 5 IU/ml, respectively, cells were equally plated in 6-well culture plates as 1.0×10^7 cells/well and were incubated for up to 48 h at 37°C in 5% CO₂. The control cell cultures were prepared in parallel with identical procedures except for using EPO-absent medium at the final step of dilution.

o-Dianisidine Staining

Differentiation of cells was confirmed by comparing the hemoglobin synthesis levels of cells collected at 0 h and 48 h after EPO-treatment through o-dianisidine staining. The staining solution [0.1 mg/ml o-dianisidine (Sigma D9154), 0.2% (v/v) SDS, 0.3% (v/v) hydrogen peroxide in PBS] was prepared freshly for each time-point. 500 µl of cell suspension was washed with 500 µl of sterile PBS by centrifugation at 250 x g x 5 min, 4°C. The supernatant was carefully aspirated and the final pellet was resuspended with 500 µl of the staining solution. After incubation at room temperature for 30 min, cells were spun down and washed with 500 µl of PBS. Cell suspensions were transferred to wells of a Lab-Tek Permanox Chamber Slide

(Nunc, Rochester, NY) and the staining was examined by light microscopy with an Axiovert microscope (Zeiss, Oberkochen, Germany).

RNA Isolation

Cells were harvested at certain time-points covering the whole 48 h differentiation. The time-course was designed to determine the temporal trends in changes of each mRNA level throughout differentiation and detect the time-point at which maximum EPO-responsiveness exists. After centrifugation at 400 x g x 5 min, 4°C, cell pellets were resuspended with 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and stored at -80°C until the entire time-course was over. When all samples were collected, cell lysates were incubated at room temperature for 5 min after thoroughly thawed. After adding 200 µl of chloroform, each sample were centrifuged at 12,000 x g x 10 min, 4°C and the top clear aqueous layer was transferred to a new sterile tube. The aqueous phase was incubated with 500 µl of isopropyl alcohol at room temperature for 10 min and centrifuged at 12,000 x g x 15 min, 4°C for RNA precipitation. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% (v/v) ethanol by centrifuging at 12,000 x g x 10 min, 4°C. After repeating the wash step at least twice, ethanol was removed and dried out from the RNA pellet at room temperature. Finally, the RNA pellet was resuspended with 50 µl of nuclease-free water and incubated in 37°C for complete dissolve. RNA solutions were treated with DNase I (Ambion, Austin, TX) to avoid any DNA contamination and the concentrations were determined spectrophotometrically (NanoDrop Technologies, Wilmington, DE).

cDNA Synthesis and Quantitative RT-PCR

Relative mRNA levels of ALAS-2, Zip10, ZnT1, MT-1 and MTF-1 were measured by quantitative real-time PCR (qRT-PCR). Primers for mouse ALAS-2 gene were designed with PRIMER EXPRESS V2.0 (Applied Biosystems, Foster City, CA) while those for others were

available from previous studies of our lab. Primers for the PCR amplification of ALAS-2 cDNA were: forward primer, 5'-CAGAGGGCAGCTCCAGAAGTT-3'; reverse primer, 5'-GCTTCGGGTGGTTGAATCC-3'. cDNA was generated by reverse transcription of total RNA (100 ng/reaction) using the high capacity cDNA archive kit (Applied Biosystems), and 1:25 diluted to be used as templates of each PCR reaction. The cDNA product of the total RNA collected at 0 h was used as the standard after 1:2 dilution and further 1:10 serial dilutions. All qRT-PCR assays were conducted with the Power SYBR Green Master Mix (Applied Biosystems) and amplification products were fluorometrically measured by the iCycler RT-PCR detection system (Bio-Rad). The specificity of each primer pair was confirmed after each qRT-PCR assay by melting curve analyses and the relative quantity was determined by normalization with respective 18s rRNA values.

Protein Isolation

Cells were treated and cultured as described above. After designated time-points of incubation, cells were spun down at 400 x g x 7 min, 4°C, and the supernatant was aspirated carefully. Total cell lysates were prepared by dissolving the cell pellets (~ 1.0 x 10⁷ cells) with 100 µl of SDS cell lysis buffer [10% (w/v) SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0 with protease inhibitor cocktail (Sigma)] and following homogenization with brief sonication. Total membrane fractions of the erythroid splenocytes were prepared by utilizing a commercial membrane protein extraction kit from BioVision (Mountain View, CA). Cell pellets (~ 9.0 x 10⁷ cells), collected after washing with PBS, were homogenized with a Dounce cell disrupter using 100 up/down strokes in the homogenization buffer provided. After spinning down the homogenate at 700 x g x 10 min, the supernatant was then further centrifuged at 1000 x g x 30 min, 4°C. The final total cellular membrane protein was dissolved in 50 µl of solubilization buffer [5 mM Tris-HCl, 0.5% Triton X-100 with protease inhibitor cocktail (Sigma)] and stored

at -80°C. The protein concentrations were determined colorimetrically with the D_C Protein Assay kit (Bio-Rad).

Affinity Purification of Antibodies

Rabbits were injected with respective antigenic peptides for production of polyclonal antibody to each Zip and ZnT transporter for previous studies (Table 3-1). Total IgG was prepared from serum using the Montage Antibody Purification PROSEP-A Kit (Millipore). Columns for affinity chromatography of each antibody were generated by immobilizing peptide to a Sulfo-link Coupling Gel (Pierce) bed. The cysteine added to the C-terminus allowed the peptide to conjugate to the Sulfo-link matrix during this process. 15~20 mg of total IgG was then incubated in the column overnight at 4°C, and purified by passing Elution Buffer (Pierce) through the column. The fractions (1 ml) with the highest protein concentrations as determined spectrophotometrically (NanoDrop Technologies) were pooled. Finally, the affinity purified IgG solution was desalted into TBS containing 0.02% sodium azide and stored at 4°C.

Western Analysis

Forty µg of each protein was denatured in a 2x denaturing buffer [125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.02% (w/v) bromophenol blue] for 15 min at 55°C. Denatured proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (Schleicher und Schuell, Dassel, Germany) or polyvinylidene difluoride membrane (PVDF) (Immobilon; Millipore, Bedford, MA). Protein transfer and equal protein loading were confirmed by Ponceau Red staining. After washing out the stain with TBS, membranes were blocked in 5% skim milk (0.5 g skim milk in 10 ml TBS-T) for 50 min on orbital shaker in room temperature. PVDF membranes were treated with methanol and Milli-Q water until they were completely wet before blocking. Blocked membranes were incubated in primary antibody solution (10 µg IgG/ml of

blocker or 1-2 μg affinity-purified IgG/ml of blocker) for additional 1 h and washed with TBS-T 4 times for 5 min each. Membranes were then incubated with anti-rabbit IgG secondary antibody (1:2000 of antibody in 5% skim milk) conjugated to horseradish peroxidase (GE Healthcare, Buckinghamshire, UK) or alkaline phosphatase (Amersham Biosciences, Arlington Heights, IL) for 50 min. Thereafter, membranes were washed twice with TBS-T and TBS for 5 min each. Membranes probed with horseradish peroxidase-conjugated antibodies were treated with an enhanced chemiluminescent substrate (SuperSignal WestPico; Pierce, Rockford, IL), and exposed to autoradiographic film for detection. Blots treated with alkaline phosphatase-conjugated antibodies were incubated with ECF chemifluorescent substrate (Amersham Biosciences) for visualization with a PhosphorImager (Storm 840 Imager; Molecular Dynamics). Specificity of bands was determined by comparing results to those obtained with primary antibody preincubated with respective peptide solution (250 μg peptide/ml of blocker).

Statistical Analysis

Data are expressed as mean \pm standard deviation and were analyzed by two-way ANOVA with time and EPO treatment as independent variables. Differences between each treatment group at respective time-points were determined by a following Bonferroni post-test (Prism 5 for Windows; GraphPad Software, San Diego, CA). The level of statistical significance was set at $P < 0.05$, and differences are annotated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3-1. Zinc transporters screened in erythrocyte ghosts and peptides used for the production and affinity purification of antibodies against each respective protein.*

Transporter	Peptide
ZnT1	CGTRPQVHSGKE
ZnT2	CHAQKDSGSHP
ZnT4	CMQLIPGSSSKWEE
ZnT5	CSPSKRGQKGTLI
ZnT6	CVAPNVLNFSDDHHVIP
Zip1	CRSGANHEASASG
Zip2	CEEEWGGTHAFGFH
Zip3	CAGLRLRELGRPG
Zip4	CAEETPELLNPETRRL
Zip10	CKRNHKCDPEKE

*Antigenic peptide sequences were designed by previous and current members of Robert J. Cousins' lab.

CHAPTER 4 RESULTS

Expression of Zinc Transporters in RBC Membranes

According to the absence of a nucleus in circulating mature erythrocytes, the zinc transporters expressed in RBCs was determined with an approach in protein levels. Membrane fractions (erythrocyte ghosts) were prepared directly from whole blood of adult male CD-1 mice. Purity and presence of the RBC population after fractionation were confirmed by light microscopy to eliminate effects of contamination by any other blood cells. Of the ZnT proteins tested (Table 3-1), ZnT1 was the only zinc exporter detectable in the erythrocyte membrane fraction. As the estimated molecular mass at 30 kDa was inconsistent with that reported by previous studies (34,35), the specificity of the affinity purified antibody to ZnT1 was further confirmed by peptide competition. Signals on the blot were successfully competed out when the antibody was preincubated with the ZnT1 peptide (Fig. 4-1A). Among the Zip proteins screened (Table 4-1), only the presence of Zip10 was confirmed with the expected band size suggested by other research groups (40 kDa) (36,37). The reliability of the Zip10 western analysis was also validated by peptide competition, and the single prominent band signal was blocked by the Zip10 peptide (Fig. 4-1B).

Confirmation of Erythroid Differentiation

Proteins in circulating RBCs are likely to be remnants of the protein components synthesized in differentiating erythroid progenitor cells. For the characterization of the responsiveness of the ZnT and Zip transporters, an *in vitro* model of late stage erythroid progenitors prepared from splenocytes of PHZ-treated anemic CD-1 mice was utilized. Cells were collected only when splenomegaly was observed as an indicator of PHZ-induced hemolytic anemia (Fig. 4-2).

For the confirmation of erythroid maturation, erythroid splenocytes were incubated with o-dianisidine prior to and after EPO-stimulation for Hb-staining. An increase in the number of Hb-positive cells was observed in the cell population incubated with EPO for 48 h (Fig. 4-3A and Fig. 4-3B). The transcript abundance of an EPO-dependent erythroid specific gene, ALAS-2, was also measured during the 48 h time-course as a more comprehensive positive marker of terminal erythroid differentiation. A temporal pattern of ALAS-2 expression, consistent to that reported by Hodges et al. (27), was detected in the EPO-treated cells. The maximum ALAS-2 mRNA abundance was observed at 24 h, with a 3-fold increase after EPO-stimulation. The significantly higher levels in differentiating cells lasted until 48 h after addition of EPO (Fig. 4-3C).

Effects of EPO on Zip10 and ZnT1 Transcript Levels

Temporal trends in transporter expression during the late stage erythroid differentiation were determined by qRT-PCR. Each value was normalized to 18S rRNA and the basal levels measured at 0 h of incubation. The transcript levels from cells cultured in the presence or absence of EPO were compared at each time-point to determine the effect of EPO per se. Zip10 mRNA levels in differentiating cells showed an approximately 2-fold increase after 6h of incubation (Fig. 4-4). However, the levels were not sustained during subsequent periods and values became less than 50% of the basal (0 h) levels after 24 h of EPO-treatment. Cells deprived of EPO failed to show any increase in Zip10 mRNA levels throughout the entire culture period. The relative mRNA abundance in differentiating cells was significantly higher than that of resting cells from the 6 h to 24 h post-incubation, while there was no significant difference detectable at the following time-points. Expression trends of ZnT1 were distinctively different with those of Zip10 when cells were differentiating. A gradual increase of ZnT1 transcripts was observed in both differentiating and resting cells until 12 h (Fig. 4-5). However, as the incubation

period reached 24 h, the mRNA abundance in differentiating cells became significantly higher than that in EPO-deprived cells, and the difference was sustained until the whole time-course was accomplished. The maximum abundance was also observed at 24 h, with a 3-fold higher level than the 0 h basal levels.

Protein Expression of Zip10 and ZnT1 during Differentiation

The effects of EPO-induction on protein levels of Zip10 and ZnT1 were initially determined by western analyses with total cell lysates. Decreased Zip10 protein abundance was detected after 48 h (Fig. 4-6A and Fig. 4-6B) regardless of the presence or absence of EPO. Even though the induction of Zip10 expression by EPO was observed in mRNA levels at early time-points (Fig. 4-4), these differences were barely detectable in protein levels with the total cell lysates. Accordingly, a pilot experiment with total cell membrane fractions was conducted to remove any potential compromising effects by excessive cytosolic proteins. Since the membrane fraction in protein samples became more concentrated, the effects of EPO on Zip10 protein expression could be detected in a consistent manner to the mRNA data (Fig. 4-6C). An increase in Zip10 abundance was observed at 9 h by EPO-induction compared to splenocytes not treated with EPO. The protein expression trend of ZnT1 in resting cells was quite similar to that of Zip10 by having a decreased expression at 48 h (Fig. 4-7). However, a different trend was observed when cells were differentiating. The abundance of ZnT1 was sustained throughout the whole time-course by the EPO-mediated terminal erythroid differentiation. Consequently, a prominent difference between the ZnT1 protein levels in the differentiating and resting cells was detectable at the final time-point, 48 h (Fig. 4-7A). In agreement with the mRNA data, these results indicate that the relative ZnT1 protein levels to those of Zip10 tend to be higher at the very late stage of terminal erythroid differentiation than during the preceding periods.

Effects of EPO on MT-1 and MTF-1 Transcript Levels

The temporal expression patterns of two zinc responsive genes involved in zinc metabolism gene expression were investigated. Little is known about the zinc metabolism during terminal erythroid differentiation. The effects of EPO-induction on MT-1 mRNA abundance were determined as this protein has been reported to be a sensitive indicator of the intracellular zinc availability in various cell types (2). It was hypothesized that MT-1 mRNA abundance would also be affected either directly or indirectly by the EPO-induced zinc transporter expressions. Even though the MT-1 transcript levels in both cell groups decreased drastically and were sustained lower than the basal level during the whole culture period, the relative abundance in differentiating cells was significantly higher than in resting cells until 24 h of incubation (Fig. 4-8).

Zinc-finger transcription factor MTF-1 is involved in the transcriptional regulation of numerous zinc-responsive genes including MT-1 and the expression of both Zip10 and ZnT1. In differentiating cells, MTF-1 mRNA abundance started to increase by 6 h of EPO-induction, and then stayed relatively higher than those measured in resting cells (Fig. 4-9). It is of interest that the expression trend of MTF-1 during differentiation revealed two peak levels, unlike trends observed in other mRNA levels. Specifically, the mRNA levels reached its first peak at 12 h, and a subsequent decrease at 18 h followed. These peaks in MTF-1 mRNA coincided with the periods when the decrease of Zip10 and the increase of ZnT1 mRNA levels occurred.

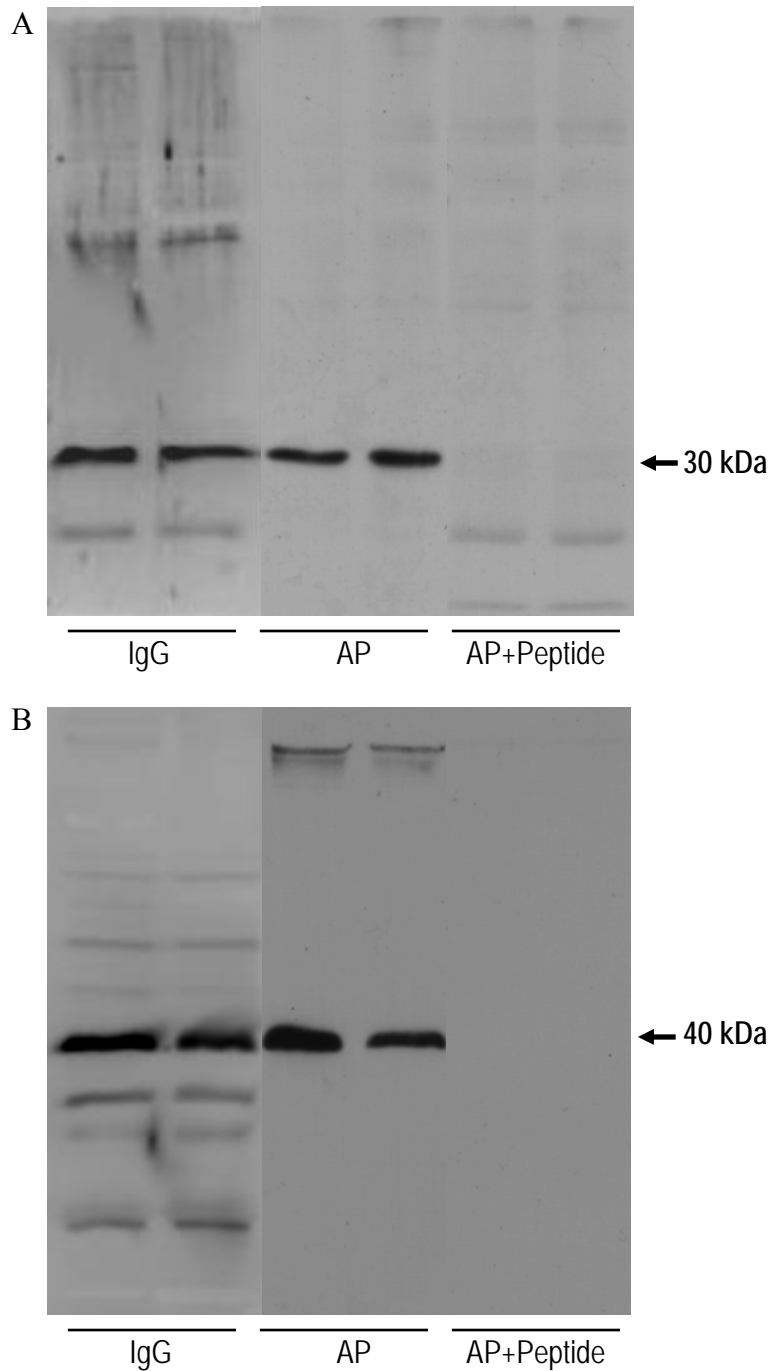


Figure 4-1. Zinc transporter expression in mature red blood cells. Erythrocyte ghosts were prepared for western analyses of zinc transporters. Among the transporters (Zip1-4, Zip10, ZnT1-2, ZnT4-6) tested, only A) ZnT1 and B) Zip10 expression were detected. The membranes were incubated with either the total IgG, affinity-purified IgG (AP), or AP that were pre-exposed to the corresponding ZnT1 or Zip10 peptide. The molecular mass of ZnT1 and Zip10 at 30 and 40 kDa, respectively, were determined with commercial molecular markers. There were no signals developed by antibodies against other zinc transporters (data not shown).

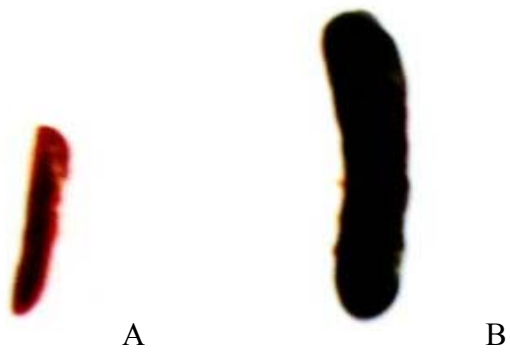


Figure 4-2. Induction of splenomegaly by phenylhydrazine-injection. CD-1 mice were treated with or without PHZ by intraperitoneal injection on day 1 and 2. Spleens were collected at day 5. A) A normal spleen and B) an enlarged spleen from PHZ-injected anemic mouse are compared.

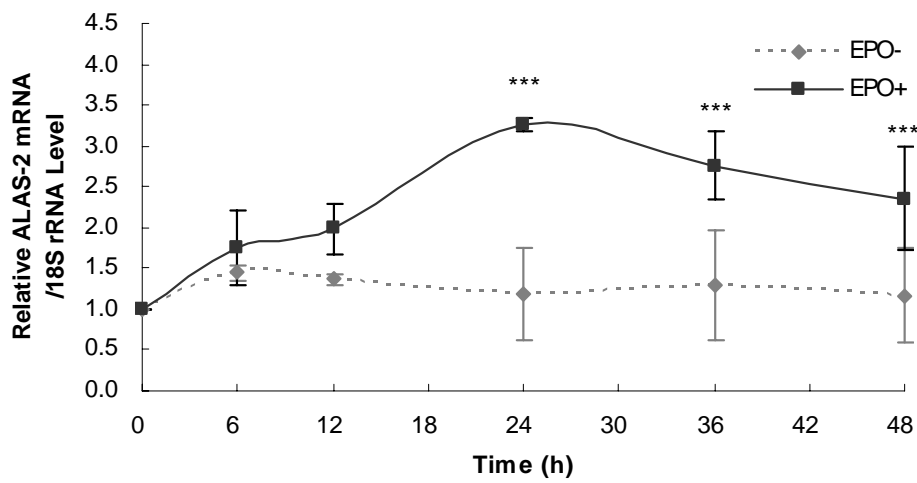
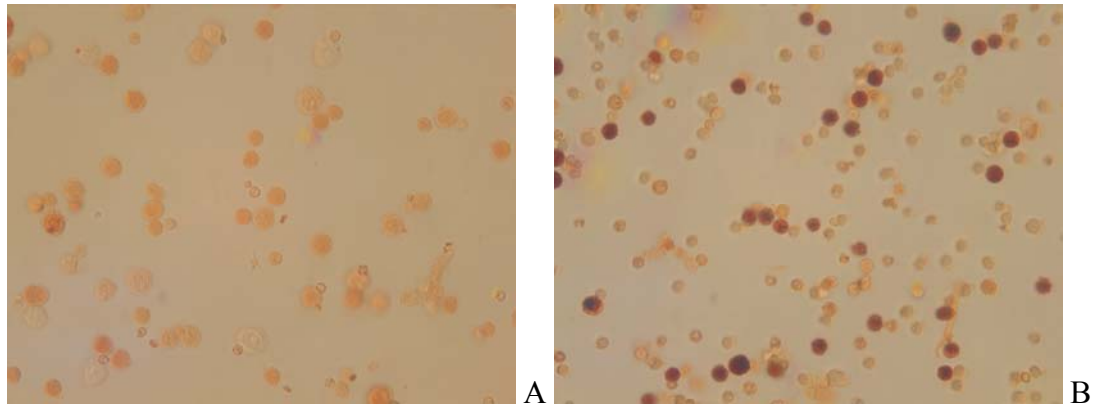


Figure 4-3. Indicators of EPO-mediated terminal erythroid differentiation *in vitro*. Hemoglobin staining of cells A) prior to and B) 48 h after EPO-treatment. C) Relative ALAS-2 mRNA abundance in EPO-treated and -deprived cells. Splenocytes were collected from spleens of two PHZ-injected mice and pooled for culture at each experiment. qRT-PCR assays were performed on duplicate total RNA samples. Values at each time-point are relative to the basal levels at 0 h. Data are expressed as mean \pm SD of four independent experiments (n = 4). Statistically significant differences between each treatment group are annotated as ***, P < 0.001.

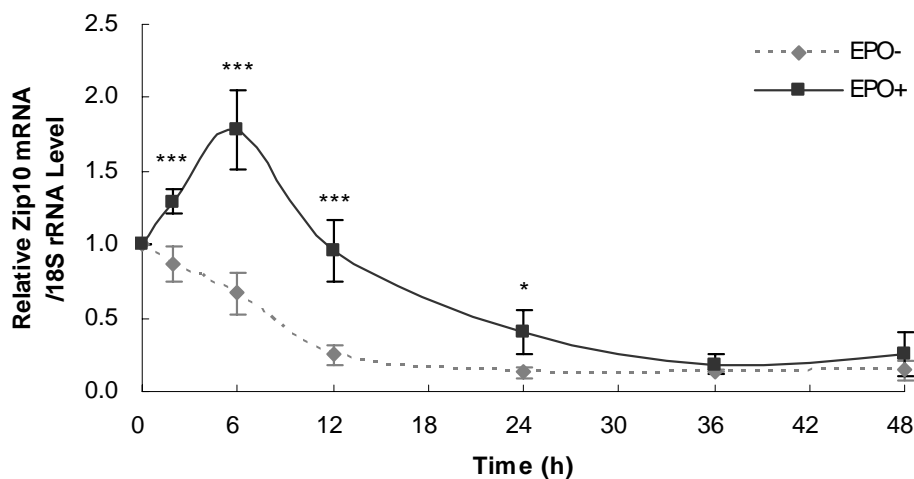


Figure 4-4. Relative Zip10 mRNA abundance during terminal erythroid differentiation. Splenocytes were collected from spleens of two PHZ-injected mice and pooled for culture at each experiment. qRT-PCR assays were performed on duplicate total RNA samples. Values at each time-point are relative to the basal levels at 0 h. Data are expressed as mean \pm SD of four independent experiments (n = 4). Statistically significant differences between each treatment group are annotated as *, $P < 0.05$; ***, $P < 0.001$.

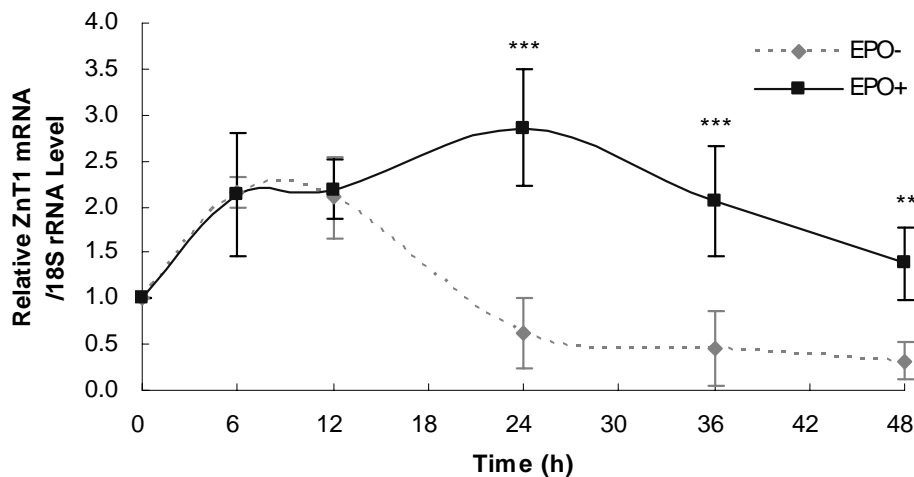


Figure 4-5. Relative ZnT1 mRNA abundance during terminal erythroid differentiation. Splenocytes were collected from spleens of two PHZ-injected mice and pooled for culture at each experiment. qRT-PCR assays were performed on duplicate total RNA samples. Values at each time-point are relative to the basal levels at 0 h. Data are expressed as mean \pm SD of four independent experiments (n = 4). Statistically significant differences between each treatment group are annotated as **, $P < 0.01$; ***, $P < 0.001$.

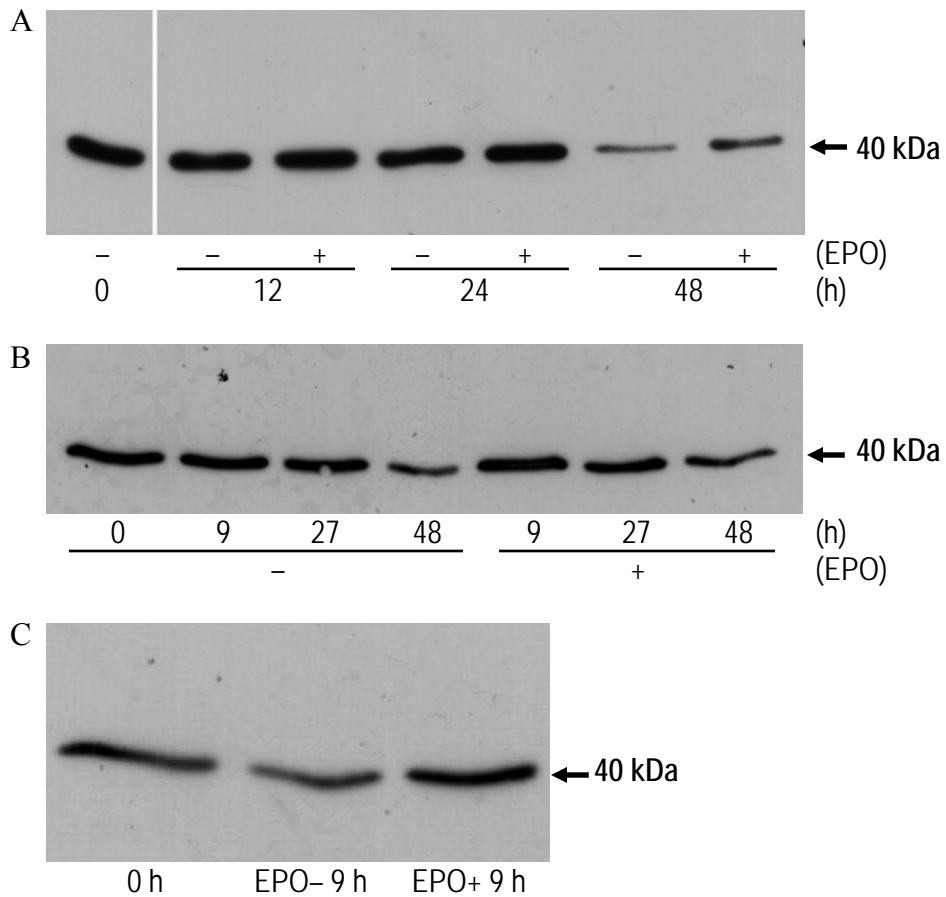


Figure 4-6. Zip10 protein expression during terminal erythroid differentiation. Cultured cells were collected at designated time-points. A,B) Western analyses from two experiments with total cell lysates reveal a decrease in Zip10 protein expression at 48 h regardless of EPO-treatment. C) EPO-induced Zip10 expression was only detectable with total membrane fractions.¹ A band with estimated molecular mass as 40 kDa was consistently observed in independent experiments.

¹ Results from the total membrane fraction reflect a pilot experiment conducted (n=1). Further assessments would be appropriate to affirm the data.

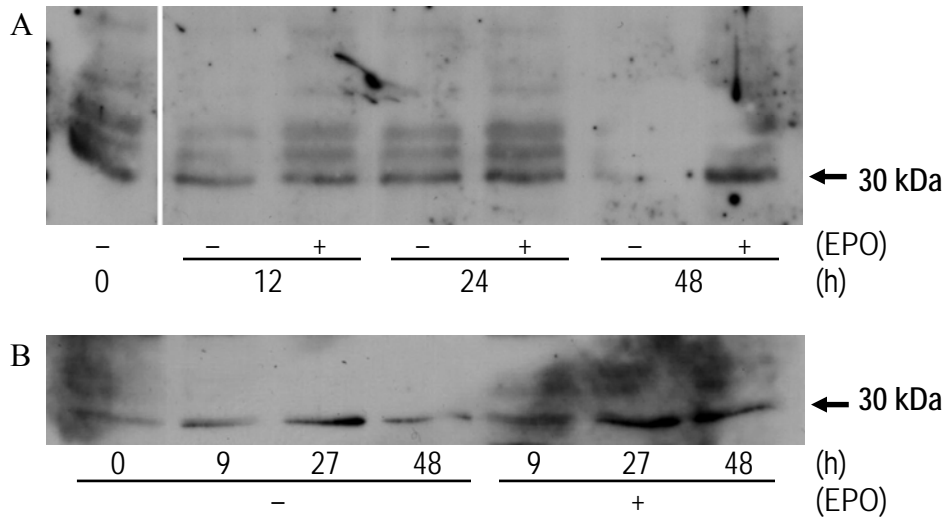


Figure 4-7. ZnT1 protein expression during terminal erythroid differentiation. Cultured cells were collected at designated time-points. A,B) Western analyses from two experiments with total cell lysates from EPO-treated cells imply a constitutive expression of ZnT1 during differentiation, while a decrease occurs at 48 h in EPO-deprived conditions. Only the band with estimated molecular mass as 30 kDa was consistently observed in independent experiments.

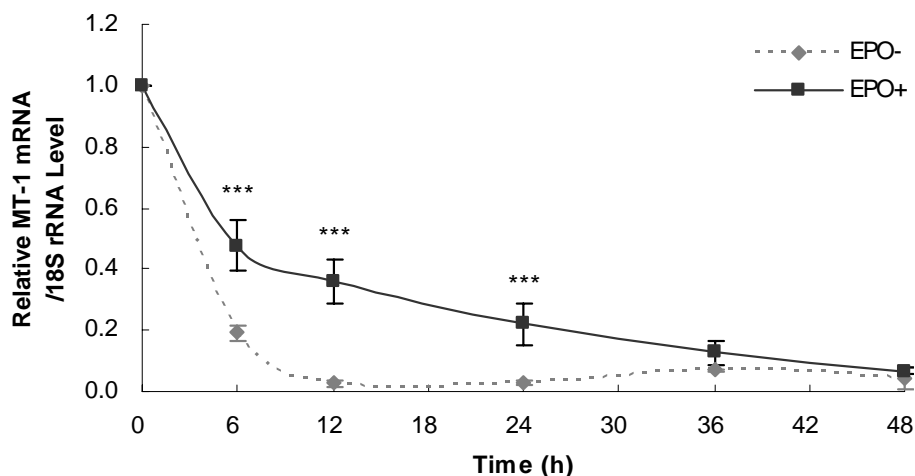


Figure 4-8. Relative MT-1 mRNA abundance during terminal erythroid differentiation. Splenocytes were collected from spleens of two PHZ-injected mice and pooled for culture at each experiment. qRT-PCR assays were performed on duplicate total RNA samples. Values at each time-point are relative to the basal levels at 0 h. Data are expressed as mean \pm SD of four independent experiments (n = 4). Statistically significant differences between each treatment group are annotated as ***, P < 0.001.

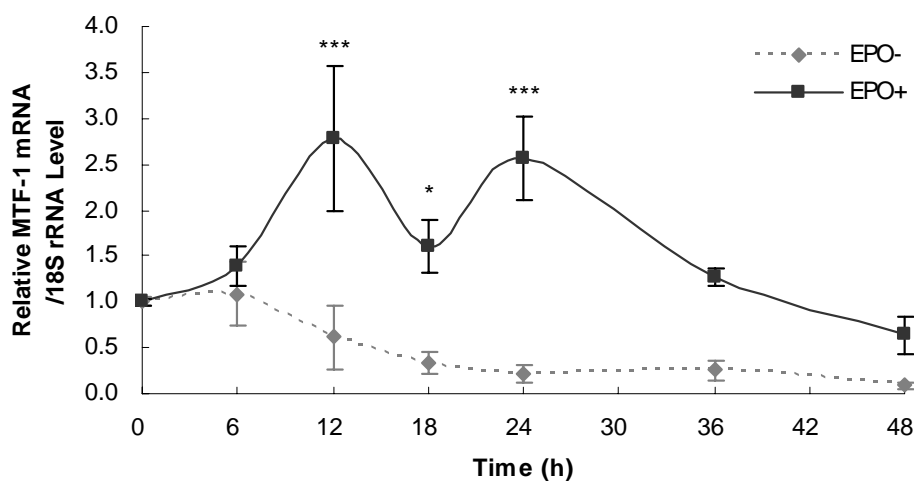


Figure 4-9. Relative MTF-1 mRNA abundance during terminal erythroid differentiation. Splenocytes were collected from spleens of two PHZ-injected mice and pooled for culture at each experiment. qRT-PCR assays were performed on duplicate total RNA samples. Values at each time-point are relative to the basal levels at 0 h. Data are expressed as mean \pm SD (n = 2 x 2).² Statistically significant differences between each treatment group are annotated as *, P < 0.05; ***, P < 0.001.

² Samples of the 18 h time-point, at which the fluctuation of MTF-1 mRNA levels was detected, were only available from two experiments. Thus, the results are represented as mean \pm SD from n = biological duplicates x analytical duplicates.

CHAPTER 5 DISCUSSION

Studies with regard of the zinc transport mechanism in various tissues and cell types have revealed two distinct gene families related to ionic zinc trafficking pathway across cellular plasma and vesicle membranes (3). Zip and ZnT proteins produced from these genes facilitate the cytosolic zinc influx and efflux, respectively, and establish the mechanism for the homeostatic regulation of intracellular zinc. Through the tissue-specific and differential expression of these transporters, the cellular zinc trafficking system can be modulated in response to various factors, such as the extracellular zinc availability, intracellular utilization, and numerous cytokines, growth factors and hormones (3).

Previous studies have consistently reported the zinc-responsiveness of the zinc trafficking system in circulating erythrocytes of animal and human subjects (9-11). Even though these may imply regulated transporter activities by dietary zinc, there has been no study to define the presence of zinc transporters in circulating RBCs. Consequently, the primary purpose of this study was to determine which transporters are expressed in mature RBCs. Each transporter was screened at the protein level utilizing the library of antibodies to numerous zinc transporters, available in our lab. The results from this experiment demonstrate that Zip10 and ZnT1 are expressed in circulating RBCs; thus, they are likely to be the zinc transporters directly involved in the homeostatic regulation of erythroid zinc metabolism. Although the estimated molecular mass of ZnT1 in RBCs (~30 kDa) conflicts with the value calculated from the amino acid composition (55 kDa), inconsistent molecular mass speculated from the migration by SDS-PAGE analysis has been reported by other ZnT1 studies as well (34,35). Possible explanations for the discrepancy in the aberrant migration of ZnT1 are well-delineated in a previous study utilizing the identical antibody for ZnT1 detection (34).

One of the most unique characteristics of circulating erythrocytes, compared to other cell types, is the absence of nucleus. In other words, the protein contents of mature cells are formed during preceding developmental stages, i.e., erythropoiesis, and the gene expression ability is deprived after maturation. Thus, the differential activity of the zinc trafficking system observed in mature RBCs in response to the host's zinc status (9-11) would be determined during the differentiation stages of earlier erythroid cell precursors. It is of note that the expression of both zinc transporters detected in mature RBC membranes have been suggested to be transcriptionally regulated in a zinc-dependent manner by the zinc-responsive activity of MTF-1; however, resulting in opposite modes (3). Even though further exploration is required to clarify these zinc effects on the RBC zinc transporters, it can be suggested that the modulated erythroid zinc uptake rate during zinc deficiency may be associated with the decreased DNA binding activity of MTF-1 that results in either the up-regulation of Zip10, down-regulation of ZnT1, or both during preceding erythroid developmental stages.

Among the available cellular models of terminal erythroid differentiation, splenocytes from PHZ-treated and FVA-infected animals have been suggested to most accurately represent the physiological aspects of *in vivo* erythroid progenitor cells (27). Accordingly, the PHZ model was selected for the characterization of zinc transporter expression during the EPO-mediated erythroid differentiation in the current study. EPO acts as a key factor for the initiation of further differentiation of late stage erythroid progenitor cells into reticulocytes either *in vivo* or *in vitro* (22,24). The properties of EPO during the RBC protein production during terminal erythroid differentiation can be categorized into two general aspects; first, the induction of *de novo* synthesis of certain proteins; second, the enhancement of an ongoing production initiated at a developmental stage prior to terminal erythroid differentiation (38). It is likely that the

expression of Zip10 and ZnT1 are extended by EPO-treatment based on the results shown in the present study. When the erythroid progenitor cells were deprived of EPO, despite a gradual increase of ZnT1 mRNA abundance at 12 h, the respective mRNA levels of Zip10 and ZnT1 generally decreased throughout the time-course examined. The final measurements, at 48 h, of both transporter mRNA levels were lower than the basal levels determined at the initial time-point when the *in vitro* culture without EPO was started. These results implicate that certain levels of Zip10 and ZnT1 mRNA expressed prior to the *in vitro* EPO-induction during developmental stages *in vivo*, could not be sustained when the differentiation process was discontinued. The temporal trend of ALAS-2 expression is known to be induced exclusively by EPO during terminal erythroid differentiation (27,29). Because of the absence of background mRNA levels from preceding differentiation stages, the ALAS-2 mRNA levels were stably sustained at the basal (0 h) level when further differentiation was blocked by EPO-deprivation.

Previous studies with *in vitro* erythroid progenitor cell models suggest that the gene expression patterns during differentiation strongly reflect the functional hierarchy of the respective protein product activities (15,27,29,38). It was of interest that the mRNA levels of Zip10 and ZnT1 revealed different temporal patterns during the EPO-mediated differentiation *in vitro*. While the EPO-dependent Zip10 expression occurred rapidly after the terminal erythroid differentiation was initiated, the EPO-responsiveness of ZnT1 gene expression was only detectable after 24 h of EPO-treatment. These results demonstrate that the zinc transporters present in mature RBCs are differentially regulated by EPO and, thus, may be involved in the homeostatic regulation of zinc in differentiating erythroid progenitor cells. The hierarchical precedence of EPO-dependent Zip10 expression to that of ZnT1 are in agreement with the zinc expenditure trend during terminal erythroid differentiation (Fig. 5-1). Specifically, various events

that involve dynamic zinc utilization, such as synthesis of zinc metalloenzymes and zinc finger transcription factors, have been shown to occur at the early stages of terminal erythropoiesis (15,27). The earlier EPO-responsiveness of Zip10 gene expression may be associated with an increased requirement of zinc supply based on the metabolic use during these events (Fig. 5-1). However, after the cells reach the very late stage of terminal erythropoiesis, the metabolic needs of zinc decrease and, additionally, free zinc ions can introduce adverse affect to heme biosynthesis by interfering with incorporation of ferrous iron into protoporphyrin (15,21). Thus, the later EPO-dependent expression of ZnT1 would be a strategic mechanism of differentiating progenitor cells to remove excessive free zinc ions and, consequently, ensure the normal hemoglobin biosynthesis at the final step of RBC maturation (Fig. 5-1).

These expression trends of Zip10 and ZnT1 were confirmed at the protein level as well. Molecular masses of Zip10 and ZnT1 in the erythroid progenitor cells, speculated from the band migration, were corresponding to those determined in mature RBCs. The ZnT1 protein expression examined with total cell lysates revealed a similar trend to that observed in mRNA levels as expected. However, the EPO-dependent elevation of Zip10 expression at the early time-points, observed at the mRNA level, was hardly detectable within these protein samples. In addition, even though a decrease in mRNA levels occurred rapidly after EPO-deprivation, the protein levels of Zip10 observed in the total cell lysates were sustained relatively longer. It is of note that these discrepancies between the mRNA and protein data were eliminated when the cytosolic protein fraction were removed from the total cellular protein content by producing a total cellular membrane fraction. This implicates that effects of certain cytosolic components, which can be either internalized Zip10 protein or other cytosolic proteins that are abundant in

erythroid progenitor cells, compromised the detectability of EPO-dependent Zip10 expression in the total cell fractions.

MT-1 mRNA levels monitored in the present study also reveal a unique temporal trend in gene expression during terminal erythroid differentiation. The zinc-responsiveness of MT-1 protein expression in differentiating erythroblasts has been confirmed by a previous study (4,12). Thus, it was presumed here that MT-1 mRNA levels may partially reflect the intracellular zinc levels regulated by the differential expression of Zip10 and ZnT1 during terminal erythroid differentiation. Although a rapid decrease occurred in both EPO-treated and -deprived cells within 6 h, MT-1 mRNA levels was sustained higher in differentiating cells than in resting cells until 24 h. These periods correspond to the time-points when the EPO-dependent Zip10 mRNA induction was observed. Thus, these results may partially indicate that an increased intracellular zinc level was introduced by the early EPO-mediated Zip10 expression.

With regard of the EPO-independent down-regulation of MT-1 mRNA abundance, possible explanations of this phenomenon can be derived from previous studies. Abdel-Mageed et al. showed that up-regulation of MT-1 expression in erythroid progenitor cells occur during the proliferation stage that precedes the EPO-mediated terminal erythroid differentiation (39). In addition, an inhibitory effect of MT-1 on the EPO-derived cell differentiation was indicated (39). Conclusively, it was proposed that the expression of MT-1 transcripts in proliferating progenitor cells should be repressed once further erythroid differentiation is committed by EPO. In another study, the dependency of MT-1 synthesis on proliferation was determined by measuring decreased MT levels by mitomycin-c treatment to K562 erythroleukemia cells (Huber et al., unpublished observation). This may imply the presence of an intrinsic factor that induces MT-1 specifically during the proliferation of erythroid progenitor cells. Thus, the rapid repression of

MT-1 mRNA levels observed in the present study would be related to the remnants from the abundant MT-1 mRNA level expressed during the proliferation *in vivo* and the absence of the proliferation-dependent MT-1 inducing factor *in vitro*.

As mentioned above, the association of MTF-1 activity with the transcriptional regulation of Zip10 and ZnT1 has been suggested by previous studies. Up-regulation of Zip10 and repression of ZnT1 expression has been observed in MTF-1^{-/-} hepatocytes and embryos, respectively (3). Thus, as both Zip10 and ZnT1 are shown to be differentially expressed in maturing erythroid progenitor cells, it was of interest to determine whether EPO-responsive of MTF-1 gene expression occurs during terminal erythroid differentiation. The results presented in the current study reveal certain interrelations of Zip10 and ZnT1 transcript levels to EPO-dependent MTF-1 mRNA abundance. Peaks observed in the temporal pattern of MTF-1 transcription in differentiating cells corresponded to the decrease and increase in Zip10 and ZnT1 mRNA levels, respectively. Although the effects of EPO on MTF-1 activity in erythroid progenitor cells need to be further explored, these results suggest that EPO-dependent transcription of MTF-1 would be involved in the regulatory mechanism of the differential Zip10 and ZnT1 expression during erythroid maturation.

Overall, the presence of erythroid zinc transporters, as Zip10 and ZnT1, has been demonstrated in the current study. Furthermore, EPO-mediated expression of these transporters was confirmed in differentiating erythroid progenitor cells. Several suggestions for future approaches, particularly, with clinical perspectives can be derived from these results. The zinc uptake rate of erythrocytes *in vitro* has been suggested to be a suitable indicator of early dietary, subclinical zinc deficiency (11). Thus, the differential expression of these transporters in RBCs, which are likely to be zinc-responsive, could be another candidate parameter for the assessment

of dietary zinc status. In addition, the expression of these zinc transporters could be connected to the rigorous modulation of RBC intracellular zinc levels during *Plasmodium falciparum* parasitemia (40,41). In other words, the abnormal zinc sequestration in malarial RBCs would be possibly caused by a transformation in the host cell zinc trafficking system, which may involve Zip10 and ZnT1 activities, by the parasite infection. Finally, to some extent, the EPO-responsive Zip10 expression observed in this study may support the suggestions from studies related to the metastasis of breast cancer. Recently, it has been shown that EPO receptors (EPO-R) are highly expressed in breast carcinoma, while the expression levels in benign mammary tissues are generally negative (42). Although the functionality of EPO-R on these cancer cells remains controversial, it has been associated with the stimulatory effect of EPO on the cell migration activity (43). Expression of Zip10 in breast carcinoma has been reported to be essential for the migratory and invasive activity of breast cancer cells (44); however, the molecular mechanism of Zip10 induction has not been understood. Based on the results of the present study and evidence mentioned above, the induction of Zip10 expression by EPO may be a possible explanation for the EPO-R mediated metastasis of breast cancer cells.

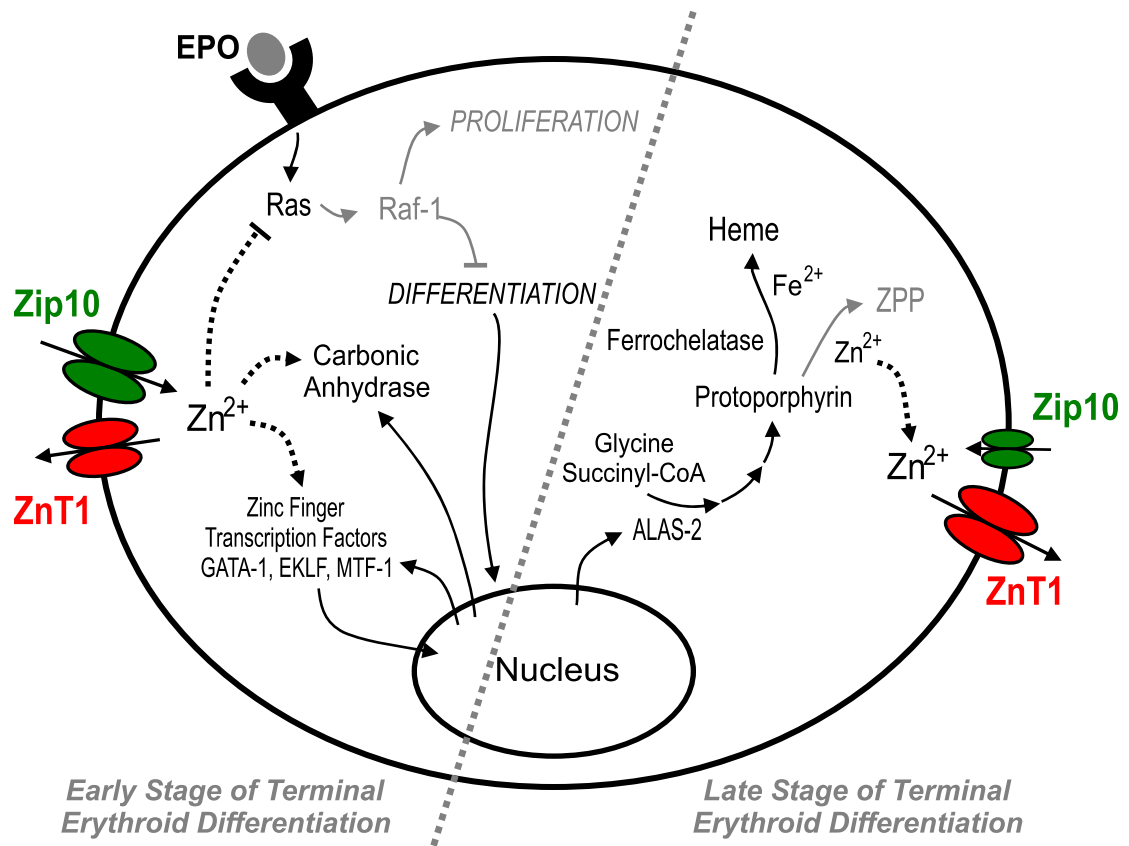


Figure 5-1. Putative model for the contribution of erythroid zinc transporters to the homeostatic regulation of zinc during terminal erythroid differentiation. EPO binds to EPO-R and induces the initiation of terminal erythroid differentiation. During the early stage of terminal erythroid differentiation Zip10 level is relatively higher than that at the late stage. Intracellular Zn^{2+} 1) inhibits Ras-Raf signaling pathway and leads EPO-mediated differentiation; 2) incorporates into CA and zinc finger transcription factors. During the hemoglobin biosynthetic pathway, down-regulation of Zip10 occurs while ZnT1 level is relatively sustained. Thus, excessive Zn^{2+} is removed and abnormal ZPP accumulation is prevented.

LIST OF REFERENCES

1. Prasad AS. Recognition of zinc-deficiency syndrome. *Nutrition*. 2001;17:67-9.
2. Cousins RJ. Zinc. In: Bowman BA, Russell RM, editors. *Present knowledge in nutrition*. 9 ed. Washington, D.C.: International Life Sciences Institute; 2006. p. 445-57.
3. Cousins RJ, Liuzzi JP, Lichten LA. Mammalian zinc transport, trafficking, and signals. *J Biol Chem*. 2006;281:24085-9.
4. Grider A, Bailey LB, Cousins RJ. Erythrocyte metallothionein as an index of zinc status in humans. *Proc Natl Acad Sci U S A*. 1990;87:1259-62.
5. Ohno H, Doi R, Yamamura K, Yamashita K, Iizuka S, Taniguchi N. A study of zinc distribution in erythrocytes of normal humans. *Blut*. 1985;50:113-6.
6. Horn NM, Thomas AL, Tompkins JD. The effect of histidine and cysteine on zinc influx into rat and human erythrocytes. *J Physiol*. 1995;489 (Pt 1):73-80.
7. Kalfakakou V, Simons TJ. Anionic mechanisms of zinc uptake across the human red cell membrane. *J Physiol*. 1990;421:485-97.
8. Simons TJ. Calcium-dependent zinc efflux in human red blood cells. *J Membr Biol*. 1991;123:73-82.
9. De KJ, Van Der SC, Veldhuizen M, Wolterbeek HT. The uptake of zinc by erythrocytes under near-physiological conditions. *Biol Trace Elem Res*. 1993;38:13-26.
10. Sasser LB, Bell MC, Jarboe GE. Influence of acute tissue injury on in vitro incorporation of Zn by sheep erythrocytes. *J Anim Sci*. 1975;41:1679-85.
11. Van Wouwe JP, Veldhuizen M, De Goeij JJ, Van den Hamer CJ. Laboratory assessment of early dietary, subclinical zinc deficiency: a model study on weaning rats. *Pediatr Res*. 1991 ;29:391-5.
12. Huber KL, Cousins RJ. Zinc metabolism and metallothionein expression in bone marrow during erythropoiesis. *Am J Physiol*. 1993;264:E770-E775.
13. Hodge D, Coghill E, Keys J, Maguire T, Hartmann B, McDowall A, Weiss M, Grimmond S, Perkins A. A global role for EKLF in definitive and primitive erythropoiesis. *Blood*. 2006;107:3359-70.
14. Ferreira R, Ohneda K, Yamamoto M, Philipsen S. GATA1 function, a paradigm for transcription factors in hematopoiesis. *Mol Cell Biol*. 2005;25:1215-27.
15. Welch JJ, Watts JA, Vakoc CR, Yao Y, Wang H, Hardison RC, Blobel GA, Chodosh LA, Weiss MJ. Global regulation of erythroid gene expression by transcription factor GATA-1. *Blood*. 2004;104:3136-47.

16. Tomoda T, Nomura I, Kurashige T, Kubonishi I, Miyoshi I, Sukenaga Y, Taniguchi T. Changes in Cu,Zn-superoxide dismutase gene during induced erythroid and myeloid differentiation. *Acta Haematol.* 1991;86:183-8.
17. Nishiyama S, Iriya K, Matsubasa T, Higashi A, Matsuda I. Zinc status relates to hematological deficits in middle-aged women. *J Am Coll Nutr.* 1998;17:291-5.
18. Nishiyama S, Kiwaki K, Miyazaki Y, Hasuda T. Zinc and IGF-I concentrations in pregnant women with anemia before and after supplementation with iron and/or zinc. *J Am Coll Nutr.* 1999;18:261-7.
19. Forman WB, Sheehan D, Cappelli S, Coffman B. Zinc abuse--an unsuspected cause of sideroblastic anemia. *West J Med.* 1990;152:190-2.
20. Fiske DN, McCoy HE, III, Kitchens CS. Zinc-induced sideroblastic anemia: report of a case, review of the literature, and description of the hematologic syndrome. *Am J Hematol.* 1994;46:147-50.
21. Bloomer JR, Reuter RJ, Morton KO, Wehner JM. Enzymatic formation of zinc-protoporphyrin by rat liver and its potential effect on hepatic heme metabolism. *Gastroenterology.* 1983;85:663-8.
22. Kaushansky K. Lineage-specific hematopoietic growth factors. *N Engl J Med.* 2006 ;354:2034-45.
23. Wojchowski DM, Menon MP, Sathyanarayana P, Fang J, Karur V, Houde E, Kapelle W, Bogachev O. Erythropoietin-dependent erythropoiesis: New insights and questions. *Blood Cells Mol Dis.* 2006;36:232-8.
24. Krantz SB. Erythropoietin. *Blood.* 1991;77:419-34.
25. Labbe RF, Rettmer RL. Zinc protoporphyrin: a product of iron-deficient erythropoiesis. *Semin Hematol.* 1989;26:40-6.
26. Alcindor T, Bridges KR. Sideroblastic anaemias. *Br J Haematol.* 2002;116:733-43.
27. Hodges VM, Winter PC, Lappin TR. Erythroblasts from friend virus infected- and phenylhydrazine-treated mice accurately model erythroid differentiation. *Br J Haematol.* 1999;106:325-34.
28. Cooper MC, Levy J, Cantor LN, Marks PA, Rifkind RA. The effect of erythropoietin on colonial growth of erythroid precursor cells in vitro. *Proc Natl Acad Sci U S A.* 1974;71:1677-80.
29. Dolznig H, Boulme F, Stangl K, Deiner EM, Mikulits W, Beug H, Mullner EW. Establishment of normal, terminally differentiating mouse erythroid progenitors: molecular characterization by cDNA arrays. *FASEB J.* 2001;15:1442-4.

30. Piao F, Yokoyama K, Ma N, Yamauchi T. Subacute toxic effects of zinc on various tissues and organs of rats. *Toxicol Lett.* 2003;145:28-35.
31. Levensgood JM, Sanderson GC, Anderson WL, Foley GL, Brown PW, Seets JW. Influence of diet on the hematology and serum biochemistry of zinc-intoxicated mallards. *J Wildl Dis.* 2000;36:111-23.
32. Witeska M, Kosciuk B. The changes in common carp blood after short-term zinc exposure. *Environ Sci Pollut Res Int.* 2003;10:284-6.
33. Lukaski HC. Low dietary zinc decreases erythrocyte carbonic anhydrase activities and impairs cardiorespiratory function in men during exercise. *Am J Clin Nutr.* 2005;81:1045-51.
34. McMahon RJ, Cousins RJ. Regulation of the zinc transporter ZnT-1 by dietary zinc. *Proc Natl Acad Sci U S A.* 1998;95:4841-6.
35. Kim AH, Sheline CT, Tian M, Higashi T, McMahon RJ, Cousins RJ, Choi DW. L-type Ca(2+) channel-mediated Zn(2+) toxicity and modulation by ZnT-1 in PC12 cells. *Brain Res.* 2000;886:99-107.
36. Kaler P, Prasad R. Molecular cloning and functional characterization of novel zinc transporter rZip10 (Slc39a10) involved in zinc uptake across rat renal brush-border membrane. *Am J Physiol Renal Physiol.* 2007;292:F217-F229.
37. Pawan K, Neeraj S, Sandeep K, Kanta RR, Rajendra P. Upregulation of Slc39a10 gene expression in response to thyroid hormones in intestine and kidney. *Biochim Biophys Acta.* 2007;1769:117-23.
38. Koury MJ, Bondurant MC, Mueller TJ. The role of erythropoietin in the production of principal erythrocyte proteins other than hemoglobin during terminal erythroid differentiation. *J Cell Physiol.* 1986;126:259-65.
39. Abdel-Mageed AB, Zhao F, Rider BJ, Agrawal KC. Erythropoietin-induced metallothionein gene expression: role in proliferation of K562 cells. *Exp Biol Med (Maywood).* 2003;228:1033-9.
40. Ginsburg H, Gorodetsky R, Krugliak M. The status of zinc in malaria (*Plasmodium falciparum*) infected human red blood cells: stage dependent accumulation, compartmentation and effect of dipicolinate. *Biochim Biophys Acta.* 1986;886:337-44.
41. Hiremath GS, Sullivan DJ, Jr., Tripathi AK, Black RE, Sazawal S. Effect of *Plasmodium falciparum* parasitemia on erythrocyte zinc protoporphyrin. *Clin Chem.* 2006;52:778-9.
42. Acs G, Zhang PJ, Rebbeck TR, Acs P, Verma A. Immunohistochemical expression of erythropoietin and erythropoietin receptor in breast carcinoma. *Cancer.* 2002;95:969-81.

43. Lester RD, Jo M, Campana WM, Gonas SL. Erythropoietin promotes MCF-7 breast cancer cell migration by an ERK/mitogen-activated protein kinase-dependent pathway and is primarily responsible for the increase in migration observed in hypoxia. *J Biol Chem.* 2005;280:39273-7.
44. Kagara N, Tanaka N, Noguchi S, Hirano T. Zinc and its transporter ZIP10 are involved in invasive behavior of breast cancer cells. *Cancer Sci.* 2007;98:692-7.

BIOGRAPHICAL SKETCH

Moon-Suhn Ryu was born on March 28, 1979 in Seoul, South Korea. He attended Yonsei University in Seoul from 1997 to 2001. Upon graduation with his Bachelor of Science in Biotechnology, Moon-Suhn joined the Republic of Korea Air Force to fulfill his military service required by the South Korean government. Since discharging his duty, Moon-Suhn worked for a beverage company, Lotte Chilsung Beverage Company Limited, in South Korea as an assistant manager in the overseas business team. He came to the United States in the fall of 2005 to start his master's study in nutritional sciences at the University of Florida. Moon-Suhn is planning to continue his graduate studies in the doctorate program for nutritional sciences at the University of Florida.