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Molecular pathogenesis of refractory anemia with ring sideroblasts (RARS): Role of the mitochondrial iron transporter gene \( ABCB7 \)

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To my family
ABSTRACT

Refractory anemia with ring sideroblasts (RARS) is characterized by anemia, erythroid apoptosis, and mitochondrial ferritin (FTMT) accumulation. Granulocyte-colony-stimulating factor (G-CSF) inhibits some of these features in vitro and in vivo and can in combination with erythropoietin normalize hemoglobin levels. The focus for this thesis was to investigate ABCB7 gene expression levels and mutational status in CD34+ cells and erythroblasts from MDS patients in order to understand mechanisms underlying the pathogenesis of RARS as well as the anti-apoptotic effects of G-CSF. Furthermore, we wanted to test the hypothesis that ABCB7 is a key mediator of aberrant iron accumulation in acquired RARS.

To dissect these mechanisms, the CD34+ compartment of RARS bone marrow, as well as erythroblasts derived from CD34+ cells in an erythroblast culture system were subjected to gene expression analysis (GEP). Erythroblasts were also analyzed after incubation with G-CSF. The mutational and DNA methylation status of ABCB7 and other key down-regulated genes was assessed. To study the ABCB7 role in aberrant iron accumulation in RARS erythroblasts, we modulated the expression of ABCB7 in several cellular systems.

ABCB7 is not mutated in RARS. However, CD34+ ABCB7 expression level was significantly lower compared to other MDS subtypes. Furthermore, there was a significant inverse relation between ABCB7 expression and the percentage of ring sideroblasts. In contrast to normal bone marrow, ABCB7 expression decreased during erythroid differentiation of RARS CD34+ cells. Other down-regulated key genes included MFN2, STAT5B, FANCC and the negative apoptosis regulator MAP3K7. Neither ABCB7, nor other down-regulated key genes in RARS showed hypermethylation. Several genes involved in erythropoiesis were significantly over-expressed in RARS CD34+ cells but showed normal or decreased expression in differentiating erythroblasts. Deregulated pathways in RARS erythroblasts included apoptosis and mitochondrial function.

Interestingly, the mitochondrial pathway including MFN2 was significantly modified by G-CSF, and several heat shock protein genes were up-regulated, as evidence of anti-apoptotic protection of erythropoiesis. However, G-CSF had no effect on the expression of iron-transport or erythropoiesis-associated genes.

ABCB7 down-regulation led to marked up-regulation of FTMT in K562 cells, while inhibiting growth and erythroid differentiation. In normal bone marrow, ABCB7 silencing reduced erythroid colony growth, and induced erythroid apoptosis and a gene expression pattern similar to that observed in RARS day 7 erythroblasts. Importantly, down-regulation led to the accumulation of mitochondrial iron, in the form of FTMT. ABCB7 up-regulation potentiated erythroid differentiation in K562 cells, and restored erythroid colony growth and decreased FTMT expression level in RARS CD34+ BM cells. Mutations in the SF3B1 gene, a core component of the RNA splicing machinery, were recently identified in a high proportion of patients with RARS. Of the nine RARS patients included in our study, 7 carried SF3B1 mutations. Interestingly, SF3B1 silencing resulted in down-regulation of ABCB7.

Our findings support an essential role of ABCB7 in the phenotype of acquired RARS and suggest a relation between SF3B1 mutations and ABCB7 down-regulation that warrants further investigation.
LIST OF PUBLICATIONS


*Equal contribution
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ALA</td>
<td>Aminolevulinic acid</td>
</tr>
<tr>
<td>ALAS2</td>
<td>Aminolevulinic acid synthase 2</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelogenous leukemia</td>
</tr>
<tr>
<td>Apo</td>
<td>Apoproteins</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFU-C</td>
<td>Colony forming unit cell</td>
</tr>
<tr>
<td>CFU-E</td>
<td>Colony forming unit erythroid</td>
</tr>
<tr>
<td>CIA</td>
<td>Cytosolic Fe-S protein assembly</td>
</tr>
<tr>
<td>CMML</td>
<td>Chronic myelomonocytic leukemia</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>DLBCL</td>
<td>Diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMT1</td>
<td>Divalent metal ion transporter 1</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FAB</td>
<td>French-American-British</td>
</tr>
<tr>
<td>FC</td>
<td>Ferrochelatase</td>
</tr>
<tr>
<td>Fdx</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>Fe-S</td>
<td>Iron-sulphur</td>
</tr>
<tr>
<td>FTMT</td>
<td>Mitochondrial ferritin</td>
</tr>
<tr>
<td>Fxn</td>
<td>Frataxin</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Gene expression profiling</td>
</tr>
<tr>
<td>GLRX5</td>
<td>Glutaredoxin</td>
</tr>
<tr>
<td>GpA</td>
<td>Glycophorin A</td>
</tr>
<tr>
<td>HRC</td>
<td>Hypochromic red cells</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cells</td>
</tr>
<tr>
<td>HUMARA</td>
<td>Human androgen receptor</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>Int-1</td>
<td>Intermediate-1</td>
</tr>
<tr>
<td>Int-2</td>
<td>Intermediate-2</td>
</tr>
<tr>
<td>IPSS</td>
<td>International Prognostic Scoring System</td>
</tr>
<tr>
<td>IRE</td>
<td>Iron responsive element</td>
</tr>
<tr>
<td>IRP</td>
<td>Iron regulatory protein</td>
</tr>
<tr>
<td>ISC</td>
<td>Iron-sulfur cluster</td>
</tr>
<tr>
<td>IWGM-MDS</td>
<td>International Working Group on Morphology of MDS</td>
</tr>
<tr>
<td>LDA</td>
<td>Low Density Array</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MCA-Meth</td>
<td>Melting curve analysis-Methylation assay</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>MLAS</td>
<td>Mitochondrial myopathy and sideroblastic anemia</td>
</tr>
<tr>
<td>NGS</td>
<td>Next-generation sequencing</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analyzes</td>
</tr>
<tr>
<td>PDC</td>
<td>Pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>PPIX</td>
<td>Protoporphyrin IX</td>
</tr>
<tr>
<td>PUS1</td>
<td>Pseudouridine synthase1</td>
</tr>
<tr>
<td>QRT-PCE</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Refractory anemia</td>
</tr>
<tr>
<td>RAEB</td>
<td>Refractory anemia with excess of blasts</td>
</tr>
<tr>
<td>RAEB-T</td>
<td>Refractory anemia with excess of blasts in transformation</td>
</tr>
<tr>
<td>RARS</td>
<td>Refractory anemia with ring sideroblasts</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>RCMD-RS</td>
<td>Refractory cytopenia with multilineage dysplasia and ring sideroblasts</td>
</tr>
<tr>
<td>RMA</td>
<td>Robust MultiChip Analysis</td>
</tr>
<tr>
<td>RNA-Seq</td>
<td>RNA sequencing</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>Scl11a2</td>
<td>Solute carrier family 11a member 2</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TfR1</td>
<td>Transferrin receptor-1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TLDA</td>
<td>Taqman Low Density Array</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XCIP</td>
<td>X-Chromosome Inactivation Pattern</td>
</tr>
<tr>
<td>XLSA</td>
<td>X-linked sideroblastic anemia</td>
</tr>
<tr>
<td>XLSA/A</td>
<td>X-linked sideroblastic anemia with ataxia</td>
</tr>
<tr>
<td>ZnPP</td>
<td>Zinc protoporphyrin</td>
</tr>
</tbody>
</table>
2. INTRODUCTION

2.1. Erythropoiesis

Human erythropoiesis is a complex multistep developmental process that begins at the level of pluripotent hematopoietic stem cells (HSCs) in the bone marrow (BM) niches and terminates with the production of erythrocytes. Erythropoiesis is regulated through key erythropoietic transcriptional factors, which in turn may be influenced by iron homeostasis, hypoxia, and oxidative stress (Tsiftsoglou, et al. 2009).

A relatively small number of lineage restricted transcription factors, including GATA-1, SCL/TAL1, LMO2, LDB1, and KLF1 mediate erythropoiesis by binding to the DNA of target genes. Furthermore, transcription factors often recruit additional regulatory proteins (co-regulators) and make critical protein-protein interactions that adjust their activities. For instance, several proteins including FOG-1, LMO2, KLF1/Sp1, p300/CBP and PU.1 have been reported to interact with GATA-1, a key erythropoietic transcriptional factor (Cantor and Orkin 2002).

In order to understand the function of co-regulators, it is useful to classify them as chromatin modifying or chromatin remodeling enzymes. Chromatin modifying enzymes catalyze histone posttranslational modifications. These modifications include acetylation, methylation, ubiquitination, and phosphorylation, which together are termed epigenetic markers. In contrast, remodeling enzymes modulate the access of transcription factors to chromatin (Fischle, et al. 2003).

The balance between positive and negative regulatory mechanisms operating within the BM niche regulates erythropoiesis. These include both soluble factors and cell-cell interactions.

The prevention of erythroid apoptosis during differentiation, and the regulation of erythroid progenitor numbers are mainly modulated by the concentration of Erythropoietin (Epo) and in some extent by SCF (kit ligand) and insulin-like growth factor I (Koury, et al. 2002). Erythropoiesis is negatively regulated by circulating cytokines, chemokines, and interleukins including; transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), and interleukin 6 (IL6). For instance, elevated levels of TNF-α inhibit erythropoiesis by inducing apoptosis through caspase-mediated cleavage of GATA-1 or/and by delaying proliferation(Chasis and Mohandas 2008).

Also cell-cell interactions within the islands play an essential role in regulation of erythropoiesis. Indeed, erythropoiesis is partly modulated by the interaction of
erythroblasts with one another and with the central macrophage within the erythroid islands (Chasis and Mohandas 2008).

In summary, erythropoiesis is a dynamic process requiring the orchestrated action of specific molecular mechanisms to strictly regulate cell proliferation and erythroid apoptosis, and to coordinate cell-cycle arrest with terminal maturation.

2.1.1. Apoptotic mechanisms in the control of erythropoiesis

There is growing evidence that apoptotic mechanisms play a key role in the regulation of erythropoiesis under physiologic and pathologic conditions.

Erythropoiesis is negatively regulated by activation of death receptors, such as Fas or TRAIL-R, or by erythropoietin deprivation. Both pathways trigger apoptosis through the activation of caspases; -3, -7 and -8, which leads to GATA-1 and Tal-1 cleavage. This cleavage results in either maturation arrest or apoptosis of erythroid cells (De Maria, et al. 1999). Interestingly, mature erythroblasts may negatively modulate the maturation rate and level of immature erythroid cells through a ligand/receptor mechanism. This model suggests that death ligands (FasL) are expressed on mature erythroid cells while the death receptors (Fas) are present on the surface of immature erythroblasts (Barcena, et al. 1996). This mechanism may account for the low but significant level of ineffective erythropoiesis due to erythroid apoptosis observed in normal subjects. Furthermore, this mechanism may function as another negative regulator of erythropoiesis, in addition to the negative feedback mechanism based on modulation of erythropoietin concentration. Moreover, it has been shown that activation of some caspases during erythroid differentiation occurs physiologically and is required for normal erythroid maturation (Zermati, et al. 2001).

The homeostasis of the erythropoietic system requires a proper balance between the rate of erythroid cell production and erythroid apoptosis, hence the collective action of both pro-apoptotic and anti-apoptotic factors.

2.2. Iron metabolism

Iron is essential for hemoglobin synthesis in maturing erythroblasts. The daily production of about 200 x 10^9 erythrocytes requires approximately 20–25 mg iron. This amount of iron is mainly provided from senescent erythrocytes recycled by macrophages, and in minimal amount (up to 1–2 mg) by intestinal iron absorption (Camaschella and Pagani 2011).
Iron has the capacity to accept and donate electrons, which makes it an effective component of oxygen-binding molecules (hemoglobin and myoglobin), cytochromes, and a variety of enzymes. However, this property also leads to toxicity. Since iron is such a crucial but potentially toxic metal, its absorption, storage and transfer are tightly regulated.

Iron is transported within the serum bound to transferrin (Tf). Tf binds to its receptor on the cell surface and this binding leads to the receptor-ligand complex internalization, which is followed by the release of iron from Tf. A protein known as divalent metal ion transporter 1 (DMT 1), transfers iron from the endosome to the cytosol. Iron can then be incorporated into cytosolic storage molecules, such as ferritin, or be exported to the mitochondria. How iron is exported to mitochondria remains unclear, but several studies indicate that Tf-containing endosomes in erythroid cells may transfer iron directly to the mitochondrion (‘kiss and run’ hypothesis) (Sheftel, et al. 2007). Finally, iron is used for heme synthesis or iron-sulphur (Fe-S) cluster biogenesis, or stored in mitochondrial ferritin within the mitochondria.

### 2.2.1. Heme biosynthesis

Heme production starts with a series of seven enzyme-catalyzed reactions to produce protoporphyrin IX (PPIX) (Figure 1). In the mitochondrial matrix, aminolevulinic acid synthase 2 (ALAS2) catalyzes the first step of the heme synthesis pathway, the condensation reaction between glycine and succinyl coenzyme A (CoA) resulting in 5-aminolevulinic acid (ALA). ALA is somehow transferred to the cytosol, where four enzyme-catalyzed reactions generate coproporphyrinogen III, which is then delivered back to the mitochondria. In the mitochondria, coproporphyrinogen III is modified to protoporphyrinogen IX and then to PPIX. The final step in the heme synthesis, the insertion of one atom of Fe\(^{2+}\) into PPIX, is catalyzed by ferrochelatase (FC), which is located at the inner mitochondrial membrane (Napier, et al. 2005).

Except for ALAS2 deficiency, the lack of any other enzymes of the heme biosynthesis pathway will lead to porphyrias. Accordingly, it is expected that the level of each enzyme downstream of ALA production is adequate to prevent accumulation of intermediates. For instance, when iron is not obtainable, FC continues to convert PPIX to metalloporphyrin, using zinc (II) as a substitute for Fe\(^{2+}\). Therefore, erythrocyte zinc protoporphyrin (ZnPP) levels are generally indicative of the availability of Fe for FC (Sheftel, et al. 2009).
2.2.2. Iron–sulphur protein biogenesis

Mitochondria are required for biogenesis of all cellular iron–sulphur proteins (Fe-S) and play therefore an essential role in Fe-S protein maturation in eukaryotes. Fe-S clusters are prosthetic groups of multiple proteins involved in several metabolic pathways and are crucial for the function of both mitochondrial (respiratory chain complexes, aconitase, succinate dehydrogenase, ferrochelatase) and cytosolic proteins (aconitase, xanthine oxidase).

Assembly of Fe-S clusters is facilitated by the mitochondrial iron-sulfur cluster (ISC) assembly machinery, which uses cysteine as a source of sulfur, combines it with iron to generate an Fe-S cluster on scaffold proteins, and finally incorporates the cluster into recipient apoproteins (Apo) (Figure 2) (Lill and Muhlenhoff 2008). The transient assembling of Fe-S clusters on the scaffold protein Isu1 requires that the two elemental components are in their reduced oxidation state, a step that is supported by the early components of the ISC assembly machinery. These components include the cysteine desulfurase complex Nfs1, which serves as the sulfur donor for cluster synthesis (Zheng and Dean 1994), the iron-binding protein Frataxin (Fxn) as the putative iron donor, and the electron transfer chain comprised of ferredoxin reductase and ferredoxin (Fdx), which receive their electrons (e−) from NADH.

The release of Fe-S clusters from Isu1 and their transfer and incorporation into recipient apoproteins is assisted by late components of the ISC assembly machinery. Through the interaction of the Hsp70 chaperone Ssq1 with Isu1, Fe-S cluster is released from Isu1. Moreover, the monothiol glutaredoxin GLRX5 is involved in this second major step (Sheftel, et al. 2009). In GLRX5 RNAi cells and GLRX5-deficient
patient cells, Fe-S cluster biogenesis is impaired, which leads to mitochondrial iron overload and relative cytosolic iron depletion (Ye, et al. 2010).

Extra-mitochondrial Fe-S protein biogenesis requires the core ISC assembly machinery and components of the mitochondrial ISC export machinery. ABCB7, the half-type ATP binding cassette transporter, exports an unknown compound (X) to the cytosol, which is used in extra-mitochondrial Fe-S protein assembly. ABCB7 mutations lead to mitochondrial iron accumulation in an inherited disease known as XLSA with ataxia (XLSA-A) (Allikmets, et al. 1999).

![Figure 2. Simplified Fe-S protein biogenesis schematic. Fxn=Frataxin; X=unknown substrate exported by ABCB7. Cfd1/Nbp35 is a cytosolic scaffold complex (Sheftel, et al. 2009).](image)

2.2.3. Iron homeostatic regulation

The expression of key proteins in iron metabolism; ALAS2, Ferritin, and TfR1, is regulated by intracellular iron levels. Special hairpin structures, called iron responsive elements (IREs) in the respective mRNAs are recognized by proteins, known as iron regulatory proteins (IRPs), in the absence of iron. Two closely related IRPs (IRP-1 and IRP-2) have been identified to date (Henderson 1996).

In mammalian cells, the assembly of Fe-S cluster on IRP1 reduces its RNA-binding affinity while increasing its aconitase activity, providing one of the mechanisms for determining intracellular iron levels. Importantly, alteration of any of the three Fe-S protein biogenesis systems; ISC assembly machinery, ISC export machinery, and the cytosolic Fe-S protein assembly (CIA) machinery influences Fe-S cluster assembly on IRP1 and modifies IRE binding activity. Another mechanism is based on iron (and heme)-dependent degradation of IRP2 (Hentze, et al. 2004).
Translation of the transcript is blocked when IRPs bind to IREs motifs in 5’-untranslated region (UTR) of the respective mRNA. By contrast, the stabilization and translation of mRNA transcripts proceeds when IRPs bind to IRE motifs in 3’-UTR of them (Figure 3) (Hentze and Kuhn 1996).

Fe-S biogenesis is also required to execute several steps in the heme synthesis, in particular when large amounts of heme are needed for hemoglobinization during erythropoiesis. Certain heme synthesis enzymes such as ferrochelatase (FC) need Fe-S for their activity. Importantly, FC is unstable without its [2Fe-2S] cluster. In addition, some other enzymes including pyruvate dehydrogenase complex (PDC) and mitochondrialaconitase require Fe-S cofactors to (potentially) provide substrates for heme synthesis. The synthesis of succinyl-CoA, a substrate for the first step of heme biosynthesis, is facilitated by PDC and mitochondrialaconitase activities. Moreover, ALAS2 can be efficiently translated only when cytosolic Fe-S biogenesis is intact (Ye and Rouault 2010).

As mentioned above, another important Fe-S protein in heme biosynthesis is IRP1, which represses synthesis of ALAS2 when it lacks an Fe-S cluster.

**Figure 3.** The presence or absence of an Fe-S cluster on IRP1 serves as a binary switch, determining the binding capacity of the protein to IREs. When the Fe-S cluster is absent, IRP1 has the ability to bind to IREs, thereby stabilizing mRNAs with 3’-UTR IREs and blocking translation of mRNAs with 5’-UTR IREs. Hence, appropriate regulation of iron handling by mammalian cells requires not only the mitochondrial ISC machineries including the substrate exported by ABCB7, but also a functional cytosolic assembly system (CIA) (Sheftel, *et al.* 2009).
2.2.4. *ABCB7*

*ABCB7* is an ATP-binding cassette (ABC) transporter located at the inner membrane of mitochondria. *ABCB7* is a functional homolog of the yeast Atm1, a mitochondrial membrane protein involved in the maturation of cytosolic Fe-S proteins. The *ABCB7* gene maps to chromosome Xq21–q22 (Shimada, *et al.* 1998).

The essential role of *ABCB7* for heme biosynthesis and erythropoiesis is revealed by the fact that *ABCB7* mutations lead to the development of XLSA/A. Mutations in this condition are missense (I400M, E433K, and V411L) and located at the border of putative transmembrane domains of the protein.

XLSA/A is a recessive disorder characterized by the onset of non-progressive cerebellar ataxia in infancy or early childhood, mild anemia with hypochromia and microcytosis, and mitochondrial iron accumulation (Allikmets, *et al.* 1999). Perhaps due to the high expression of *ABCB7* in cerebellum in addition to in BM, patients with mutations also have ataxia.

*ABCB7* function is required for erythropoiesis, as XLSA/A patients develop anemia. It is so far unclear at which step *ABCB7* influences heme biosynthesis. It has been suggested that *ABCB7* physically interacts with ferrochelatase and somehow supports its activity (Taketani, *et al.* 2003). Another possible mechanism where *ABCB7* may exert its effect is through the inhibition of *ALAS2* translation. Mouse embryonic stem cells and male mouse embryos expressing a conditionally deleted *Abcb7* allele are not viable. Furthermore, using X-chromosome inactivation assays and tissue-specific deletions it was shown that *Abcb7* is essential for the development and function of several other cell types and tissues. In liver, *ABCB7* deficiency results in mild mitochondrial injury, impaired cytosolic Fe-S cluster assembly, and modified iron sensing by IRP1. This contributes to dysregulation of hepatocyte iron metabolism and increased total liver iron (Pondarre, *et al.* 2006).

Interestingly, complete loss of *abtm-1*, the *C. elegans* functional homologue of *ABCB7*, is lethal, as is the loss of *Abcb7* in mice. Partial loss of *abtm-1* function leads to arrested embryos and adults with elevated Fe3+ levels, higher oxidative stress, and a phenotype characteristic of mutations that cause mitochondrial dysfunction (Gonzalez-Cabo, *et al.* 2011).

*ABCB7* silencing in HeLa cells results in cell proliferation decrease, and a cytosolic iron deprivation accompanied by large iron depositions in the mitochondria (Cavadini, *et al.* 2007).
Fe-S cluster and heme levels in Atm1p-depleted mitochondria from Saccharomyces cerevisiae cells were dramatically decreased in comparison to wild type mitochondria. Atm1p depletion also led to a gradual increase of non-heme Fe 2+ ions in the mitochondria and an increased oxidative damage (Miao, et al. 2009).

In human cells, the unknown substrate transported by ABCB7 appears to be required for the maintenance of iron homeostasis in cytosol, which may in turn affect the Fe-S cluster biogenesis process. When production or export of this unknown substrate is diminished, the cell responds as though mitochondria were iron depleted, and tries to modify the misperceived state of mitochondrial iron depletion, which leads to mitochondrial iron overload (Ye and Rouault 2010).

2.2.5. Mitochondrial ferritin

Mitochondrial ferritin (FTMT) is encoded by an intronless gene on chromosome 5q23. It is structurally and functionally similar to the ubiquitous cytosolic ferritin, with a ferroxidase activity necessary for storing and detoxifying cellular iron.

Unlike cytoplasmic ferritin, FTMT mRNA lacks IRE and may be transcriptionally regulated by iron. The expression of FTMT is higher in tissues that have high numbers of mitochondria (eg, testis) but lower in tissues involved in iron storage (eg, the liver) (Levi, et al. 2001; Santambrogio, et al. 2007).

While normal erythroblasts do not express FTMT, ring sideroblasts in the sideroblastic BM express significant amounts of the protein (Cazzola, et al. 2003). FTMT expression is also detected in peripheral blood reticulocytes of patients with X-linked sideroblastic anemia (XLSA), suggesting that FTMT may be a clinically useful marker of sideroblastic anemia. FTMT may protect the mitochondria from the damage caused by iron accumulation. The expression of FTMT gene in cultured cells actively sequesters iron at the expense of the cytosolic iron and results in mitochondrial iron accumulation (Corsi, et al. 2002).

Expression of human FTMT in frataxin-deficient yeast cells, a model of mitochondrial iron overload and oxidative damage, rescues the respiratory deficiency caused by the loss of frataxin protecting the activity of Fe-S enzymes. Furthermore, inhibition of mitochondrial iron overload by FTMT expression preserves mitochondrial DNA integrity and increases cell resistance to H$_2$O$_2$ (Campanella, et al. 2004).
2.3. The sideroblastic anemias

Initially, Kaplan introduced the term “sideroblast” to describe an erythroblast with a cytoplasm in which siderotic granules could be detected by ordinary microscopy. Later, Douglas and Dacie illustrated the presence of large siderotic granules surrounding the nucleus in the form of collar; ringed sideroblasts (Douglas and Dacie 1953). Electron microscopy demonstrated that these granules were localized to mitochondria.

The common feature of all sideroblastic anemias is the presence of “ring” sideroblasts, i.e. basophilic and polychromatophilic erythroblasts characterized by mitochondrial iron accumulation that form a perinuclear ring, detectable with Perl's stain (Figure 4) (Fontenay, et al. 2006). This morphological feature characterizes not only the inherited forms but also the acquired refractory anemia with ring sideroblasts (RARS), a type of myelodysplastic syndrome (MDS), as well as sideroblast formation due to toxins or drugs.

![Figure 4. Ring sideroblasts](image)

2.3.1. Hereditary sideroblastic anemias

The inherited sideroblastic anemias are rare genetic disorders characterized by reduced heme biosynthesis and mitochondrial iron accumulation in the erythroblasts. Depending on the original function(s) of the mutated genes, the clinical features of sideroblastic anemias differ. To date, several mutations responsible for hereditary sideroblastic anemia have been identified. The resulting disorders are X-linked or autosomal recessive, while some are not yet characterized at the genetic level (Table 1) (Sheftel, et al. 2009).

The anemia in these conditions varies from mild to severe, with most patients having moderate degrees. Due to blood transfusions or as a result of increased iron
absorption secondary to ineffective erythropoiesis, serum iron, transferrin saturation, and serum ferritin levels are often elevated (Camaschella 2009).

The hereditary forms are much less common than primary sideroblastic anemias associated with MDS. However, it is important to identify the function of the mutated genes, as this will improve our knowledge of mitochondrial iron metabolism. Furthermore, if the mechanism of mitochondrial iron overload is similar in hereditary and acquired sideroblastic anemia, observations in the former group could be helpful to understand mechanisms underlying the pathogenesis of acquired sideroblastic anemia (Harigae and Furuyama 2010).

Table 1. Hereditary sideroblastic anemias

<table>
<thead>
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<th>Table 1. Hereditary sideroblastic anemias</th>
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<tr>
<td>A. X chromosome-linked</td>
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<tr>
<td>(1) ALAS2 deficiency</td>
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<tr>
<td>(2) ABCB7 mutations</td>
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<tr>
<td>B. Autosomal</td>
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<tr>
<td>(1) MLASA (PUS1 mutations)</td>
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<tr>
<td>(2) GLRX5 deficiency</td>
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<tr>
<td>(3) SLC25A38 mutations</td>
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<tr>
<td>C. Mitochondrial</td>
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<td>(1) Pearson marrow-pancreas syndrome</td>
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2.3.1.1. X chromosome-linked hereditary sideroblastic anemias

X-linked sideroblastic anemia (XLSA) and X-linked sideroblastic anemia with ataxia (XLSA/A) are two well characterized hereditary sideroblastic anemias.

XLSA was first described by Cooley in 1945 and is caused by mutations in ALAS2 gene (Furuyama, et al. 2006). ALAS2 deficiency causes hyperferremia and potential death from hemochromatosis which can be partially rescued by pyridoxine therapy in some patients (Napier, et al. 2005).

XLSA/A was first reported in members of two families with non-progressive spinocerebellar syndrome and sideroblastic anemia (Pagon, et al. 1985). Later, (Allikmets, et al. 1999) demonstrated an ABCB7 gene mutation in a family with five affected males with sideroblastic anemia with ataxia, (refer to 2.2.4. ABCB7). Mutations are missense and lead to loss of function. Nonsense mutations have not been
reported, probably because a complete loss of \textit{ABCB7} would be lethal, as in the Abcb7-deficient mouse model (Pondarre, \textit{et al.} 2006).

\subsection*{2.3.1.2. Autosomal hereditary sideroblastic anemias}

Mitochondrial myopathy and sideroblastic anemia (MLASA) is a rare autosomal recessive oxidative phosphorylation disorder specific to skeletal muscle and bone marrow (Bykhovskaya, Casas \textit{et al.} 2004), caused by a homozygous missense mutation in pseudouridine synthase-1 gene (\textit{PUS1}). \textit{PUS1} enzyme may play an important role in tRNA function and in stabilizing the secondary and tertiary structure of many RNAs (Chen and Patton 1999).

Another type of autosomal inherited sideroblastic anemia was described in shiraz (sir) zebrafish mutants. These mutants have a deficiency in Grx5 encoded by a gene (\textit{GLRX5}), whose product is required for Fe-S assembly. This deficiency blocks \textit{ALAS2} translation through elevated IRP1 binding to the IRE located in the 5´-UTR of \textit{ALAS2} transcript (Wingert, \textit{et al.} 2005). Interestingly, Camaschella, \textit{et al.} identified a homozygous mutation in the \textit{GLRX5} gene in a male patient with autosomal recessive pyridoxine-refractory sideroblastic anemia (Camaschella, \textit{et al.} 2007).

Guernsey \textit{et al.} identified 11 different homozygous or compound heterozygous mutations in the \textit{SLC25A38} gene in 18 patients with autosomal recessive pyridoxine-refractory sideroblastic anemia. \textit{SLC25A38} is a member of the mitochondrial carrier family which may be involved in ALA production by transferring glycine to mitochondria or by exchanging glycine for ALA across the mitochondrial inner membrane (Guernsey, \textit{et al.} 2009).

\subsection*{2.3.1.3. Mitochondrial hereditary sideroblastic anemia}

In 1979, Pearson \textit{et al.} described a new syndrome of refractory sideroblastic anemia with vacuolization of marrow precursors and exocrine pancreatic dysfunction (Pearson, \textit{et al.} 1979). In Pearson marrow-pancreas syndrome, severe, transfusion-dependent, macrocytic anemia begins in infancy. The genetic defects in Pearson marrow-pancreas syndrome are deletions in the mitochondrial DNA. The most common deletion reported is a 4977 base pair deletion identified in 80\% of affected children. Deletion of mitochondrial DNA may result in deficiency of respiratory chain complexes, including respiratory complex I (NADH dehydrogenase), complex
IV (cytochrome c oxidase), and complex V (ATP synthase) (Harigae and Furuyama 2010).

2.3.2. Acquired sideroblastic anemias

Acquired sideroblastic anemias are either primary, clonal diseases, representing one subtype of MDS, or secondary due to some chemicals, drugs, lead poisoning, copper deficiency, or chronic neoplastic disease (Hellstrom-Lindberg, et al. 2006; Sheftel, et al. 2009).

2.3.2.1. Primary sideroblastic anemias

RARS is a subtype of MDS, a clonal hematopoietic disorder. Different aspects of RARS are reviewed under the subtitle 2.5.

2.3.2.2. Secondary sideroblastic anemias

Drugs and chemicals, which are associated with sideroblastic anemia include Isoniazid, Pyrazinamide, Cycloserine, Chloramphenicol, lead poisoning, and copper deficiency. Many of these are pyridoxine antagonists. Therefore, although discontinuation of the causing agent should always be the first step, administration of pyridoxine may improve anemia (Hellstrom-Lindberg, et al. 2006; Sheftel, et al. 2009). The presence of ring sideroblasts in BM can be a feature of lead poisoning. In one case, massive lead poisoning led to severe erythroid dysplasia with 40% ring sideroblasts in BM (Hellstrom-Lindberg, et al. 2006).

2.4. The myelodysplastic syndromes

The term myelodysplastic syndrome is used to describe a heterogeneous group of disorders that are characterized by clonal and ineffective hematopoiesis, morphological dysplasia, peripheral blood cytopenias and progressive BM failure. MDS transforms to acute myeloid leukemia (AML) in approximately 30% of cases. Survival following a diagnosis of MDS varies from a few months to more than ten years.

This highly variable prognosis underscores the importance of a classification system, supplemented by a prognostic index, to predict the survival of patients with MDS and the likelihood of transformation to AML (Mufti, et al. 2008).
2.4.1. MDS classification

In 1982, the French-American-British (FAB) cooperative group classified five subgroups of MDS: refractory anemia (RA), refractory anemia with ring sideroblast (RARS), refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMML) (Bennett, et al. 1982). This classification based on morphological criteria was revised in 2001 and 2008, resulting in the 2008 World Health Organization (WHO) classification (Table 2), (Swerdlow, et al. 2008).

RARS is a subgroup of MDS which is defined by the presence of more than 15 % ring sideroblasts of erythroid precursors, and less than 5% myeloblasts in BM. According to the new WHO classification of MDS, RARS was redefined to be limited to erythroid dysplasia and less than 10% dysplastic changes in myeloid and megakaryoid lineages. Refractory cytopenia with multilineage dysplasia and ring sideroblasts (RCMD-RS) is similar to RARS but has more than 10% dysplastic changes in non-erythroid lineages.

The most widely used prognostic scoring system used for MDS is the International Prognostic Scoring System (IPSS). This system was developed by Greenberg et al. and allowed a unified approach to the care of patients with MDS (Greenberg, 1997). Based on percentage of BM blasts, number of cytopenias, and karyotype, the IPSS stratifies patients into four risk categories: low, intermediate-1 (Int-1), intermediate-2 (Int-2), and high. Most patients with RARS belong to the low risk IPSS subgroup of MDS.

Table 2. 2008 WHO classification of MDS (Swerdlow et al. 2008).
2.5. Refractory anemia with ringed sideroblasts (RARS)

RARS is a myelodysplastic syndrome characterized by isolated anemia, erythroid dysplasia only, less than 5% myeloblasts, and 15% or more ring sideroblasts in the BM. Since the vast majority of patients with this syndrome have no cytogenetic abnormalities, the clonal nature of RARS has been questioned. However, X-chromosome inactivation studies in female patients have suggested that RARS derives from the clonal proliferation of a multipotent hematopoietic stem cell with the potential for myeloid and lymphoid differentiation (Malcovati, *et al.* 2009; Cazzola and Invernizzi 2011).

2.5.1. Historical background of RARS

The presence of siderocytic granules surrounding the nucleus in the form of a collar, the ring sideroblast, was illustrated first by Douglas and Dacie (Douglas and Dacie 1953). However, the first definitive description of a chronic refractory anemia with ring sideroblasts was provided by Björkman. He reported four cases of severe refractory anemia with ring sideroblasts and hyperplastic BM. Three patients had benign anemia, but the forth patient died of myeloblastic leukemia (Björkman 1956).

2.5.2. Clinical and morphological diagnosis of RARS

The initial phase of RARS is characterized by erythroid hyperplasia and ineffective erythropoiesis, which is usually stable for many years but may be followed by a phase of marrow failure, and occasionally by leukemic transformation (Cazzola and Invernizzi 2011).

Pancytopenia and acute leukemic transformation is infrequent in RARS patients compared to other MDS subtypes. Moreover, hepatomegaly and splenomegaly is also uncommon in RARS (Mufti, *et al.* 2008).

The main clinical problems for patients with RARS are severe anemia, chronic transfusion need, and a subsequent risk of iron overload (Bennett, *et al.* 1982; Greenberg, *et al.* 1997).

The International Working Group on Morphology of MDS (IWGM-MDS) recommends that ring sideroblasts should be defined as erythroblasts with a minimum of five siderotic granules covering at least one third of the circumference of the nucleus (Mufti, *et al.* 2008). The red cells are hypochromic, and commonly have a dimorphic appearance in the blood film and usually big in size.
Ring sideroblasts are found exclusively in pathological conditions, and they should be distinguished from ferritin sideroblasts, which are observed in normal BM.

2.5.3. Prognosis of RARS

As is shown in Figure 5, approximately 15% of patients with MDS are diagnosed with RARS/RCMD-RS. The cumulative risk of AML evolution was 0% in RARS and 12% in RCMD-RS (Germing, et al. 2006).

The mean survival time based on FAB classification is approximately 35 months for the RARS subtype (Bennett 2005). However, in a study of 88 patients with MDS, significantly longer median survival was seen in RARS patients with uni-lineage dysplasia relative to multiple-lineage dysplasia (36.3 months and 14.9 months, respectively) (Dunkley, Manoharan et al. 2002).

![Figure 5. Patient distribution and estimated survival across categories in the FAB classification system of MDS (Bennet, 2005).](image)

2.5.4. Treatment of RARS

The ineffective hematopoiesis in RARS responds less well to Epo than other low-risk subgroups of MDS (7.5% vs. 21%). However, it has been shown that the combination of granulocyte colony-stimulating factor (G-CSF) and Epo induces the highest response rate and most durable responses in patients with RARS (Hellström-Lindberg, et al. 1998; Jädersten, et al. 2005). Also, a pronounced in vivo synergy between G-CSF and Epo was observed in this group. Erythroid improvement was associated with a decrease in the number of apoptotic BM precursors (Hellström-Lindberg, et al. 1997).
In a series of studies, it was shown that G-CSF strongly inhibited apoptosis in differentiating RARS erythroblasts *in vitro* as well as *in vivo*, through a marked inhibition of mitochondrial cytochrome c release and a subsequent decrease in caspase-9 and caspase-3 activity (Schmidt-Mende, *et al.* 2001; Tehranchi, *et al.* 2003; Tehranchi, *et al.* 2005). By contrast, G-CSF did not affect the accumulation of mitochondrial ferritin at any measured time point of differentiation (Tehranchi, *et al.* 2005). Moreover, Epo is also a well-known inhibitor of apoptosis (Koury and Bondurant 1990).

As expected, patients with RARS and IPSS low-risk showed the lowest cumulative incidences of AML. Intriguingly, patients with RARS have significantly higher erythroid response rate to Epo±G-CSF than patients with RCMD-RS (unpublished observations, Nordic MDS Group). This difference correlates well with the predictive model for erythroid response, since most RARS patients belong to the good predictive group, while most RCMD-RS patients belong to the intermediate predictive group. Furthermore, RARS shows significantly longer median survival compared with RCMD-RS (Jadersten, *et al.* 2008).

### 2.5.5. Cytogenetic aberrations in RARS

Cytogenetic abnormalities are seen in 10 % of patient with RARS and 50% of RCMD-RS (Komrokji and Bennett 2003) and are largely the same as for MDS in general.

### 2.5.6. Molecular alterations in RARS

The molecular mechanism underlying the pathogenesis of RARS has until recently been unrevealed. It was initially thought that the hallmark of RARS, mitochondrial iron overload in erythroblasts, is due to an enzyme defect in heme biosynthesis pathway. In the case of impaired portoporphyrin IX (PPIX) synthesis, the imported iron to mitochondria would lack its partner and accumulate in the matrix. However, PPIX is elevated rather than reduced in RARS and *ALAS2* gene mutations are not seen in RARS. Therefore, an important enzyme defect in heme synthesis pathway was excluded (Steensma, *et al.* 2007; Sheftel *et al.* 2009).

Next, alterations in the last step of heme synthesis, namely incorporation of iron into PPIX, could be suggested. This step is catalyzed by ferrochelatase (FC), however, FC concentration is normal or even elevated (Pasanen, *et al.* 1981) and somatic
missense and splicing mutations of FC are very uncommon in patients with RARS (Sternberg, et al. 2005).

FC accepts only ferrous iron (Fe2+) for heme synthesis while iron accumulates in the ferric form (Fe3+) in sideroblastic anemia. Gattermann suggested that iron is not in the right chemical form in sideroblastic anemia, and therefore cannot incorporate into heme and accumulates in the mitochondrial matrix (Gattermann 2000).

In one study, RARS patients demonstrated an elevated percentage of hypochromic red cells (HRC) as a sign of insufficient iron incorporation into heme (Murphy, et al. 2006). As mentioned above, iron and zinc compete for the metal binding site of FC, therefore, actual or functional iron deficiency leads to abnormal zinc incorporation into PPIX and ZnPP accumulation. Interestingly, this study showed a strong correlation between the percentage of HRC and red cell ZnPP levels in RARS patients (Murphy, et al. 2006).

Mutations of the subunit I of cytochrome c oxidase gene, encoded by mtDNA, were identified in some RARS cases, but it is not clear yet that these mutations result in defective iron reduction and mitochondrial iron overload in sideroblastic anemia (Gattermann 2000). ABCB7 and PUS1 genes mutated in hereditary sideroblastic anemia syndromes, are not mutated in acquired cases (Steensma, et al. 2007).

2.5.7. **SF3B1 mutations in RARS**

Mutations in the **SF3B1** gene, a core component of the RNA splicing machinery, have recently been identified in a high proportion of patients with RARS (Papaemmanuil, et al. 2011 Accepted). It is recognized that initial splicing occurs as the nascent RNA molecule is being transcribed. This is an integrated process in which the spliceosome is in continuous cross talk with the initiation, elongation and termination phases of the transcription cycle. **SF3B1** mutations could potentially influence either splicing itself or interactions with the transcriptional complex.

It was observed that CD34+ cells from **SF3B1**-positive patients show under-expression of several key biological pathways, including those involved in mitochondrial function, with a striking lack of up-regulated gene pathways (Papaemmanuil, et al. 2011 Accepted). This implies that the net effect of the mutation is systematic under-expression of many genes, although a mechanistic understanding of how this leads to the specific phenotype will require detailed biochemical studies of the mutant protein.
2.6. Gene expression profiling

Gene expression profiling (GFP) gives an image of the transcriptional activity of specific genes at a specific time point. The application of microarray technology has developed our understanding of complicated mechanisms underlying malignant processes in general and of hematologic malignancies in particular.

Interestingly, the study of gene expression in diffuse large B-cell lymphoma (DLBCL) led to the classification of patients according to their expression profiles. As a result, DLBCL were split into two different subtypes: germinal center B-cell-like DLBCL and activated B-cell-like DLBCL, which differ in the proliferation rate, differentiation level, response to therapy and prognosis (Alizadeh, et al. 2000).

GEP studies are assumed to reflect the activity of the resulting protein product. However, the relationship between the expression level of one specific gene and the level of its functional protein is not necessarily linear. Several kinetic mechanisms can result in a highly skewed mRNA distribution. For instance, the stability of a protein plays an important role in gene expression level. Proteins that degrade slowly don’t require continues transcription; while fast degradation needs increased mRNA synthesis.

Furthermore, the post-translational modifications including phosphorylation, acetylation, methylation, sumoylation and ubiquitination may modulate protein function. Protein activity is also under the control of microRNAs, which have different expression in hematopoietic disorders and may modify protein translation without altering the expression level of the gene (Galili and Raza 2009).

2.6.1. cDNA microarray

The global patterns of gene expression can be studied using high-density cDNA microarrays. One of the most frequently utilized methods in studying of the gene expression is Affymetrix GeneChip® technology (Santa Clara, CA, USA). Affymetrix' GeneChip® technology was invented by a team of scientists led by Stephen P.A. Fodor in the late 1980's.

Re-sequencing of rather long DNA segments by hybridization, and detection of a large number of DNA sequence variations are facilitated using Affymetrix GeneChips®. In this technology, the oligonucleotide probes are synthesized directly on the surface of quartz wafers. The synthesized probes could also include large genomic DNA fragments, cDNA, RNA, or proteins. In some types of microarrays, oligonucleotides are pre-synthesized and spotted on a solid surface (Bilitewski 2009).
The latest generations of GeneChips interrogate over 40,000 transcripts (Genome arrays), analyze individual exons of genes separately (exon arrays), or even more advanced, the entire genome for transcriptional activity without focusing on predicted genes (tiling arrays) (Nasedkina, et al. 2009).

2.6.2. Gene expression profiling in MDS

Exploration of GEP in MDS could potentially be very useful considering the heterogeneity nature of the disease. However, the application of microarray in these patients is more challenging compared to many other disorders.

Variability is a difficult problem that complicates the study of gene expression in MDS. MDS BM cellular components are multi-lineage, and each lineage includes cells in different states of maturation. Therefore, it is not possible to isolate a single cell population as ‘the’ MDS cell. Furthermore, while MDS patients are classified in different subtypes, each subtype is composed of a heterogeneous group of patients who may indicate different responses to the treatment. Therefore, the number of microarray studies in patients with MDS is still limited (Galili and Raza 2009).

Several groups have studied the GEP of purified CD34+ cell fractions of BM of MDS patients (Hofmann, et al. 2002; Chen, et al. 2004; Sternberg, et al. 2005; Pellagatti, et al. 2006). A lack of certain defensive proteins in CD34+ cells from low-risk MDS patients may lead to the increased susceptibility to cell damage (Hofmann, et al. 2002).

Another study showed that GEP of MDS CD34+ cells, particularly RARS cells, is similar to that observed in IFN-γ-induced CD34+ cells from healthy individuals. That study also demonstrated that RARS patients show a specific gene expression pattern characterized by over-expression of mitochondrial-related genes, in particular genes involved in heme synthesis (eg, ALAS2) (Pellagatti, et al. 2006).

2.6.3. Pathway analysis

The studying of deregulated gene pathways in malignancies can lead to a better understanding of the molecular pathogenesis of the diseases. The identification of deregulated key pathways and functional groups in MDS HSCs has been facilitated by application of global gene expression analysis combined with detailed annotated pathway analyzes and gene ontology analyzes (Pellagatti, et al. 2010). Immunodeficiency, apoptosis and chemokine signaling are the most deregulated
pathways in early MDS, while advanced MDS is characterized by deregulation of DNA damage response and checkpoint pathways.

Considering these observations, Pellagatti et al. suggested a model for MDS in which early MDS cells is characterized by immune deregulation and activation of apoptosis pathways, consistent with the clinically observed ineffective hematopoiesis. Subsequently, DNA damage checkpoints are impaired in advanced MDS leading to an increase in the error rate of DNA repair, defective genomic instability, and development towards AML (Pellagatti, et al. 2010).

2.7. Low Density Array (LDA)

The expression levels of many thousands of genes can be studied simultaneously using microarrays technology. However, the key end-points for understanding the molecular basis of malignancies require the quantification of gene expression. The common methods for studying the gene expression include northern-blot, semi-quantitative RT PCR and quantitative real time-PCR (QRT-PCR). Application of a technology, which could combine the ability of microarray analysis to measure the expression levels of many genes in a single sample while retaining the sensitivity and quantitative capacity offered by QRT-PCR could be very informative (Lu, et al. 2008).

Recently, Applied Biosystems (Foster City, CA, USA) introduced LDA, a novel TaqMan QRT-PCR-based method. TaqMan LDA (TLDA) is a miniaturized TaqMan quantitative PCR based on micro-fluidic systems.

In contrast to conventional QRT-PCR, several genes in a single sample can be studied simultaneously using LDAs. Furthermore, its ease of use and cost implications should be considered. Loading and setting up the LDA cards is straightforward, with no requirement for robotic liquid handling. Additionally, LDAs require a very low reaction volume, which means that there are large savings in master mix, and RNA sample per study (Goulter, et al. 2006).

However, when analyzing LDA results of low copy genes, caution is recommended due to limited reproducibility of LDA for the genes with high Ct values (Lu, et al. 2008).

In conclusion, LDA technology provides a sensitive and reproducible approach to study gene expression. Additionally, this novel technique produces comparable results to those produced by conventional QRT-PCR.
2.8. Whole Transcriptome Analysis (RNA sequencing)

The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition. Studying the transcriptome can be informative for understanding the functional elements of the genome and revealing the molecular components of cells and tissues, and also for understanding development of diseases.

By application of transcriptomics, all types of transcripts including mRNAs, non-coding RNAs and small RNAs could potentially be identified, leading to the identification of splicing patterns and post-transcriptional modifications as well as to quantification of the modifying expression levels of each transcript (Wang, et al. 2009).

Next-generation sequencing (NGS) technologies are now being employed not only to study static genomes, but also dynamic transcriptomes in an approach called RNA sequencing (RNA-Seq). RNA-Seq employs recently developed deep sequencing technologies. In general, a population of RNA is converted to a library of cDNA fragments with adaptors attached to one or both ends. Each molecule is then sequenced in a high-throughput manner to obtain short reads (sequences). The resulting reads, then, are aligned to a reference genome to generate a genome-scale transcription map including both the transcriptional structure and/or level of expression for each gene (Figure 6) (Marguerat and Bahler 2010).

Several comparisons of RNA-seq and microarray data have been made to date, which state that sequencing has higher sensitivity and dynamic range, coupled with lower technical variation. Moreover, RNA-seq gives details about transcriptional features, such as novel transcribed regions, allele-specific expression, RNA editing and a comprehensive capability to capture alternative splicing that arrays cannot (Oshlack, et al. 2010).

However, RNA-Seq is not without its challenges. The crucial step of RNA-seq is library preparation, since it determines how closely the cDNA sequence data reflect the original RNA population. Large RNA molecules must be fragmented into smaller pieces (200–500 bp). Different fragmentation methods namely RNA fragmentation and cDNA fragmentation create a different bias in the outcome. Some manipulations during library construction also complicate the analysis of RNA-Seq results. For instance, many identical short reads can be obtained from cDNA libraries that have been amplified. These could be a real reflection of abundant RNA species, or they could be PCR artifacts.
Another important issue is sequence coverage, i.e. the percentage of transcripts surveyed. To detect a rare transcript or variant, considerable depth is needed. Greater coverage requires more sequencing depth which increases the costs and may be a limiting factor for some studies.

Like other high-throughput sequencing technologies, RNA-Seq faces several informatics challenges, including the development of efficient methods to store, retrieve and process large amounts of data (Wang, et al. 2009).

In summary, although RNA-Seq is still in the early stages of use, it has clear advantages over previously developed transcriptomic methods. As the cost of sequencing continues to fall, RNA-Seq is expected to replace microarrays.

**Figure 6.** Flowchart of a typical RNA-seq experiment

### 2.9. Exome sequencing

Despite the continued increases in next-generation sequencing, the cost of obtaining and analyzing full genome sequences on a large number of human individuals is limiting. Therefore, large scale human genetic studies relies on techniques that select and enrich chromosomal regions of interest before performing sequencing (Hedges, et al. 2009).

Exome sequencing is an efficient technology to selectively sequence the coding regions of the genome in order to detect novel genes associated with rare and common disorders. Sequencing of the 'exome' involves fragmentation of the genome into millions of pieces and capturing and sequencing only selected DNA from exon regions.
(Teer and Mullikin 2010). Exome sequencing differs from RNA sequencing by focusing on DNA rather than the expressed RNA and it is cheaper than whole-genome sequencing.

Targeted sequencing methods generally increase sequence coverage of interesting regions at lower cost and higher throughput compared with random shotgun sequencing. Exome, the exons of whole genome, represents only approximately 1% of the genome (about 30 Mb). Therefore, higher sequence coverage with considerably less raw sequence and cost can be readily achieved using next generation sequencing technology. However, the targeting process can be challenging. For instance, uneven capture efficiency across exons can mean that not all exons are sequenced and some off-target hybridization can occur (Meyerson, et al. 2010).

In summary, the higher coverage of the exome that can be achieved for a large number of samples makes exome sequencing highly suitable for mutation discovery in cancer samples of mixed purity.
3. AIM OF THE THESIS

The overall aim of the thesis was to investigate and understand mechanisms underlying the pathogenesis of RARS.

Specific aims were as follows:

**Study I:** To investigate the expression levels and mutational status of the \( ABCB7 \) gene in CD34+ cells and erythroblasts from MDS patients and healthy controls

**Study II:** To explore the molecular mechanisms underlying the ineffective erythropoiesis and aberrant iron accumulation in RARS erythroblasts, as well as the anti-apoptotic effects of G-CSF

**Study III:** To test the hypothesis that \( ABCB7 \) is a key mediator of aberrant iron accumulation in acquired RARS

**In addition** we wanted to explore upstream events leading to \( ABCB7 \) down-regulation and aberrant iron accumulation in RARS
4. MATERIAL AND METHODS

4.1. General comments

The techniques used in this study are described in details in papers I-III. Here, material and methods will be listed and briefly commented upon.

4.2. Patients and healthy volunteers (Paper I-III)

122 patients with MDS (35 RA, 33 RARS and 54 RAEB) and 16 healthy controls were included in paper I. MDS patients were classified according to the FAB criteria. In paper II, the diagnostic procedure was performed according to the WHO 2008 classification (Swerdlow, et al. 2008) and routines previously reported by the Nordic MDS Group (Jädersten, et al. 2005). In paper II, Seven patients with either RARS (n = 3) or RCMD-RS (n = 4) and 6 healthy volunteers were included. In addition, a larger cohort of RARS and RCMD-RS (n = 19) and normal controls (n = 10) were assessed for methylation status of identified candidate genes. In paper III, 9 patients with MDS with <5% myeloblasts and ≥15% ring sideroblasts (the term RARS is hereafter used both for RARS and RCMD-RS) and 8 healthy individual were included. For detailed patient information, the reader is referred to the corresponding paper.

4.3. CD34+ cell separation and erythroblast culture (Paper I-III)

CD34+ cells were isolated from BM samples of MDS patients and healthy controls using magnetic-activated cell sorting (MACS) columns according to the manufacturer’s recommendations. Following separation, CD34+ cells were cultured (0.1x10⁶/ml) for 14 days in Iscove’s medium supplemented with 15% BIT9500 serum substitute (containing bovine serum albumin, bovine pancreatic insulin and iron/saturated human transferrin and recombinant human interleukin (rh-IL)-3 (10 ng/ml), rh-IL-6 (10 ng/ml), rh-stem cell factor (rh-SCF; 25 ng/ml), 1% penicillin and streptomycin and 1% l-glutamine. Epo (2 iu/ml) was added to the medium at day 7, and fresh medium supplemented as above (plus Epo) was added at day 9 and 11. Phenotype and erythroid maturation of cells was, as previously validated and reported, analyzed at day 4, 7 and 14 using CD36 and glycophorin A (GpA) antibodies (Tehranchi, et al. 2003; Tehranchi, et al. 2005).
In paper I, an aliquot of cells harvested at day 7 were treated with 100 ng/ml G-CSF for 4 hours, e.g. the same concentration and exposure time that were used in previous experiments (Tehranchi, et al. 2003; Tehranchi, et al. 2005).

4.4. K562 cultures and erythroid differentiation (Paper III)

The human myeloid leukemia cell line K562 was cultured in RPMI1640 containing 10% FBS, at 37 C° in a humidified atmosphere of 5% CO2 in air. Cultures were split every 4 days to maintain an exponential growth before experiments. For erythroid differentiation, K562 cells were treated by Hemin 50 µM in NaOH 0.1 N for 72 h. Differentiation of K562 cells was confirmed by assaying the expression level of γ-globin and glycophorin A by QRT-PCR or flow cytometry.

4.5. RNA extraction (Paper I-III)

Total RNA from primary or cell lines was extracted using TRIZOL following the protocol supplied by the manufacturer and yields were determined spectrophotometrically at 260 nm and RNA integrity assayed using Agilent Bioanalyzer 2100.

4.6. Microarray experiments and data analysis (Paper I and II)

GEP experiments were performed on CD34+ cells and cultured cells harvested at day 7. Extracted RNA was amplified and labelled according to the manufacturer’s recommendations. Labelled fragmented cRNA was hybridized to oligonucleotide probes on an Affymetrix Human Genome U133 Plus 2.0 GeneChip. Hybridization occurred for 16 h at 45 C°. The arrays were scanned using a GeneChip Scanner 3000.

Scanned GeneChip images were processed using GeneChip Operating Software (GCOS). Data analysis was performed by GeneSpring 7.3. Quality control was performed within the GCOS software. Affymetrix CEL files were uploaded and pre-processed using Robust MultiChip Analysis (RMA). Differentially expressed genes (t-test, P < 0.05, Benjamini–Hochberg multiple testing correction) between conditions were identified using Gene-Spring and used for pathway analysis using Ingenuity 5.0. Hierarchical clustering was performed with GeneSpring software using Pearson correlation.
4.7. QRT-PCR (Paper I-III)

QRT-PCR was performed for selected genes. The expression level of the housekeeping gene *GAPDH* or *beta-2-microglobulin* was used to normalize for differences in input cDNA. QRT-PCR was carried out using TaqMan gene expression assay in 7500 Real-Time PCR system (Applied Biosystems) or in a LightCycler 480 instrument (Roche Diagnostics) (paper III). The expression ratio was calculated using the $\Delta\Delta^C_T$ method (Livak and Schmittgen 2001).

4.8. DNA sequencing (Paper I)

Direct sequencing of *ABCB7* was performed on DNA from patients using Applied Biosystems Big dye terminator kit

4.9. Promoter methylation studies (Paper I)

The *ABCB7* promoter was predicted using PromoterScan and CpGPlot software. Then, methylation status of the genomic DNA was studied using bisulphate sequencing method. Primers to amplify the modified DNA were designed using Primer3 software. The PCR products were purified and cloned using the pGEM-T Easy cloning system and sequenced with Applied Biosystems Big dye terminator kit v1.1 and the *ABCB7* forward primer.

4.10. HUMARA analysis (Paper II)

Clonality of cultured erythroblasts was evaluated through the study of the X-Chromosome Inactivation Pattern (XCIP) by both DNA methylation and differential allelic expression analysis of the human androgen receptor (*HUMARA*) gene. DNA methylation status analysis was performed as previously described (Tonon, *et al.*. 1998). The expression clonality assay was based on a nested primer RT-PCR as reported by Busque *et al.* (Busque, *et al.*. 1994). Amplified bands were subjected to density analysis by Molecular Imager FX software.

4.11. Western blotting (Paper II)

Cells were lysed with lysis buffer and total protein concentration was determined using the BCA Protein Assay kit. 25 micrograms of total protein was separated on a 7.5–10% acrylamide gel and transferred to nitrocellulose membrane. Membranes were probed with antibodies that recognize HSPA1B, MFN2 and GAPDH. After
incubation with appropriate secondary antibodies, specific proteins were detected using enhanced chemiluminescence reagents.

4.12. DNA extraction, bisulfite modification and methylation (Paper II)

Genomic DNA was extracted from myeloid cell fractions using Gene Elute mammalian genomic extraction kit. Bisulfite modification of genomic DNA was carried out using EZ DNA methylation GoldTM kit following standard protocol.

Melting curve analysis-Methylation assay (MCA-Meth) was performed as described by (Lorente, Mueller et al. 2008). Primers for ABCB7, MFN2, FANCC and FOXO3 were designed using Methprimer software (Li and Dahiya 2002). Both amplification reaction and melting curve analysis were carried out using an ABI 7500 FAST real time PCR system (Applied Biosystems).

4.13. Lentiviral vector construction & lentivirus preparation (Paper III)

4.13.1. pLKO.1-GFP-shABCB7

The shRNA constructs were designed to include a hairpin of 21 base pair sense and antisense stem and a 6 base pair loop. Each hairpin sequence was cloned into the lentiviral vector (pLKO.1) and sequence verified

4.13.2. pRRL-TRE-ABCB7-IRES-GFP-PGK-rtTA

IRES-GFP was amplified from MSCV-IRES-GFP (Persons, et al. 1997) and inserted into pSIN-TREmSEAP-hPGKrtTA2S resulting in pRRL-TRE-IREs-GFP-PGK-rtTA. Human ABCB7 gene was amplified by PCR and cloned into pRRL-TRE-IREs-GFP-PGK-rtTA.

4.13.3. Prrlsin-cPPT-PGK-IRES-YFP-WPRE and pRRLSIN-ABCB7-IRES-YFP-WPRE

The region containing IRES-YFP was amplified from MSCV-IRES-YFP (Persons, et al. 1997) by PCR. This fragment was ligated into pRRLSIN-cPPT-PGK-GFP-WPRE from which the GFP was removed resulting in the vector pRRLSIN-cPPT-PGK-IREs-YFP-WPRE. Human ABCB7 gene was amplified by PCR and cloned into pRRLSIN-cPPT-PGK-IREs-YFP-WPRE.
4.13.4. Lentivirus production by 293FT cells

293FT cells (Invitrogen) were plated in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with L-glutamine and 10% FBS. The next day, 20µL Lipfectamine™2000 (Invitrogen) was added to 500 µL Opti-MEM medium and incubated for 5 min at room temperature (RT). Then it was added to the mixture containing 24 µg DNA (12 µg vector containing gene of interest or control vector, 8 µg pΔR8.9 and 4 µg pMDG) in 500 µL Opti-MEM and incubated again for 20 min at RT.

The medium was taken away from the 293FT cell dishes, Opti-MEM as well as the prepared mixture were added to the cells and incubated at 37°C, 5% CO2. The next day, the medium was replaced by fresh DMEM complete medium and incubated again. After 24 and 48 h, viral supernatant was harvested, filtered and frozen. The viral supernatant was concentrated and titrated before transduction of the cells.

4.14. Lentiviral transduction of BM CD34+ cells (Paper III)

BM CD34+ cells were cultured in Iscove’s medium supplemented with 15% BIT 9500 serum substitute and recombinant human interleukin (rh-IL)-3 (10 ng/ml), rh-IL-6 (10 ng/ml), rh-stem cell factor (rh-SCF; 25 ng/ml), and 1% L-glutamine. After 16 h, the CD34+ cells were transferred to 24-well plates, pre-coated with the human fibronectin fragment CH-296 blocked with 2% BSA in phosphate buffered saline (PBS) for 30 min at RT, and pre-loaded with viral supernatant. Subsequently, the cells were added to the wells in serum-free medium and with cytokines as above and spinoculation for 90 min at 25°C.

4.15. Colony forming unit cell (CFU-C) assay (Paper III)

Lentiviral transduced-BM CD34+ cells were plated on day 3 after initiation of culture in triplicate in 35 mm dishes in H4230 methylcellulose. The cells were cultured at 37°C in a humidified atmosphere with 5% CO2. The number of erythroid colonies (CFU-E and BFU-E), myeloid colonies, YFP+/GFP+ erythroid, and YFP+/GFP+ myeloid colonies were counted on day 14 after transduction.

4.16. Immunohistochemical investigation for FTMT and ABCB7 (Paper III)
K562 cells and erythroblasts on cyto-centrifuged slides were fixed by 4% formaldehyde and analyzed for FTMT and ABCB7 with specific related polyclonal antibodies. Bound antibody was detected by an immuno-alkaline phosphatase method. A negative control was performed by replacing the primary antibody with PBS. Finally, slides were counterstained with Hematoxylin. Slides were studied and analyzed in Olympus BH-2 light microscope.

4.17. Flow Cytometry (Paper II and III)

In paper II, the purity of CD34+ separated cells was assessed by flow cytometry using anti-CD34 monoclonal antibody. Phenotype and erythroid maturation was analyzed at day 4, 7, 11 and 14 using CD36 and GpA antibodies. In paper III, transduced cells were incubated with antibodies against APC-conjugated anti-Glycophorin-A, APCCy7-conjugated anti-CD36, PECy5-conjugated C-kit and PECy7-conjugated CD34. Apoptosis was detected using Annexin V-Pacific Blue. All analyzes were performed on a LSRII-Fortessa.

4.18. Mutational analysis of SF3B1 (Paper III)

The coding exons of SF3B1 were screened using massively parallel pyrosequencing of DNA pools from selected samples. A total of 27 oligonucleotide primer pairs were designed using Primer 3 v.0.4.01, targeting all protein coding exons for transcript CCDS 33356, as annotated by Ensembl genome browser. Selected samples were individually amplified and indexed. DNA pools for massively parallel sequencing were prepared and high throughput sequencing of pooled products was performed. Individual sample sequencing information was deconstructed as previously described (Varela, et al. 2011), mapping of individual sample FLX 454 reads to the human genome (Build 37) was done using BWA and to facilitate variant calling pileup files were constructed with Samtools (Li and Durbin 2010). All novel sequence variants were verified using conventional PCR based Sanger sequencing.

4.19. RNAi knockdown of SF3B1 in K562 cells (Paper III)

For transfection of siRNA, 2×10^6 K562 cells were electroporated in an Amaxa Nucleofector I, using the Amaxa cell optimization kit V. The transfection efficiency was assessed after 24 h by checking the percentage of GFP-positive cells. Three non-
overlapping siRNAs targeting SF3B1 and two different scramble sequences with GC content similar to the siRNA sequences were used in two independent experiments. Expression levels of SF3B1 and ABCB7 were assessed by QRT-PCR.

4.20. Transcriptome analysis of normal and RARS progenitors during erythroid differentiation (Preliminary)

To further explore the molecular mechanisms in RARS, we examined the transcriptional profile associated with early erythroid maturation in NBM and RARS using RNA-Seq. BM CD34+ cells from one patient and one control were cultured for 4 days to allow for erythroid maturation. cDNA libraries were prepared from RNA extracted from these two time points (0 and 4 days), and thereafter sequenced on Life Technology's next generation sequencing platform SOLiD. Approximately 80 million reads were obtained for each library. Two sets of analyzes were made; one blinded for position to allow for an unbiased analysis of data and one comparing position profiles with raised previous GEP findings. Differential gene expression analysis was done using the bioconductor package DESeq. We compared samples pairwise using a negative binomial statistical model to assess significant differential expression.

4.21. TLDA (Preliminary)

Each LDA card includes 384 wells and each well contains specific, user-defined primers and probes, capable of detecting a single gene. In this study, two samples were loaded per array (in triplicate) and 64 genes including three housekeeping genes, GAPDH, beta-2-microglobin and HPRT were analyzed simultaneously. Genes were chosen based on transcriptome preliminary results, previous GEP findings (Nikpour, et al. 2010) and literature. In this study, we included 11 RARS (D0), 7 RARS (D4), 4 NBM (D0) and 4 NBM (D4).

Each cDNA sample was added to TaqMan Universal PCR Master Mix (Applied Biosystems) and mixed gently. The mixture was then transferred into a loading port on a TLDA card (Applied Biosystems). After centrifugation of arrays, the cards were sealed and PCR was performed. Expression values were calculated using the comparative C_T method as previously described (User Bulletin No. 2, Applied Biosystems).
5. RESULTS

5.1. ABCB7 expression levels and percentage of bone marrow ring sideroblasts (Paper I)

The first study was based on a previous GEP analysis from CD34+ cells from a variety of MDS patients (Pellagatti, et al. 2006). The expression levels of ABCB7 in BM CD34+ cells were lowest in the RARS group compared to RA, RAEB and healthy controls (Figure 7-A).

MDS cases were divided in three groups by percentage of BM ring sideroblasts (0–14%, 15–40% and ≥41%). Interestingly, there was a strong relationship between increasing percentage of bone marrow ring sideroblasts and decreasing ABCB7 gene expression levels (Figure 7-B).

To further explore gene expression in RARS, we then did a second GEP analysis on cultured day 7 erythroblasts. Compared to CD34+ cells, erythroblasts showed an even lower ABCB7 expression. Again, expression in RARS was significantly lower than in RA and healthy controls.

![Figure 7. ABCB7 expression levels.](image)

(A) ABCB7 expression levels in the CD34+ cells from healthy controls and MDS patients with RA, RAEB and RARS. (B) ABCB7 expression levels and percentage of ring sideroblasts in BM.

5.2. ABCB7 gene sequencing and promoter methylation (Paper I)

Direct sequencing of ABCB7 showed 80% methylation in all samples, with no significant difference between RARS patients and healthy controls.
5.3. RARS erythroblasts are clonal (Paper II)

All day 7 RARS erythroblasts showed a skewed XCIP (corrected allelic ratios: 4.28, 5.62, 8.19 and complete inactivation of one allele in two cases), indicative of a clonal erythroid progenitor population. Three of these were also analyzed at the start of culture (CD34+ cells), and all showed skewed XCIP (complete inactivation of one allele in two cases).

5.4. Differentially expressed genes in RARS and normal erythroblasts (paper II)

6228 probe sets were significantly different between day 7 RARS and normal erythroblasts. From these, 3960 probe sets were up- and 2268 probe sets were down-regulated. For detailed information, the reader is referred to the corresponding paper. Our data demonstrated that several genes involved in cellular iron metabolism and mitochondrial function including \textit{ABCB7}, \textit{MFN2} and \textit{Sfxn1} were de-regulated in RARS erythroblasts.

Interestingly, \textit{ABCB7} gene was suppressed throughout erythroid maturation in RARS, with more pronounced suppression in mature erythroblasts, in contrast to the increasing expression over time in normal erythroblast cultures (Figure 8).

\textbf{Figure 8}. ABCB7 expression level in differentiating RARS and normal erythroblasts, with ABCB7 expression normalized to that of day 0 healthy controls (results shown as mean ± SD).

We also found that erythropoiesis-related genes were not over-expressed in RARS erythroblasts, which was in contrast to previous finding in RARS CD34+ cells. For instance, the over-expression of \textit{ALAS2} in RARS erythroblasts was much lower than that in RARS CD34+ cells. The up-regulation seen in the RARS CD34+ progenitors
may reflect early compensatory mechanisms in these cells as well as the expansion of immature CD34+ erythroblasts.

Moreover, we observed deregulation of several genes involved in cell survival and apoptosis, including \textit{MAP3K7} and two members of the heat shock protein 70 family, \textit{HSPA1B} and \textit{HSPA9} genes.

Deregulation of several interferon induced genes including \textit{IRF2}, \textit{IRF6}, \textit{IRF2BP2}, \textit{TOR1AIP2}, \textit{IFNA17}, \textit{AEN} and \textit{ISG20L2} were observed also in RARS erythroblasts.

\section*{5.5. Pathway analysis of RARS compared to normal erythroblasts (Paper II)}

Functional classification revealed 25 significantly deregulated pathways in RARS compared to normal erythroblasts (P < 0.01). RARS erythroblasts showed a marked deregulation of several important pathways including integrin, PI3K/AKT and VEGF signaling, protein ubiquitination, apoptosis, DNA damage checkpoint regulation, mitochondrial function, and the JAK/Stat pathway.

\section*{5.6. Potential mechanisms behind the anti-apoptotic effects of G-CSF in RARS (Paper II)}

1153 probe-sets were significantly different (P < 0.05) between G-CSF treated and untreated RARS erythroblasts. Importantly, several genes, which in RARS were altered in a direction of enhanced apoptosis, were reverted back to the normal range by G-CSF. The reduced \textit{MFN2} expression was reverted to normal range. However, the expression level of \textit{MAP3K7} was not altered by G-CSF. The expression level of \textit{HSPA9} and \textit{HSPA1B}, which were slightly up-regulated in RARS, further increased after G-CSF treatment (Figure 9).

Furthermore, pathway analysis revealed 17 significantly deregulated pathways in G-CSF treated and un-treated RARS erythroblasts (P < 0.05). Interestingly, the ‘mitochondrial dysfunction’ pathway, one of the main deregulated pathways in RARS erythroblasts, was significantly modulated after G-CSF treatment (P = 0.04)
5.7. Methylation analysis (Paper II)

In order to investigate whether down-regulation of key genes was caused by hypermethylation, the methylation status of some candidate genes was determined. The MFN2, FANCC and FOXO3 genes were not methylated in the RARS samples. Regarding ABCB7, there was 50% methylation in females (both RARS and controls), which is in line with the fact that one allele of ABCB7 is inactivated because of its location on chromosome X (Figure 10).

5.8. The aberrant gene expression pattern in RARS is accentuated during late erythroid differentiation (Paper III)

The expression pattern during late erythroid differentiation is markedly altered in RARS, which parallels the time of ring sideroblast formation in vivo. ABCB7 expression showed a continuous decrease in the RARS cultures, while increasing in
normal cultures. Moreover, \textit{ALAS2} and \textit{GATA1} were down-regulated during late differentiation.

5.9. Down-regulation of \textbf{ABCB7} in K562 cells leads to erythroid apoptosis and accumulation of \textit{FTMT} (Paper III)

Down-regulation of \textit{ABCB7} in K562 led to a six times over-expression of \textit{ALAS2}, in analogy with the pattern observed in RARS CD34+ cells. We also observed up-regulation of \textit{FTMT} after \textit{ABCB7} silencing in K562 cells.

Next, the role of \textit{ABCB7} in K562 cells was studied during erythroid differentiation. Importantly, \textit{ABCB7} silenced K562 cells showed impaired erythroid differentiation and increased \textit{FTMT} expression (Figure 11).

![Figure 11](image)

**Figure 11.** Down-regulation of \textbf{ABCB7} in K562 cells interferes with erythroid differentiation and leads to accumulation of ferritin mitochondrial. \textbf{A.} K562 cells transduced with scrambled-shRNA or shRNA specific for ABCB7 were mixed with untransduced K562 cells and cultured in vitro for 4 days. GFP+ cells % was analyzed daily by flow cytometry. \textbf{B.} Analysis of gene expression of ABCB7 and ALAS2 in transduced K562 cells on day3 post transduction. \textbf{C} and \textbf{D.} After successful transduction of K562 cells, erythroid differentiation was induced by hemin for 72 h and the expression level of \textit{γ-globin} and \textit{FTMT} was analyzed. \textbf{E.} K562 cells were transduced with shABCB7 and treated with hemin for 72 h to induce erythroid differentiation. The percentage of FTMT positive K562 cells was analyzed by IHC and quantitated. The arrow depicts the FTMT positive K562 cells.
5.10. Down-regulation of *ABCB7* in normal progenitors reduces erythroid survival and colony growth and leads to accumulation of *FTMT* (Paper III)

*ABCB7* silencing significantly reduced erythroid colony growth. Moreover, the survival of *ABCB7* silenced cells decreased during the latter part of differentiation.

To further explore the gene expression pattern during differentiation, shRE1-GFP+ transduced NBM CD34+ cells were sorted two days after transduction and expression levels of the candidate genes were analyzed. Even a moderate down-regulation of *ABCB7* for 2 days of differentiation caused a two-fold increase of *FTMT* expression.

Furthermore, studying the expression level of some candidate genes after erythroblast culturing of the sorted cells indicated marked reduction of *ALAS2, GATA-1, FOXO3A* and *FANCC*.

5.11. Over-expression of *ABCB7* promotes erythroid differentiation in K562 cells (Paper III)

*ABCB7* over-expression did not affect cell growth of K562 cells and the expression levels of our candidate genes. However, up-regulation of *ABCB7* potentiated erythroid differentiation of K562 cells indicating a role in the erythroid differentiation process.

5.12. Over-expression of *ABCB7* in RARS restored erythropoiesis and reverted gene expression towards the normal range (Paper III)

Importantly, *ABCB7* up-regulation decreased the expression level of *FTMT* in all transduced cases. Moreover, *ABCB7*- transduced CD34+ cells showed clearly enhanced erythroid colony growth compared with the cultures transduced with mock vector. The increase mainly consisted of YFP expressing colonies (Figure 12).
Figure 12. Up-regulation of ABCB7 restores erythroid function in RARS progenitors. A. Colony growth in RARS CD34+ cells (n=4), transduced by either mock or ABCB7-vector. B. Depicted is one representative colony experiment showing both total colony numbers and percentage of YFP+ CFU-Cs. C and D. Transduced cells were cultured in erythroblast cultures for 14 days and expression of ALAS2 and FMTM in RARS erythroblasts was analyzed on day 10.

5.13. Mutations of *SF3B1* in the RARS patient cohort (Paper III)

Of the nine RARS patients included in the study, 7 carried *SF3B1* mutations, one was wild-type and one was not analyzed.


The expression levels of *ABCB7* were down-regulated in *SF3B1*-silenced cells compared with the scramble controls, and *ABCB7* expression levels were also restored at day 10 post-transfection.

5.15. Transcriptome analysis of RARS (Preliminary results)

The number of differentially expressed genes ranged from 10 (comparison Control D4-RARS D4) to 294 (comparison Control CD34+-RARS CD34+). Based on correlation of gene expression and the number of differentially expressed genes, samples could be clustered into two pairs, Control CD34+-RARS CD34+ and Control D4-RARS D4. Interestingly, several non-coding RNAs (SNORDs) involved in eg
miRNA processing were significantly down-regulated in both RARS positions, compared to NBM.

We also identified dramatic dysregulation of two putative zinc finger transcription factors during erythroid differentiation. A selenoprotein gene, SEPP1 involved in protection against oxidative stress, was markedly dysregulated during differentiation (Table 3).

**Table 3.** Top twenty genes for RARS/Ctrl comparison (Day 4)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>log2FC (RARS/Ctrl)</th>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP11-184E9.1</td>
<td>-8.95</td>
<td>chr 5</td>
<td>No protein product</td>
</tr>
<tr>
<td>RP11-549K20.1</td>
<td>-6.54</td>
<td>chr 5</td>
<td>No protein product</td>
</tr>
<tr>
<td>SNORD114-13</td>
<td>-5.93</td>
<td>14q32</td>
<td>?</td>
</tr>
<tr>
<td>SNORD113-4</td>
<td>-5.76</td>
<td>14q32.31</td>
<td>?</td>
</tr>
<tr>
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<td>-5.18</td>
<td>14q32</td>
<td>?</td>
</tr>
<tr>
<td>SNORD113-7</td>
<td>-4.92</td>
<td>14q32.31</td>
<td>?</td>
</tr>
<tr>
<td>SNORD114-23</td>
<td>-4.82</td>
<td>14q32</td>
<td>?</td>
</tr>
<tr>
<td>RUNX1T1</td>
<td>-4.72</td>
<td>8q22</td>
<td>Zinc finger transcription factor and oncoprotein.</td>
</tr>
<tr>
<td>ADCYAP1</td>
<td>-4.63</td>
<td>18p11</td>
<td>Activation of adenylate cyclase activity</td>
</tr>
<tr>
<td>SNORD114-26</td>
<td>-4.49</td>
<td>14q32</td>
<td>?</td>
</tr>
<tr>
<td>SYCP2</td>
<td>-4.13</td>
<td>20q13.33</td>
<td>A major component of the synaptonemal complex</td>
</tr>
<tr>
<td>SNORD113-9</td>
<td>-4.06</td>
<td>14q32.31</td>
<td>?</td>
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<tr>
<td>SNORD113-6</td>
<td>-3.75</td>
<td>14q32.31</td>
<td>?</td>
</tr>
<tr>
<td>DOK6</td>
<td>-3.62</td>
<td>18q22.2</td>
<td>Involved in the RET signaling cascade</td>
</tr>
<tr>
<td>RNU5D</td>
<td>-3.53</td>
<td>chr 1</td>
<td>?</td>
</tr>
<tr>
<td>GPC6</td>
<td>-3.26</td>
<td>13q32</td>
<td>A putative cell surface coreceptor for growth factors, extracellular matrix proteins, proteases and anti-proteases.</td>
</tr>
<tr>
<td>AC016683.6</td>
<td>5.3</td>
<td>chr 2</td>
<td>?</td>
</tr>
<tr>
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<td>chr 1</td>
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</tr>
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<td>ZFP57</td>
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<td>6p22.1</td>
<td>Zinc finger transcription factor</td>
</tr>
</tbody>
</table>

**5.16. TLDA (Preliminary results)**

TLDA analysis confirmed the transcriptome data, further underlying that several of the most upregulated genes in RARS are involved in oxidative stress. A family of nuclear receptors functioning as tumor suppressors is also up-regulated during
erythroid differentiation. These results constitute the basis for recently initiated studies in the research group (Figure 13).

**Figure 13.** Principal component analyzes (PCA) of TLDA, positive value of each axis means highly contributed gene to distinguish between NBM and MDS with down-regulation trend in MDS. Negative value means also highly contribution with up-regulation trend in MDS. Zero means no contribution (no difference between NBM and MDS).
6. DISCUSSION

Acquired refractory anemia with ring sideroblasts (RARS) was described by Björkman more than 50 years ago (Björkman 1956). However, the molecular basis of RARS has until recently been unrevealed. In this thesis, we investigated and tried to understand mechanisms underlying the pathogenesis of RARS.

The hypothesis under consideration was that hereditary XLSA/A and acquired RARS may share a molecular basis. That leads to the study of the role of $ABCB7$ in the pathogenesis of RARS. $ABCB7$ is required for maturation of cytosolic Fe–S proteins, such as iron regulatory protein 1, which regulates protein levels of the major iron homeostasis genes (Cairo and Recalcati 2007). Hence, disruption of Fe-S cluster biogenesis pathways can result in maladaptive changes in iron metabolism.

In the first step, we investigated the mutational status and the expression levels of the $ABCB7$ gene in CD34+ cells and erythroblasts from MDS patients and healthy controls.

Direct sequencing of $ABCB7$, including the promoter region, was performed on DNA from 13 RARS patients, but no mutations were identified. Similarly, in a recent study, Steensma et al. found no coding mutations of $ABCB7$ in 37 patients with RARS (Steensma, et al. 2007).

Interestingly, the expression levels of $ABCB7$ were significantly lower in RARS CD34+ cells than in healthy controls, RA or RAEB. Moreover, there was a significant inverse relation between $ABCB7$ expression and the percentage of ring sideroblasts, with the lowest expression in patients with $>$40% ring sideroblasts in BM.

Variability is a difficult problem that complicates the study of gene expression in MDS. The cellular components of the MDS BM encompass all cell lineages, and each lineage is present at various stages of maturation. Thus there is no single cell population that can be isolated and studied as ‘the’ MDS cell. Even the CD34+ compartment may reflect different cell mixes in different MDS subtypes (Kanter-Lewensohn, et al. 1996). Considering the typical morphology of RARS, it is likely that mRNAs from the CD34+ progenitor population mirror a higher proportion of erythroblasts compared to e.g. 5q-syndrome CD34+ cells. To address this problem, we used cultured erythroblasts for the microarray analyzes using a well-validated culture method. Importantly, $ABCB7$ gene expression was lower in RARS erythroblast than in CD34+ cells. While, in normal erythroblast cultures, $ABCB7$ expression increased during differentiation; it decreased.
gradually from day 0 to day 14 in RARS cultures, supporting a critical role of ABCB7 also in the aberrant iron accumulation in acquired RARS.

Gene expression profiling of RARS erythroblasts revealed an unexpected finding. Several genes involved in erythropoiesis, which were significantly over-expressed in RARS CD34+ cells showed normal or nearly normal expression in erythroblasts. For instance, while ALAS2 over-expression in the CD34+ compartment was 12.77-fold the normal level, it was only 1.37-fold the normal level in intermediate day 7 erythroblasts. The up-regulation in the RARS CD34+ progenitor compartment is likely to reflect early compensatory mechanisms in these cells as well as the expansion of immature CD34+ erythroblasts characteristic of the RARS marrow. Therefore, this data supports the strength of assessing gene expression during differentiation.

The single most down-regulated gene in RARS erythroblast was NSMCE4A, which is involved in DNA damage repair and hence may be associated with DNA instability in RARS (Hu, et al. 2005; Novotna, et al. 2008). Moreover, FANCC, a key mutated gene in Fanconi anemia and involved in DNA damage repair, was also markedly suppressed. Finally, apoptosis of RARS erythroblasts was paralleled by MAP3K7 down-regulation. MAP3K7 deletion leads to massive hematopoietic cells apoptosis in mice (Tang, et al. 2008).

The identification of deregulated gene pathways in tumor can lead to a better understanding of the molecular pathogenesis of disease and identify new targets for treatment. Several pathways were deregulated in RARS erythroblasts compared to normal erythroblasts, including mitochondrial function, apoptosis and JAK/Stat signaling, all key elements of functional erythropoiesis. Deregulation of JAK/Stat signaling pathway, particularly STAT5 down-regulation, is in line with the impaired response to Epo in RARS patients (Hoefsloot, et al. 1997).

However, RARS erythroblasts induced by G-CSF are able to undergo terminal differentiation due to the dispensable role of STAT5B in G-CSF-R signaling (Millot, et al. 2001). This may be a relevant explanation for the particularly good synergistic in vivo and in vitro effects of G-CSF and Epo on the anemia and erythroid apoptosis in RARS (Tehranchi, et al. 2003; Jadersten, et al. 2005; Tehranchi, et al. 2005).

To further address the anti-apoptotic role of G-CSF in RARS cells, we assessed the genes, whose expression levels were returned towards the normal range by G-CSF. G-CSF restored MFN2 gene and protein expression and had also a significant effect on the whole mitochondria pathway in RARS cells. MFN2 participates in the mitochondrial pathway and its silencing leads to enhanced apoptosis in HeLa cells.
(Sugioka, et al. 2004) and reduced mitochondrial membrane potential (Bach, et al. 2003). The reduced MFN2 expression in RARS erythroblasts may therefore link to cytochrome c release in these cells, and G-CSF-induced re-expression of MFN2 to the inhibitory effect of the growth factor on cytochrome c release and erythroid apoptosis (Tehranchi, et al. 2003). Furthermore, G-CSF up-regulated HSPA1B, a member of the HSP70 family, which protects GATA-1 from caspase-3-mediated proteolysis during differentiation (Ribeil, et al. 2007). This may constitute another mechanism for G-CSF, which inhibits caspase-3 activation in RARS cells (Schmidt-Mende, et al. 2001).

In contrast to its anti-apoptotic effects, G-CSF does not seem to affect sideroblast formation or mitochondrial iron accumulation, as shown by the ABCB7 results and the unaffected accumulation of mitochondrial ferritin (Tehranchi, et al. 2005). Interestingly, RARS erythrocytes from patients responding to growth factors develop a more abnormal phenotype with increased cell size and hypochromatic cytosol, which supports this interpretation (Ljung, et al. 2004). We therefore suggest that G-CSF promotes erythroblast survival until the erythrocyte stage, despite impaired cellular function.

Although, ABCB7 expression has been shown to be down-regulated in CD34+ cells and differentiating erythroblasts from RARS bone marrow; several other genes are also down-regulated in RARS, and a functional relation between ABCB7 and ring sideroblast formation had not been established in acquired RARS. To answer the question if ABCB7 deficiency contributes to the pathogenesis of RARS, we modulated its expression in several cellular systems.

ABCB7 down-regulation using the moderately effective vectors used in our NBM experiments inhibited erythropoiesis, and increased apoptosis and decreased survival of erythroblasts. In addition, ABCB7 down-regulation reduced growth, erythroid differentiation and survival of K562 cells.

ABCB7 up-regulation had virtually no effect on K562 cells in standard culture and competition experiments. However, hemin treatment of K562 cells over-expressing ABCB7 potentiated erythroid differentiation and γ-globin expression, suggesting that forced erythropoiesis requires an increased production of the protein. Interestingly, ABCB7 over-expression rescued the defective erythroid growth of RARS CD34+ in four patient samples. These data are to our knowledge the first to show that modification of a single gene could rescue erythropoiesis in MDS.

As aforementioned, ALAS2 gene is over-expressed in RARS CD34+ cells and erythroblasts. Similarly, ABCB7 suppression in K562 cells leads to ALAS2 over-
expression (6-fold). However, after \( ABCB7 \) silencing and erythroid differentiation of normal progenitors, \( ALAS2 \) expression level decreased dramatically after 7 days. When \( ABCB7 \) was up-regulated in RARS CD34+ cells, \( ALAS2 \) expression was unchanged or decreased. Hence, \( ALAS2 \) dysregulation seems to be a consequence of \( ABCB7 \) down-regulation.

Importantly, \( ABCB7 \) down-regulation in NBM, followed by sorting of GFP positive and negative cells, erythroblast culturing, and analysis after 7 days of transduction induced a gene expression pattern similar to that observed in RARS day 7 erythroblasts. The anti-apoptotic \( MAP3K7 \) gene was suppressed, together with \( FOXO3A, GATA-1 \) and \( FANCC \).

To link \( ABCB7 \) deficiency to the RARS phenotype, it is necessary to show its association with accumulation of mitochondrial iron in human progenitors. Although, the erythroblast culture model does not produce ring sideroblasts; \( FTMT \) can be used as a marker for aberrant mitochondrial iron accumulation. \( FTMT \) expression increased in hemin-treated K562 and NBM cells after \( ABCB7 \) silencing. On the other hand, in spite of relatively moderate transduction frequencies, \( FTMT \) expression decreased in three RARS samples after \( ABCB7 \) over-expression.

Recently, \( SF3B1 \) gene mutations have been identified in high proportion of RARS patients. Interestingly, we found that \( SF3B1 \) silencing resulted in down-regulation of \( ABCB7 \), suggesting a direct link between the two genes. Whether this relationship forms part of the pathophysiology of RARS remains to be determined.

In summary, our findings that \( ABCB7 \) up-regulation can restore erythroid growth and survival of RARS progenitors while decreasing \( FTMT \) expression support the essential role of \( ABCB7 \) in the phenotype of RARS.
7. CONCLUSION

- *ABCB7*, the mutated gene in hereditary XLSA/A, was identified as one of the most down-regulated genes in RARS. In contrast to normal bone marrow, *ABCB7* expression decreased during erythroid differentiation of RARS CD34+ cells. There was a significant inverse relation between *ABCB7* expression and the percentage of BM ring sideroblasts.

- Other down-regulated key genes in RARS erythroblasts included *MFN2, STAT5B, FANCC*, and the negative apoptosis regulator *MAP3K7*. Several genes involved in erythropoiesis were significantly over-expressed in RARS CD34+ cells but showed normal expression in erythroblasts. Deregulated pathways encompassed apoptosis, DNA damage repair, mitochondrial function and the JAK/Stat pathway.

- *ABCB7* is not mutated in acquired RARS. Neither *ABCB7*, nor other down-regulated key genes in RARS showed hypermethylation.

- G-CSF had no effect on the expression of iron-transport or erythropoiesis-associated genes. The mitochondrial pathway including *MFN2* was significantly modified by G-CSF, and several heat shock protein genes were up-regulated.

- *ABCB7* silencing in K562 cells reduced growth, erythroid differentiation and survival. In normal bone marrow, *ABCB7* down-regulation reduced erythroid colony growth, and induced erythroid apoptosis and a gene expression pattern similar to that observed in RARS day 7 erythroblasts. Importantly, down-regulation led to the accumulation of iron, in the form of mitochondrial ferritin (*FTMT*).

- *ABCB7* up-regulation potentiated erythroid differentiation in hemin-treated K562 cells, rescued erythroid colony growth of RARS CD34+ cells, and reverted gene expression, including *FTMT*, towards to normal range.

- High-quality transcriptome analysis at different time points during erythroid maturation added significant new information compared to conventional gene expression profiling. TLDA analysis confirmed the transcriptome data, further underlying that several of the most up-regulated genes in RARS are involved in the oxidative stress pathway.
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9. REFERENCES


