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Development of novel therapies for Diamond-Blackfan Anemia

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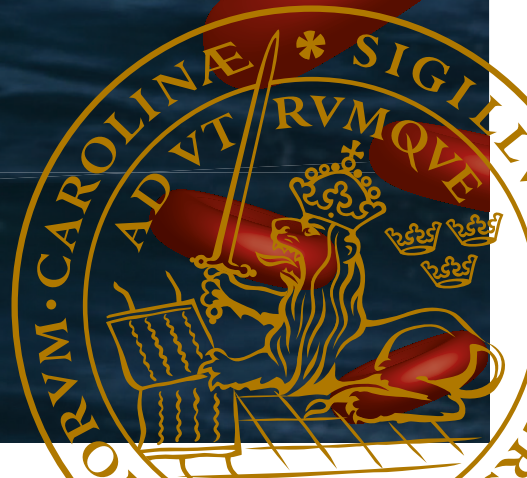
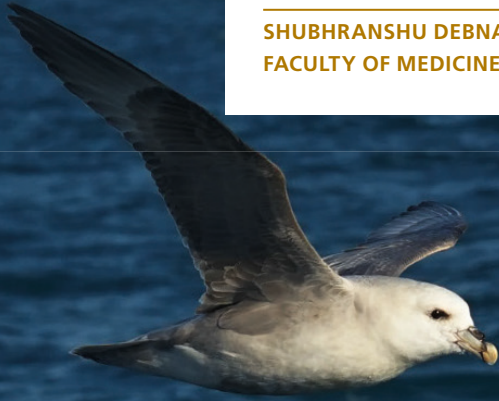
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Development of novel therapies for Diamond-Blackfan Anemia

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Shubhranshu Debnath



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DOCTORAL DISSERTATION

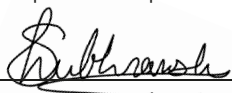
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Abstract Diamond-Blackfan anemia is a congenital erythroid hypoplasia manifesting early in life. In at least 60-70% of cases, DBA is caused by a functional haploinsufficiency of genes encoding for ribosomal proteins. Approximately, 25% percent of patients have mutations in the gene encoding ribosomal protein S19 (RPS19). The hematological profile of DBA patients shows macrocytic anemia with reticulocytopenia, normal or decreased levels of neutrophils and variable platelets counts. DBA patients also exhibit various non-hematological manifestations such as physical abnormalities and cancer predisposition. Corticosteroids are the main therapeutic option in DBA. Around 80% of the patients initially respond to corticosteroids, but only 40% of patients sustain the therapeutic response and the remaining 40% of patients need chronic blood transfusion. Twenty% of patients go into spontaneous remission and maintain an acceptable hemoglobin level without therapeutic intervention. The only curative treatment available for DBA patients is allogeneic bone marrow transplantation. This work focuses on understanding the disease pathogenesis and development of novel therapies for DBA. In Article-I we sought to understand the physiological relevance of the 5S RNP-Mdm2-p53 pathway for generation of the erythroid defect upon RPS19 deficiency. In Article-II we aimed to evaluate the therapeutic effect of the amino acid L-Leucine in the treatment of DBA. In Article-III and IV we examine the feasibility of RPS19 gene therapy in the treatment of RPS19 deficient Diamond Blackfan Anemia. In summary, this work focuses on basic and translational research towards evaluating novel therapies and understanding molecular mechanisms for DBA.		
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Development of novel therapies for Diamond-Blackfan Anemia

Understanding molecular mechanism and efficacy of
novel treatment in mice

Shubhranshu Debnath



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Illustration on cover: “*The hope for cell and mechanism based therapies for Diamond-blackfan anemia*”. The bird represents knowledge and information, flying towards red cell in a challenging environment. Photography by *Shubhanshu Debnath*. Graphic design by *Karolina Komorowska* and *Daniel Tornero*.

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To my family, friends, teachers and well wishers

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Abbreviations

4E-BP	Eukaryotic initiation factor 4E binding protein
ADA	Adenosine deaminase
AGM	Aorta-gonad-mesonephros
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
Baso EB	Basophilic erythroblast
BFU-E	Burst-forming unit-erythroid
BM	Bone marrow
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU-E	Colony-forming unit-erythroid
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DBA	Diamond-Blackfan anemia
eEF	Eukaryotic elongation factor
EFS	Elongation factor 1 α promoter short
eIF	Eukaryotic initiation factor
Epo	Erythropoietin
EpoR	Erythropoietin receptor
EB	Erythroblast
Env	Envelope glycoprotein
FA	Fanconi anemia

FACS	Fluorescence-activated cell sorting
FL	Fetal Liver
FOG1	Friend of GATA1
Gag	Group-specific antigen
GC	Glucocorticoid
GCR	Glucocorticoid receptor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-Macrophage colony-stimulating factor
GMP	Granulocyte macrophage progenitor
GVHD	Graft versus host disease
HIF-1 α	Hypoxia-induced factor 1 α
HIV-1	Human immunodeficiency virus 1
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem/progenitor cell
HDM2	Human double minute 2
IBMFS	Inherited bone marrow failure syndrome
IL	Interleukin
IRES	Internal ribosome entry site
JAK2	Janus kinase 2
KLF	Krüppel-like factor
KD	Knockdown
KO	Knockout
LCR	Locus control region
Lin	Lineage
LMO2	LIM-only transcription factor 2
LM-PCR	Ligation-mediated PCR
LSK	Lineage-negative Sca-1-positive
LTR	Long terminal repeat

Mdm2	Mouse double minute 2
MDS	Myelodysplastic syndrome
MEF	Mouse embryonic fibroblast
miRNA	Micro RNA
MPP	Multipotent progenitor
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
mTORC	Mechanistic target of rapamycin complex
NK	Natural killer cell
Ortho EB	Orthochromatic erythroblast
pA	Polyadenylation signal
PB	Peripheral blood
PI3K	Phosphoinositide-3-kinase
Pol	Polymerase/reverse transcriptase
Poly EB	Polychromatic erythroblast
Pre-rRNA	Precursor ribosomal RNA
Pro EB	Proerythroblast
Rev	Regulation of viral RNA-splicing and export
RP	Ribosomal protein
RRE	Rev-responsive element
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
S6K	Ribosomal protein S6 kinase
Sca-1	Stem cell antigen 1
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SCID-X1	X-linked severe combined immunodeficiency
shRNA	Short hairpin RNA
SIN	Self-inactivating

siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
SFFV	Spleen focus-forming virus
SLAM	Signaling lymphocytic activation molecule
STAT5	Signal transducer and activator of transcription 5
TIF-1A	Transcriptional initiation factor 1A
TOP	Terminal oligopyrimidine tract
Tpo	Thrombopoietin
tRNA	Transfer RNA
UTR	Untranslated region
VSV-G	Vesicular stomatitis virus glycoprotein

Articles included in the Thesis

- I. Jaako, P., **S. Debnath**, K. Olsson, Y. Zhang, J. Flygare, M. S. Lindstrom, D. Bryder and S. Karlsson (2015). "Disruption of the 5S RNP-Mdm2 interaction significantly improves the erythroid defect in a mouse model for Diamond-Blackfan anemia." *Leukemia* 29(11): 2221-2229.
- II. Jaako, P., **S. Debnath**, K. Olsson, D. Bryder, J. Flygare and S. Karlsson (2012). "Dietary L-leucine improves the anemia in a mouse model for Diamond-Blackfan anemia." *Blood* 120(11): 2225-2228.
- III. Jaako, P., **S. Debnath**, K. Olsson, U. Modlich, M. Rothe, A. Schambach, J. Flygare and S. Karlsson (2014). "Gene therapy cures the anemia and lethal bone marrow failure in a mouse model of RPS19-deficient Diamond-Blackfan anemia." *Haematologica* 99(12): 1792-1798.
- IV. **Debnath, S.**, P. Jaako, K. Siva, M. Rothe, J. Chen, M. Dahl, H. B. Gaspar, J. Flygare, A. Schambach and S. Karlsson (2017). "Lentiviral Vectors with Cellular Promoters Correct Anemia and Lethal Bone Marrow Failure in a Mouse Model for Diamond-Blackfan Anemia." *Mol Ther*.

Abstract

Diamond-Blackfan anemia is a congenital erythroid hypoplasia manifesting early in life. In at least 60-70% of cases, DBA is caused by a functional haploinsufficiency of genes encoding for ribosomal proteins. Approximately, 25% percent of patients have mutations in the gene encoding ribosomal protein S19 (RPS19). The hematological profile of DBA patients shows macrocytic anemia with reticulocytopenia, normal or decreased levels of neutrophils and variable platelets counts. DBA patients also exhibit various non-hematological manifestations such as physical abnormalities and cancer predisposition. Corticosteroids are the main therapeutic option in DBA. Around 80% of the patients initially respond to corticosteroids, but only 40% of patients sustain the therapeutic response and the remaining 40% of patients need chronic blood transfusion. Twenty% of patients go into spontaneous remission and maintain an acceptable hemoglobin level without therapeutic intervention. The only curative treatment available for DBA patients is allogeneic bone marrow transplantation.

This thesis focuses on understanding the disease pathogenesis and development of novel therapies for DBA. In Article-I we sought to understand the physiological relevance of the 5S RNP-Mdm2-p53 pathway for generation of the erythroid defect upon RPS19 deficiency. In Article-II we aimed to evaluate the therapeutic effect of the amino acid L-leucine in the treatment of DBA. In Article-III and IV we examine the feasibility of RPS19 gene therapy in the treatment of RPS19 deficient Diamond Blackfan Anemia.

In summary, this work focuses on basic and translational research towards evaluating novel therapies and understanding molecular mechanisms for DBA.

Hematopoiesis

Hematopoiesis (“To create blood” in Greek) is the continuous and dynamic process of blood formation in the body. Regarded as a liquid organ, the hematopoietic system provides all the necessary functions required for the survival of the individual. An adult human produces one trillion blood cells per day. The process of blood production occurs in the bone marrow (BM) of an adult and fetal liver (FL) in the embryo. A small group of highly-specialized cells known as the hematopoietic stem cells (HSC), have the ability to generate different types of blood cells through a complex multi-step differentiation process (Ogawa, Porter et al. 1983, Ogawa 1993).

Mature blood cells

Blood is mainly composed of liquid plasma and numerous different types of cells with highly specialized functions. Mature blood cells can be broadly classified by their functions of immune response, wound healing and transport of essential molecules. Erythrocytes (RBC; Red blood cells) facilitate the transport of oxygen to all organs and tissues in the body (Morera and MacKenzie 2011, Sender, Fuchs et al. 2016). Thrombocytes (Platelets) are essential for blood clotting (Heemskerk, Bevers et al. 2002). Leukocytes (WBC; White blood cells) make up the immune system, which can be further divided into innate and adaptive systems (Dempsey, Vaidya et al. 2003, Warrington, Watson et al. 2011). Granulocytes, macrophages and dendritic cells operate in the innate immune system. They recognize foreign substance and comprehensively mediate the immediate induction of inflammatory response as well as the process of phagocytosis of foreign entities (Warrington, Watson et al. 2011). In contrast to the innate immune system, the adaptive immune system involves the generation of highly effective response against specific substances. The adaptive immune response creates long lived memory cells that signal a rapid immune response upon re-exposure to specific substances. The cells of the adaptive immune system are known as lymphocytes and can be classified into T-lymphocytes (killers of infected cells) and B-lymphocytes (Producers of antibodies) (Warrington, Watson et al. 2011).

The hematopoietic hierarchy

Most blood cells have a relatively short lifespan and require continuous replacement in order to remain in constant numbers. Hematopoietic development is a hierarchical process. At the top of the hierarchy HSC represent a rare population of cells that can be defined by their capacity for self-renewal, multi lineage differentiation and long-term repopulation capacity of recipients that have been lethally irradiated (Morrison, Wandycz et al. 1997, Yang, Bryder et al. 2005) (Figure 1). In this hierarchical procedure HSC give rise to progenitor cells that progressively lose their self-renewal capacity, become restricted in lineage potential and undergo extensive proliferation. This process ultimately amplifies the cellular output originating from each HSC division.

Hematopoietic cells can be distinguished and isolated using Fluorescence-activated cell sorting (FACS) technology. This process makes use of fluorochrome-conjugated antibodies to label different populations of bone marrow cells depending on the presence or absence of cell surface proteins.

In mice HSC are found in a population that lack specific markers of lineage, positive expression of stem cell antigen-1 and positive expression of c-kit markers (LSK) (Ikuta and Weissman 1992, Okada, Nakauchi et al. 1992). The LSK population is heterogeneous in terms of their self-renewal potential and mainly comprised of multi potent progenitors (MPP). The HSC population can be further purified based on their lack of CD34 (Osawa, Hanada et al. 1996) and FLT3 expression (Adolfsson, Borge et al. 2001) or based on the expression of signaling lymphocyte activation molecule (SLAM) markers (Kiel, Yilmaz et al. 2005). The first lineage restriction occurs after MPPs and comprises the choice between common myeloid (CMP) and the common lymphoid (CLP) progenitor cells (Kondo, Weissman et al. 1997, Akashi, Traver et al. 2000). CLPs are the precursors of lymphocytes and give rise to B-lymphocytes, T-lymphocytes and NK-cells on further differentiations. CMPs differentiate into granulocytes-macrophages progenitor (GMP) and pre-megakaryocytes-erythroid (preMegE) progenitor cells (Akashi, Traver et al. 2000). The cells at the bottom of the hierarchy are mature blood cells (Orkin 2000, Weissman 2000).

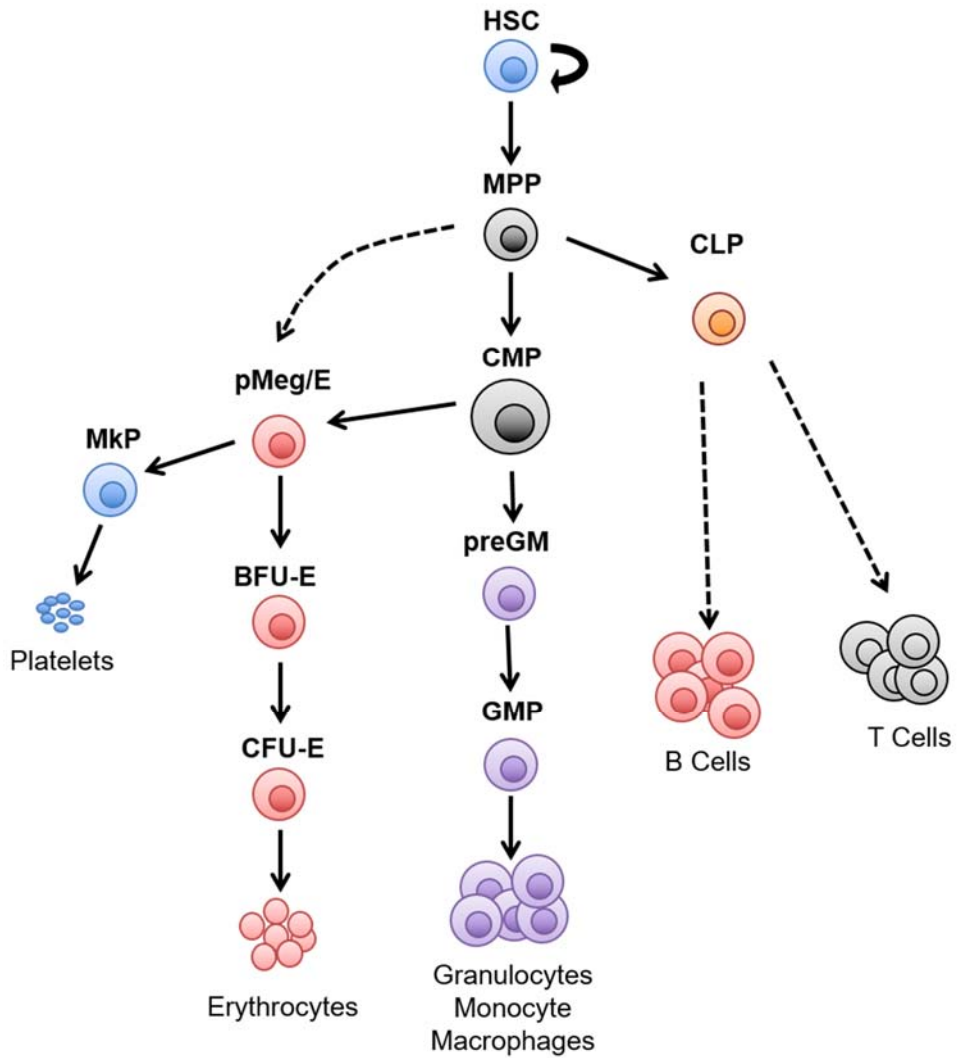


Figure 1. An overview of hematopoietic system. Hematopoiesis is a hierarchical process in which HSCs give rise to all mature blood cell types through a series of progressively lineage-restricted progenitor and precursor cells.

Erythropoiesis

Erythrocytes are the most abundant cell types found in blood. An average adult human has approximately 25 trillion red blood cells. Mature erythrocytes have a lifespan of 120 days in healthy humans and 55 days in mice to maintain the steady state environment. Approximately 2 million new erythrocytes are generated every second, this is a very high rate of cell generation and requires a continuous supply of iron for hemoglobin synthesis. The process of generation of erythrocytes (red blood cells) is known as erythropoiesis. Erythropoiesis for the most part takes place inside bone marrow of healthy adult humans. However, in order to maximize red blood cell production in cases of chronic stress or illness, erythropoiesis may take place in extramedullary locations such as the spleen and liver. During embryo development erythropoiesis occurs at different anatomical locations - it starts from the yolk sac then migrates to the aorta gonad mesonephros region (AGM), fetal liver and finally before birth to the BM (Mikkola and Orkin 2006, Palis, Malik et al. 2010).

The erythroid compartment

Similar to all other hematopoietic cells, erythrocytes also develop from HSC. The committed erythroid lineage consists of erythroid progenitors and precursor cells (Figure 2). Depending on their colony forming potential *in-vitro* erythroid progenitor cells can be divided into early and late progenitor cells. The first solely committed erythroid cells are the early progenitor cells known as burst-forming unit-erythroid (BFU-E) which also possess limited self-renewal capacity and generate enormous multi-clustered colonies (Testa 2004). The proliferation and survival of BFU-E is primarily based on SCF and IL3 signaling. The second solely committed erythroid progenitors are colony forming unit-erythroid (CFU-E). In comparison with BFU-E, CFU-E are more differentiated and erythropoietin (Epo) is alone adequate for proliferation and survival.

The CFU-E further differentiate into more definitive erythroid precursors. In the order of hierarchy, first recognizable precursor cells are proerythroblast, after undergoing further 3-5 cell divisions it give rise to basophilic, polychromatic and

orthochromatic erythroblast respectively (Daugas, Cande et al. 2001, Zermati, Garrido et al. 2001). During terminal erythroid differentiation, erythroblasts progressively undergo major morphological changes characterized by chromatin activation, proteolytic activation and cell size reductions. In the final maturation process orthochromatic erythroblasts undergo cell cycle arrest and the nucleus is extruded in order to form reticulocytes. Subsequently, reticulocytes lose mitochondria and ribosomes to give rise to mature erythrocytes (Testa 2004).

Mature erythrocytes have a lifespan of around 120 days in humans (around 55 days in mice) after which they undergo phagocytosis and are systematically removed by macrophages in the liver or spleen.

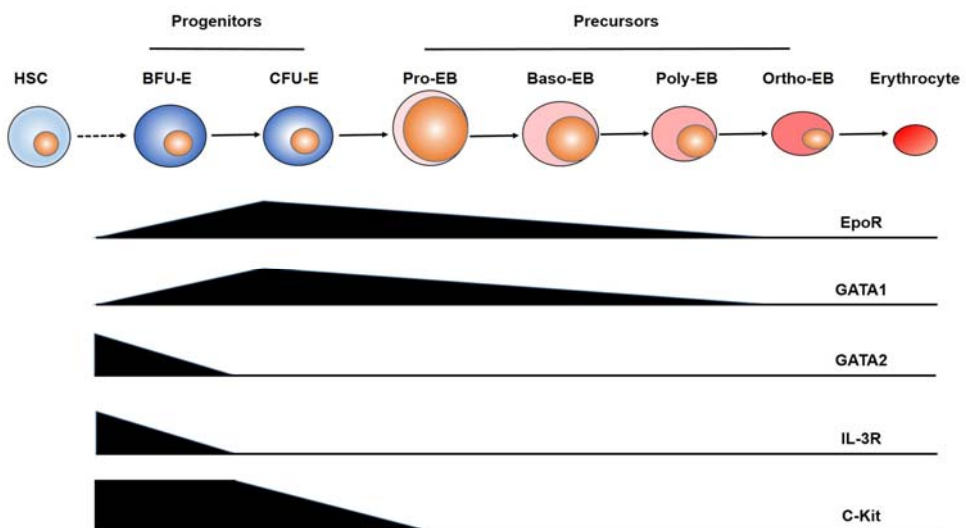


Figure 2. Schematic representation of erythroid differentiation. The committed erythroid lineage consists of erythroid progenitors and precursor cells. Based on their differential growth factor requirements and colony-forming capacity *in vitro* progenitor cells are distinguished, SCF and IL-3 signaling dependent BFU-E, and CFU-E progenitor cells, which are solely dependent on Epo. In divergence to the erythroid progenitor cells, erythroid precursor cells are recognized based on their morphology, reflected by accumulation of erythroid-specific proteins, decrease in size and nuclear condensation. GATA1 is the core transcription factor driving the terminal erythroid maturation.

Regulation of erythropoiesis

Extrinsic regulation

Erythropoiesis is a dynamic process and it is controlled by many key regulators. Erythropoietin (Epo) is the core enzyme involved in erythropoiesis and is synthesized by the kidney in response to hypoxia. Epo interacts with its receptor (EpoR) bearing cells, That is to say, all the cells in definitive erythropoiesis with the exception of reticulocytes expressed EpoR. Epo signaling is not necessary for erythroid lineage commitment or generation of CFU-E. It is however, required for CFU-E and later definitive progenitor survival (Koury and Bondurant 1990, Broudy, Lin et al. 1991, Wu, Liu et al. 1995)

During stress erythropoiesis, Epo collaborates with stem cell factor (SCF) and glucocorticoids (GC) to mobilize and distinguish into more mature erythroid cells (von Lindern, Zauner et al. 1999). SCF signaling facilitates proliferation and causes delay in erythroid maturation. Where Epo is required for erythroid maturation, both act synergistically to maximize blood production (Bakker, van Dijk et al. 2007). Epo and SCF activate common downstream signaling pathways such as PI3K. While Epo acts through the Jak2/STAT5 kinase pathway (Constantinescu, Ghaffari et al. 1999), it further activate anti apoptotic protein Bcl-XL (Socolovsky, Nam et al. 2001). Bcl-XL levels are increased in the erythroblast compartment, thus shielding the erythroblasts from apoptosis (Kieslinger, Woldman et al. 2000, Dolznig, Grebien et al. 2006). However, SCF acts with the help of its receptor c-kit which is highly expressed in both BFU-E and CFU-E (Wu, Klingmuller et al. 1995, Broudy, Lin et al. 1996). GCs are hormones that activate GC receptors (GCR). One of the essential transcriptional targets of GCR is c-Myb, which has been associated in the regulation of c-Kit expression (Ratajczak, Perrotti et al. 1998). Many of the downstream targets of GCR contain binding sites for hypoxia-induced factor 1 (Hif-1). Hif-1 implicates that all these factors act synergistically to promote erythroid progenitor self-renewal (Flygare, Rayon Estrada et al. 2011).

Transcriptional regulation

Erythroid lineage commitment is orchestrated by a series of transcription factors, mainly, GATA1 and GATA2. The targeted disruption of these factor blocks erythroid differentiation at different stages of maturation shown in mice (Cantor and Orkin 2002)

GATA1 is expressed at low levels in the MPP stage. Here, it drives the cells towards erythroid and megakaryocytic lineage commitments. GATA1 is later expressed in high quantities during definitive erythroid differentiation and helps in maturation (Zhang, Behre et al. 1999, Zhang, Zhang et al. 2000). It interacts and regulates EpoR, c-Kit, Bcl-XL, CD71 and as well as itself during BFU-E and CFU-E stages

(Zhao, Kitidis et al. 2006, Shimizu, Trainor et al. 2007). GATA1 interacts through proteins including Friend of GATA1 (FOG1) and Erythroid Krüppel-like factor (EKLF). GATA1 deficient mice are embryonic lethal due to failure of erythroid progenitors to mature beyond the erythroblast stage (Tsang, Fujiwara et al. 1998).

Another GATA family member, GATA2 is also significant for erythroid differentiation and it is expressed at high levels in HSPCs. GATA2-deficient mice are embryonic lethal due to an early hematopoietic defect characterized by the absence of practically all hematopoietic lineages (Tsai, Keller et al. 1994).

Inherited Bone Marrow Failure Syndromes

Inherited bone marrow failure syndromes (IBMFS) are rare genetic disorders that are characterized by aplastic anemia (failure of the bone marrow to produce mature blood cells). While the bone marrow is hypo-productive and causes cytopenia in all IBMFS, these syndromes are comprised of diverse groups of disorders and are traditionally classified according to the lineage affected. Some IBMFS, such as Fanconi anemia, usually develop pancytopenia. In others; for example Kostmann neutropenia or Diamond Blackfan anemia one lineage is mainly involved. The underlying cause of bone marrow failure is unknown in the majority of IBMFS cases. However, it can be triggered in a sub-group of patients as a result of environmental factors such as exposure to viruses, drugs and toxins (Jones 1976).

One third of the IBMFS that manifest early in life (congenital) are inherited and caused by alteration in genetic component resulting in bone marrow dysfunction (Shimamura and Alter 2010). Many congenital IBMFS are associated with a wide range of physical anomalies and predisposition towards cancers. Current advances in the field of genetics have resulted in a profound understanding of IBMFS pathophysiology. Remarkably, most of the genes altered or mutated in IBMFS encode for critical components of fundamental cellular processes such as ribosome biogenesis and assembly, oxidative and DNA damage repair, telomerase maintenance, etc. Additionally, as demonstrated in many recent studies, disruptions in fundamental cellular processes lead to the activation of p53 which acts as a primary cause for the hematological manifestations in IBMS.

Fanconi anemia

Fanconi anemia (FA) is a rare classical marrow failure disorder, typically inherited as an autosomal recessive manner with a prevalence of one case per hundred thousand child births. A cardinal feature of FA is the gradual onset of pancytopenia. The majority of patients show short stature and other physical abnormalities (Jones 1976). Predisposition towards cancer is quite high in Fanconi anemia patients; mostly hematologic malignancies such as AML, MDS and ALL (Kutler, Singh et al.

2003). So far, genetic analysis has identified seventeen FA associated genes. These genes play a crucial role in DNA damage repair pathways (Walden and Deans 2014). Allogenic bone marrow transplantation is the only curative therapy available for the hematological abnormalities in FA, where HLA matched sibling BM transplantation is preferred.

Dyskeratosis Congenita

Dyskeratosis congenita (DKC) is a rare disorder characterized by ectodermal abnormalities, bone marrow failure and an elevated risk of cancer. The initial hematological manifestation includes thrombocytopenia, anemia or both followed by pancytopenia in later stages. The DKC is caused by a mutation in a gene that encodes for vertebrate telomerase RNA component (*TERC*) or in genes associated with *TERC*. *TERC* is responsible for telomerase maintenance (Vulliamy, Marrone et al. 2001, Wong and Collins 2006, Gourronc, Robertson m et al. 2010)

Shwachman-Diamond syndrome

Shwachman-Diamond syndrome (SDS) is rare autosomal recessive disorder characterized by neutropenia, exocrine pancreatic dysfunction and bone marrow failure. SDS patients exhibits short stature, skeletal abnormalities and hepatic impairment. SDS is caused by mutation in the *SBDS* gene which is associated with mRNA transcription (Boocock, Morrison et al. 2003, Shammas, Menne et al. 2005).

Diamond Blackfan anemia

Diamond Blackfan anemia (DBA) is a rare inherited congenital bone marrow failure disorder with pure red blood cell aplasia manifesting early in life (Campagnoli, Garelli et al. 2004, Lipton 2006). DBA typically presents at 2-3 months of age and patients are most often diagnosed during their first year of life (Lipton 2006). However, in extreme rare cases DBA may present in adulthood (Willig, Niemeyer et al. 1999, Lipton 2006). The occurrence of DBA is estimated to be around 5-7 cases per million live births without biased sex ratio (Willig, Niemeyer et al. 1999).

Clinical features

The primary hematological manifestation at the time of diagnosis in DBA patients is macrocytic anemia with reticulocytopenia and selective absence of erythroid precursors in bone marrow (Diamond, Wang et al. 1976, Halperin and Freedman 1989). The bone marrow is otherwise normocellular, and myeloid and megakaryocytic lineages are mostly unchanged (Halperin and Freedman 1989). Additional clinical features include, elevated levels of fetal hemoglobin (hemoglobin F) and the vast majority of DBA patients exhibit elevated erythrocyte adenosine deaminase (eADA) activity (Gahr and Schroter 1982, Glader and Backer 1988, Vlachos and Muir 2010). Along with anemia, some patients show mild thrombocytopenia and neutropenia, with disease progression these become increasingly more common features (Giri, Kang et al. 2000).

Similar to other IBMFS, physical abnormalities have been reported in approximately 40-50% of the DBA patients (Halperin and Freedman 1989, Vlachos and Muir 2010). The majority of those with the defect exhibit short stature, craniofacial dysmorphisms, finger and upper limb defects as well as urogenital and heart defects (Vlachos, Ball et al. 2008, Vlachos and Muir 2010). With respect to the general population, DBA patients have 5.4 fold higher relative risk of developing malignancies (Vlachos and Muir 2010, Vlachos, Rosenberg et al. 2012).

Disease management

To maintain a sufficient level of hemoglobin the patients that do not respond to corticosteroids require chronic transfusion therapy (Lipton 2006). Complete correction of the hemoglobin level by transfusion is not advisable as it may prevent endogenous erythropoiesis. Iron chelation therapy must be combined with chronic transfusion therapy to prevent accumulation of iron in the liver, heart, kidneys and other vital organs. During the progression of the disease approximately 20% of the patients enter spontaneous remission with a physiologically acceptable hemoglobin level. The underlying mechanism behind remission is still elusive and relapse of the disease occurs in approximately 15% of the patients.

Allogenic bone marrow transplantation is the only curative treatment available for DBA. Young transfusion dependent patients (≤ 10 years) are normally considered if a healthy matched sibling donor is accessible (Lipton 2006, Vlachos, Ball et al. 2008).

Many other alternative therapies have been tested in the treatment of DBA. Few patients with no response to corticosteroids were sustainably treated with the immunosuppressant drug cyclosporine (Alessandri, Rogers et al. 2000, Bobey, Carcao et al. 2003). Treatment with recombinant human growth factors such as IL3 and Epo have been tested in patients, with no long-term benefits (Niemeyer, Baumgarten et al. 1991, Ball, Tchernia et al. 1995). Treatment using amino acid L-leucine or histone deacetylase inhibitor valporic acid also demonstrated complete remission in isolated cases (Jabr, Aoun et al. 2004, Pospisilova, Cmejlova et al. 2007).

A higher degree of fatalities in DBA are treatment-related and patients with corticosteroids have a significantly higher survival advantage over chronic transfusion dependent patients. Deaths are mainly the result of complications caused by iron overload and infection and are less due to bone marrow transplantation, leukemia or other cancers. The overall median survival for DBA patients is around 65 years.

Genetics aspects

The pathogenic mechanism in DBA has been associated with ribosomal biogenesis due to deficiency of ribosomal proteins. Mutation in genes encoding for ribosomal proteins have been identified in 60- 70% of all DBA patients (Cmejla, Cmejlova et al. 2007, Gazda, Sheen et al. 2008, Doherty, Sheen et al. 2010, Farrar, Vlachos et al. 2011, Gazda, Preti et al. 2012, Landowski, O'Donohue et al. 2013). Around 10

to 15 ribosomal protein genes have been associated with DBA pathogenesis. However the definitive role of these genes in disease pathogenesis is still elusive (Gazda, Sheen et al. 2008, Gazda, Preti et al. 2012). All reported mutations are heterozygous with autosomal dominant inheritance resulting in functional haploinsufficiency.

The most common gene mutated in DBA is the gene that encodes for Ribosomal protein S19 (RPS19). This gene accounted for twenty five percentage of the cases (Draptchinskaia, Gustavsson et al. 1999). Approximately 120 different mutations have been identified - most of these mutations resulted in no protein production.

Recent studies have discovered that two genes, erythroid transcriptional factor GATA1 and TSR2, a direct binding partner of RPS26, can cause the DBA phenotype (Sankaran, Ghazvinian et al. 2012, Gripp, Curry et al. 2014, Ludwig, Gazda et al. 2014). In spite of advances with genome wide exome sequencing, the underlying mechanism of pathogenesis in remaining DBA patients is still elusive. However it has been speculated that these patients may carry mutations in genes encoding for proteins which are associated with ribosomal biogenesis (Farrar, Vlachos et al. 2011, Gazda, Preti et al. 2012).

All the mutations associated with DBA genes caused premature termination, disruption in splicing mechanism or frame shifting, supporting functional haploinsufficiency as basis of DBA pathogenesis.

Pathophysiology in DBA

Hematopoietic defect in DBA

The BM from DBA patients is characterized by a nearly complete absence of erythroid precursors. The hematopoietic defect in DBA seems to be related to the intrinsic nature of HSCs and selectively limits their ability to differentiate towards the erythroid compartment. In the BM of young DBA patients erythroid precursors are generally present in normal number. This signifies that the main underlying defect resulted from compromised terminal erythroid differentiation (Lipton, Kudisch et al. 1986, Casadevall, Croisille et al. 1994). Clonogenic assay of DBA marrow cells typically shows a low number of BFU-E and CFU-E progenitors (Nathan, Clarke et al. 1978, Zivny, Jelinek et al. 2003, Flygare, Kiefer et al. 2005, Vlachos, Ball et al. 2008). The erythroid defect in DBA has been located at the onset of Epo-dependent terminal erythroid differentiation (Ohene-Abuakwa, Orfali et al. 2005).

Along with erythroid defects, some DBA patients develop neutropenia and thrombocytopenia over time. This gradually leads to hypocellular BM (Casadevall,

Croisille et al. 1994, Giri, Kang et al. 2000). Even though the frequency of HSC and progenitor cells appears to be normal, their capacity to proliferate is compromised when compared to healthy controls (Giri, Kang et al. 2000, Hamaguchi, Flygare et al. 2003, Flygare, Kiefer et al. 2005, Jaako, Flygare et al. 2011).

Role of p53 in DBA

As studies using down regulation of RPS19 and RPS14 in primary human BM cells show, the underlying erythroid defect is due to p53 activation (Dutt, Narla et al. 2011). It has been shown that rapidly dividing erythroblasts require a high rate of protein synthesis to produce large quantities of hemoglobin. However, in DBA, defective translational mechanism leads to severe cellular stress and leads to activation of the p53 pathway (Cmejlova, Dolezalova et al. 2006, Dutt, Narla et al. 2011). Recent studies by Jakko and co-workers have shown anemia and BM failure in RPS19 deficient mice are completely rescued in the p53 null background mice (Jaako, Flygare et al. 2011).

Ribosome Biogenesis

Ribosomes are the macromolecular machineries that are responsible for mRNA translation into proteins. The eukaryotic ribosome (80S ribosome) consists of two subunits, the large 60S subunit and small 40S subunit along with 46 ribosomal proteins. The large 60S subunit is made of 28S, 5.8S and 5S mature rRNAs and the small 40S subunit consist of 18S rRNA and 33 ribosomal proteins (Thomson, Ferreira-Cerca et al. 2013).

Ribosome assembly

The process of the assembly of the ribosome is known as ribosome biogenesis, which takes place in the nucleolus (Figure 3). Ribosome synthesis is one of the most energy-demanding cellular processes, which starts from active transcription of rRNA genes 18S, 5.8S and 28S by RNA polymerase I to form the 47S large precursor. The 5S precursor is transcribed by RNA polymerase III and imported to the nucleolus. The 47S pre-RNA then associates with ribosomal proteins to form 90S pre ribosome which later undergoes series of alteration wherein it transforms to 18S, 28S and 5.8S mature rRNAs (Hadjiolova, Nicoloso et al. 1993). The remodeling, facilitated by small nucleolar ribonucleoparticles (snoRNPs), involves endonucleolytic and exonucleolytic cleavage, modifications such as methylation and the uridinylation of approximately 200 nucleotides residue. (Hadjiolova, Nicoloso et al. 1993, Maden and Hughes 1997, O'Donohue, Choesmel et al. 2010, Thomson, Ferreira-Cerca et al. 2013).

Ribosomal proteins and small nucleoproteins facilitate ribosome processing, nuclear export and cytoplasmic maturation in a hierarchical manner. Most of the ribosomal proteins in mammals are encoded by single copy genes (Kenmochi, Ashworth et al. 1998). Ribosomal proteins are synthesized in huge quantities and are controlled mainly at the translational level (Lam, Lamond et al. 2007, Perry 2007)

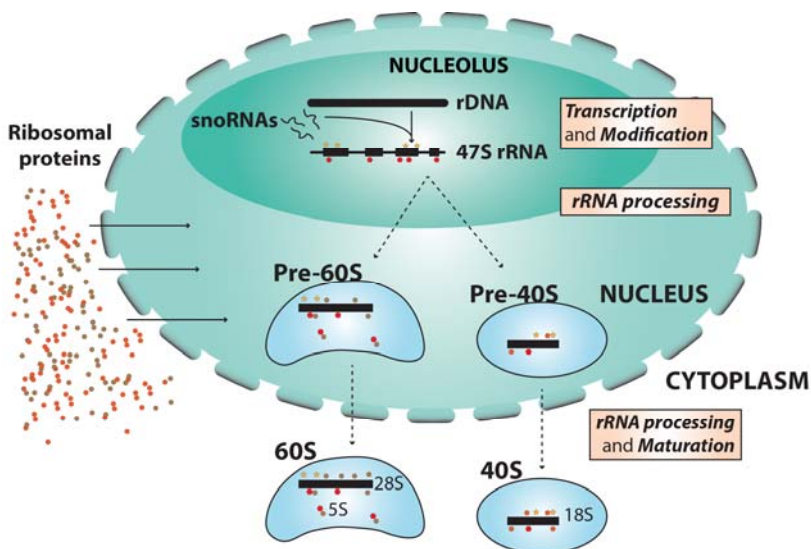


Figure 3. An overview of Ribosome biogenesis. During the process of ribosomal assembly, transcription of rDNA is carried out by RNA polymerase I to give rise 47S rRNA precursor, which associates with trans-acting factors that mediate a series of chemical modifications and nucleolytic cleavages. This results in the formation of pre-40S and pre-60S ribosomal subunits that are exported into the cytoplasm for the final maturation.

Regulation of ribosome biogenesis

Ribosome biogenesis is a major energy-dependent cellular process and requires extra cellular nutrients and growth factors while suppressing intracellular stress. The mechanistic target of rapamycin (mTOR) pathway is a key signaling pathway that regulates signals for ribosome biogenesis. mTOR is a serine/threonine protein kinase that belongs to the PI3K kinase family (Laplane and Sabatini 2012, Pan and Finkel 2017). mTOR assemble into two distinct protein complexes, mTORC1 and mTORC2. mTOR1 is the primary nutrient sensing complex, regulating ribosome biogenesis by ribosomal protein translation and rRNA gene transcription. This is accomplished primarily through phosphorylation of two downstream effector proteins, the ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor (eIF) 4E-binding protein (4E-BP).

Downstream of mTORC1, the protein 4E-BP binds and represses eIF4E. eIF4E is involved in cap-dependent translation. This is done by the phosphorylation of 4E-BP which leads to the dissociation of the eIF4E - 4E-BP complex, allowing eIF4E to bind cap-binding complex. On the other hand, the phosphorylation of S6K leads to multiple downstream signaling cascades including the transcriptional initiation

factor 1A (TIF-1A), RPS6 and eEF2. The TIF-1A is a co-factor necessary for the active functioning of RNA polymerase 1, thus directly promoting the expression of rRNA genes (Mayer and Grummt 2006, Sengupta, Peterson et al. 2010).

Ribosomal Stress

The tumor suppressor protein p53 is a preliminary member of the stress response mechanism. Stimulation of cellular stress activates the p53 which in turn starts a cascade of downstream signaling pathways leading to cell cycle arrest, senescence and apoptosis. In a steady state condition the p53 activity is regulated by Mdm2 (in humans Hdm2). In the absence of cellular stress, Mdm2 binds to p53 and direct it towards proteosomal degradation. However, cellular stress interrupt the interaction between Mdm2 and p53, consequently activating the p53 response.

During ribosomal stress it has been demonstrated that RPL11 has the ability to interact with Mdm2, subsequently inhibiting p53 proteosomal degradation (Lohrum, Ludwig et al. 2003, Zhang, Wolf et al. 2003) (Figure 4). It has been demonstrated that, by inhibition of rRNA synthesis by actinomycin D or serum starvation, RPL11-MDM2 interaction is enhanced (Bhat, Itahana et al. 2004). Several other studies also show that RPL5, RPL23 and RPS7 interact with Mdm2 in response to impaired ribosome biogenesis (Dai and Lu 2004, Dai, Zeng et al. 2004, Gilkes and Chen 2007)

Ribosomopathies

Ribosomopathies are disorders caused by alterations in the structure or function of ribosomal proteins, rRNA genes, or other genes associated in ribosome biogenesis. There are several known ribosomopathies such as Diamond Blackfan anemia, 5q myelodysplastic syndrome, X linked dyskeratosis congenita and Treacher Collins syndrome (Nakhoul, Ke et al. 2014).

5q-myelodysplastic syndrome

Myelodysplastic syndromes (MDS) is a heterogeneous group of clonal disorders characterized by dysplastic bone marrow and cytopenia. 5q-MDS is a subtype of MDS where part of the long arm of chromosome 5 has been deleted. It is characterized by macrocytic anemia, normal or elevated platelet counts, dysplastic megakaryocytes and an elevated risk of AML (Giagounidis, Germing et al. 2004). The deletion region of chromosome 5q encodes for forty different proteins

(Boulton, Fidler et al. 2002). Using short hairpin RNA (shRNA) to systematically target each gene in the deleted part Ebert and co-workers identified RPS14 as the critical gene for erythroid manifestation in MDS patients (Ebert, Pretz et al. 2008). Haploinsufficiency of RPS14 leads to defective rRNA biogenesis causing p53 activation (Ebert, Pretz et al. 2008, Dutt, Narla et al. 2011, Narla, Dutt et al. 2011)

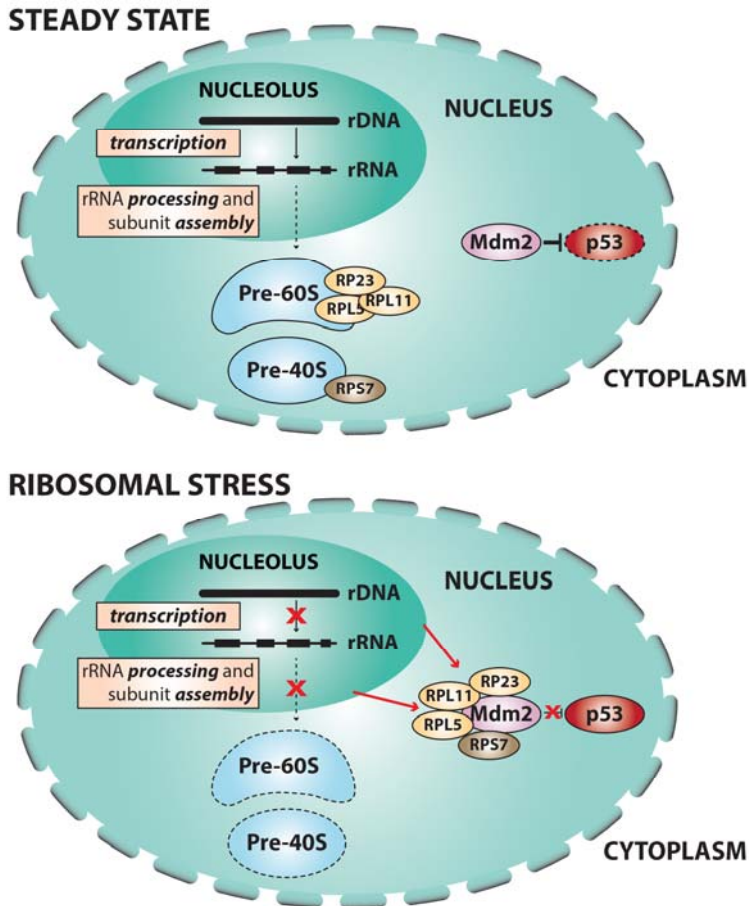


Figure 4. Schematic representation of Ribosomal stress. In the steady state condition, the levels of p53 are kept low by Mdm2, which binds and ubiquitinates p53. Subsequently, this leads to its proteosomal degradation. Impaired rRNA synthesis or processing leads to nuclear accumulation of free ribosomal proteins, which then bind to Mdm2 and inhibit its ubiquitin ligase function, resulting in the activation of p53.

Gene Therapy

The fundamentals of gene therapy encompass the rectification of a disease process by reinstating or amending cellular processes through the introduction of genetic information into target cells. Conversely, based on the rationale of replacing a defective gene with its functional counterpart in monogenic hematological disorders, gene therapy offers an alternative therapy option when there is no availability of a matching donor. The approach requires *ex vivo* gene correction of autologous HSCs and progenitor cells and subsequent re-infusion of the corrected cells (Figure 5). The long-term multi potentiality of HSCs and stable integration of therapeutic gene into the genome of target HSCs, allowing a constant expression of the therapeutic gene in HSCs and their progenitors and providing lifelong gene correction and restoration of normal biological processes of the patient's hematopoietic system.

Retroviral based vector systems

By employing the inherent property of retroviruses to reverse-transcribe their single-stranded RNA genome into double-stranded DNA (dsDNA) and insert the viral DNA into the host genome (Verma and Weitzman 2005), stable integration of an transgene can be achieved in the host cells making the viral DNA an inheritable part of the host cell. In order to replicate, retroviruses use the host cell's mechanism for transcription. All retroviruses contain three critical genes; *Gag*, *Pol* and *Env*, which encode for viral core protein, enzymes and envelop proteins, required for replication and assembly of the virus. In addition to these genes, the proviral DNA is made up of long terminal repeat sequences (LTR). The LTR is comprises of U3, R and U5 regions; the 5' U3 region act as viral promoter and enhancer required for transcription of viral DNA.

The retrovirus family can be divided into two subfamilies: Orthoretrovirinae and Spumaretrovirinae. The orthoretrovirinae subfamily consists of alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus and lentivirus (Gabriel, Schmidt et al. 2012). The inherent properties of retroviruses are used in

the production of viral vectors where majority of the genes encoding for viral replications are removed to produce replication defective vectors.

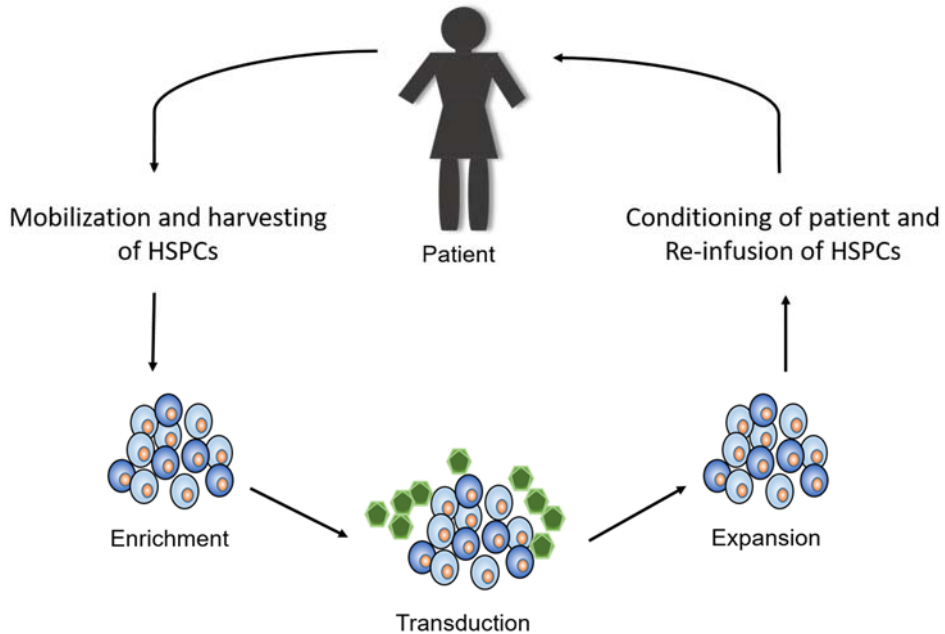


Figure 5. A simplified overview of ex vivo gene therapy for monogenic hematopoietic disorders. The process involves harvesting and enrichment of hematopoietic stem/progenitor cells (HSPCs). HSPCs are transduced with viral vectors and the patient is treated with a conditioning regimen (for example, chemotherapy) prior to re-infusion of transduced HSPCs.

Gammaretroviral and Lentiviral vector

The gammaretroviral vectors based on the murine leukemia virus have the ability to integrate into the host genome in a stable fashion. Replication-defective vectors are most commonly choice of virus capable of infecting their target cells and delivering their viral payload into the target cell. However, they fail to continue the typical lytic pathway that leads to cell lysis and death. The primary disadvantage to use gammaretroviruses is their requirement for target cells to be actively dividing as they are dependent on the disassembly of the nuclear membrane during cell division in order to reach the nucleus of host cells. As a consequence, quiescent cells are resistant to infection and transduction by gammaretroviruses. The

gammaretroviruses have been employed in most of the clinical trials for HSC gene transfer, although recently, lentiviral vectors are increasingly used in the clinic.

On the other hand, lentiviral vectors are based on the lentivirus and they have unique ability to integrate into the genome of quiescent cells along with the dividing cells. Members of the lentivirus are the immunodeficiency viruses such as human immunodeficiency virus1 (HIV-1). Gammaretroviral vectors along with lentiviral vectors have been significantly used as a research tool to study and investigate the hematopoietic system after gene marking.

Lentiviral vector construction

Lentiviral vectors are based on the backbone of the wild type human immunodeficiency virus (HIV-1) (Figure 6). HIV-1 genes have been divided into cis- and trans-acting gene sequences. The Trans acting genes were only needed to be expressed in a producer cell line and are packaged into three different plasmids, containing the *Gag/Pol* gene, the *Rev* gene and the *Env* gene. The cis expressing sequences will be integrated into the host genome and contain the transgene, packaging sequences, and rev-response element (RRE) necessary for mRNA nuclear export to the cytoplasm. It is also comprised of the woodchuck post-transcriptional regulatory element (wPRE) known to increase transgene expression and central polypurine tract (cPPT) to improve the PIC (ABBREVIATION) nuclear import efficiency (Schambach, Bohne et al. 2006). Additionally, the 3' and 5'UTR have been deleted and are denoted as self-inactivating (SIN) vectors. To minimize the risk of insertional activation of neighboring genes they also contain an internal promoter of choice (Miyoshi, Blomer et al. 1998, Zufferey, Dull et al. 1998). The four plasmid system is referred to as the third generation lentiviral system (Dull, Zufferey et al. 1998). To yield lentiviral particles, the plasmids are transfected into 293T cells. To allow broader target cell types vesicular stomatitis virus glycoprotein (VSV-G) Env is often used to pseudo-type viral particles (Ory, Neugeboren et al. 1996).

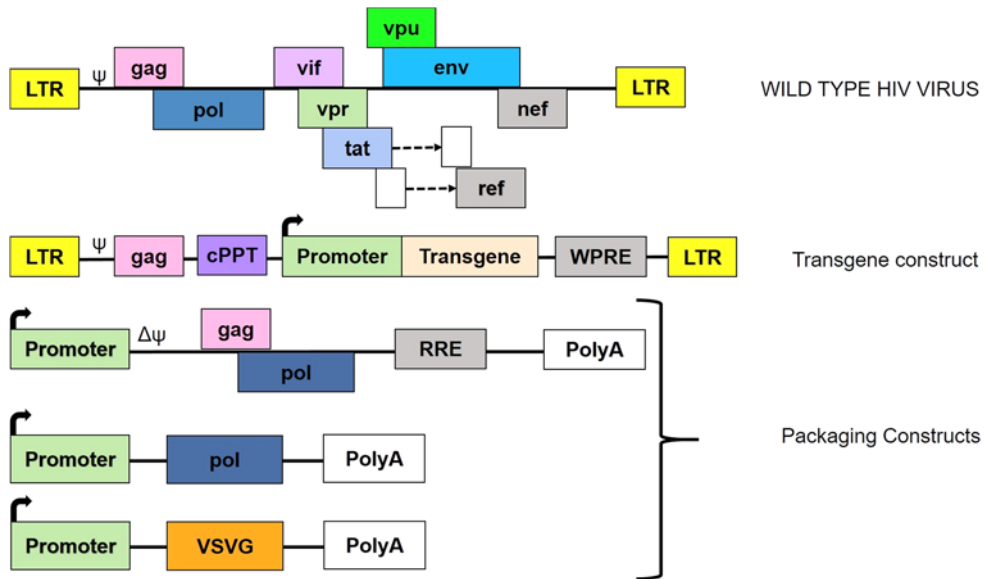


Figure 6. Lentiviral vector construction. Lentiviral vectors are based on the wild type human immunodeficiency virus 1 (HIV-1) backbone without any of the virulence genes.

Clinical gene Therapy for hematological disorders

Severe combined immunodeficiency syndrome (SCID) has been the primary clinical target disease in the field of gene therapy. SCID patients possess defects in differentiation and maturation of T-lymphocytes, B-lymphocytes and natural killer (NK) cells; the genetic variation in different types of SCID is well characterized making them suitable candidate for first clinical gene therapy trails (Qasim, Gaspar et al. 2009). Two patients were completely cured in the clinical trials for X-linked SCID (SCID-X1) in the year 2000 and this marked the first real success of gene therapy (Cavazzana-Calvo, Hacein-Bey et al. 2000). Successively, two patients with ADA-deficient SCID were also reported to be cured with gene therapy (Aiuti, Slavin et al. 2002). Regrettably, five patients out of twenty in SCID-X1 trials treated with gammaretroviral vectors developed T cell leukemia due to insertional mutagenesis. As a result, this has raised the safety concern of such vectors. Despite of preclinical trials predicted low risk of insertional mutagenesis (Kohn, Sadelain et al. 2003), in four out of five patients diagnosed with T cell leukemia, the integration of viral vector near the proto-oncogene *LMO2* was the genotoxic cause. Further studies conclude that *LMO2* seems to be the preferred site of gammaretroviral vectors (Hacein-Bey-Abina, von Kalle et al. 2003, Hacein-Bey-Abina, Von Kalle et al. 2003). So far 90 patients have been successfully treated with HSC gene therapy

(Aiuti, Cattaneo et al. 2009, Gaspar, Aiuti et al. 2009, Gaspar, Cooray et al. 2011, Gaspar, Cooray et al. 2011).

In comparison with gammaretroviral vectors, SIN lentiviral vectors show a potentially safer integration pattern (Modlich, Navarro et al. 2009). So far, SIN lentiviral vectors have been efficaciously used in the clinic for treating β -thalassemia, X-linked adrenoleukodystrophy (ALD) and Wiskott - Aldrich syndrome (Cartier, Hacein-Bey-Abina et al. 2009, Cavazzana-Calvo, Payen et al. 2010, Hacein-Bey Abina, Gaspar et al. 2015). To this point no adverse effects concerning genotoxicity have been identified using lentiviral vectors (Cartier, Hacein-Bey-Abina et al. 2009, Biffi, Bartolomae et al. 2011).

Extensive preclinical testing of vectors for efficacy of disease correction, together with integration site analysis are prerequisite before commencement of any clinical trials.

Hematopoietic stem cells in the clinic

The events that unfolded in the aftermath of the horrific nuclear bombing on Hiroshima and Nagasaki during the Second World War led to the important discovery of bone marrow as one of the highly radiation sensitive tissues. These findings resulted in the significant discovery that transplanting BM cells could revert the detrimental effects of lethal irradiation (Lorenz, Uphoff et al. 1951, Ford, Hamerton et al. 1956). The first bone marrow transplantation in the clinic was successfully performed in the year 1959 (Thomas, Lochte et al. 1959), despite the fact that it took more than a decade to completely understand the importance of human leukocyte antigen (HLA) matched donors (Thomas 2000). The seminal work performed by pioneering stem cell scientists Till, McCulloch and others shown that transplanted BM cells home to spleen and are also capable of multi lineage colonies in irradiated mice (Till and Mc 1961). These colonies were also capable of generating all blood lineage in secondary mouse recipients led to the concept of hematopoietic stem cell with capacity of self-renewal and differentiation into all other types of blood cells (Becker, Mc et al. 1963, Siminovitch, McCulloch et al. 1963). All these discoveries led to the establishment of HSC transplantation as a treatment method for treating hematopetic disorders.

Source of HSC

Hematopetic stem cells can be collected either from bone marrow or from peripheral blood (PB). The BM aspiration is done traditionally from the iliac crest, followed by a mononuclear cell count to assess the yield. HSPC can also harvested from PB although the yield is less than from BM. The HSPC harvest from PB causes less discomfort for the donor than the aspiration from the BM. To maximize the yield of HSPC in PB, HSPC are mobilized from BM by using the Granulocyte colony stimulating factor (G-CSF) or AMD3100 (Korbling and Anderlini 2001, Korbling and Freireich 2011, Motabi and DiPersio 2012)

HSC transplantation can be performed either by using the patient's own cells, known as autologous HSC transplantation, or by transplanting stem cells from a matched HLA donor. The success of *allogenic* BM transplantation from a genetically non-identical individual depends on the degree of HLA matching between donor and patient. The role of HLA molecules is to direct T-lymphocytes

response and are specific to each individual so the immune cells use HLA to distinguish self-cells from foreign one. In allogenic BM transplantation the graft rejection or *Graft-versus-host-disease* (GVHD) is a serious complication and this is due to the attack of transplanted donor cells that recognize the patient's cells as non-self and launch an immunological response against them (Copelan 2006). Surprisingly in hematological malignancies the graft attacked the remaining leukemic cells and can be beneficial to the patients, this phenomenon is denoted as *Graft-versus-leukemia effect* (GVL) (Kolb 2008).

Umbilical cord blood is a promising alternative source of HSPCs, with the more transient benefit of easy transplantation across HLA barriers (Rubinstein, Carrier et al. 1998). Umbilical cord-blood derived HSC has been successfully transplanted to children, however the number of HSC per blood unit is often too low for treating adult patients (Brunstein, Miller et al. 2011). Consequently, a major interest in stem cell research is to expand HSC *ex vivo* by understanding regulatory mechanisms with the aim to improving HSC based therapies.

Conditioning

Prior to the transplantation of HSC, patients need to undergo chemotherapy, total-body irradiation or a combination of both in a procedure called conditioning. The conditioning regime leads to the eradication of the patient's own hematopoietic and immune system resulting in cell apoptosis. Consequently, this paved the way for infused HSPCs to gradually re-establish hematopoiesis in patients. Cyclophosphamide and busulfan are two chemotherapeutic agents commonly used for conditioning. These inhibit DNA replication and induce apoptosis. For disorders such as hematopoietic malignancies where full restoration of genetic defect is required, conditioning helps in better engraftment of donor HSPCs by eliminating host HSPCs in BM and creating space for the transplanted cells (Quesenberry, Dooner et al. 2005, Bhattacharya, Ehrlich et al. 2008).

For hematopoietic disorders where there is the selective advantage of healthy or gene corrected cells, there is a shift from myeloablative to non-myeloablative conditioning. Non-myeloablative conditioning does not require complete ablation and is less toxic and has fewer side effects. The non-myeloablative conditioning approach has been effectively applied in gene therapy trials for ADA-SCID (Aiuti, Slavin et al. 2002, Gaspar, Bjorkegren et al. 2006)

Summary of Present studies

Article - I

Disruption of the 5S RNP-Mdm2 interaction significantly improves the erythroid defect in a mouse model for Diamond-Blackfan anemia.

Aim

To understand the physiological relevance of the 5S RNP-Mdm2-p53 pathway for generation of the erythroid defect upon RPS19 deficiency.

Summary

Diamond-Blackfan anemia (DBA) is a congenital erythroid hypoplasia caused by haploinsufficiency of genes encoding ribosomal proteins (RPs). Disturbed ribosome biogenesis in DBA leads to a p53-mediated ribosomal stress response. However, the mechanisms of p53 activation and its relevance for the erythroid defect remain vague. Previous studies have discovered that activation of p53 is triggered by the inhibition of mouse double minute 2 (Mdm2), the main negative regulator of p53, by the 5S ribonucleoprotein particle (RNP).

We asked whether this mechanism is solely mediated through the p53-dependent pathway in DBA. To study this question, we crossed our mouse model for RPS19-deficient DBA with Mdm2C305F knock-in mice that have a disrupted 5S RNP-Mdm2 interaction. Upon induction of the Rps19 deficiency, Mdm2C305F reduced the p53 response enhanced expansion of hematopoietic progenitors *in vitro*, and the anemia was improved. In the present study, we shown that the 5S RNP-Mdm2 interaction has a dominant role in mediating the p53 activation upon Rps19 deficiency and its disruption partially recovers the erythropoiesis of Rps19-deficient mice *in vivo*. Unexpectedly, we also found that the disruption of 5S RNP-Mdm2 interaction causes mild anemia by triggering a p53 signature in erythroid progenitor cells that led to a selective defect in erythropoiesis.

Our results indicate that, 5S RNP-Mdm2 disruption improves the reconstitution capacity of serially transplanted hematopoietic stem cells (HSCs), suggesting that

the 5S RNP-Mdm2-p53 pathway might play vital role in the functional decline of the hematopoietic system upon replicative stress in a cell-autonomous manner over time.

Article- II

Dietary L-leucine improves the anemia in a mouse model for Diamond-Blackfan anemia.

Aim

To evaluate the therapeutic effect of the amino acid L-leucine in the treatment of DBA.

Summary

RPS19 deficiency was induced by administering doxycycline containing food pellets. Simultaneously 1.5% (weight/Volume) L-leucine was administered in drinking water to double the concentration of L-leucine in serum. After two weeks of Doxycycline induction, mice treated with L-leucine exhibit significant increase in erythrocyte number and hemoglobin concentration compared to RPS19 deficient mice without L-leucine administration

However, no difference in stimulatory effect of L-leucine was observed in wild type mice on erythroid recovery upon sub-lethal irradiation or phenyl hydrazine-induced hemolytic anemia.

Immunophenotypic analysis of the various components of myeloerythroid compartment of untreated and L-leucine treated RPS19-deficient mice shows that L-leucine treatment decreases the frequencies of hematopoietic stem and progenitor cells. L-leucine treatment also reduced the frequencies of erythroblasts. However, the frequency of reticulocytes was similar in both L-leucine treated and untreated mice, which indicates that L-leucine enhances the differentiation potential of RPS19-deficient erythroid precursor into fully functional erythrocytes.

Our study shows that L-leucine treatment did not influence the activation of mTOR kinase by quantifying two downstream components of the pathway, phospho-RPS6 and phosphor-4E-BP1, in myeloid progenitors and erythroid precursor cell using flow cytometry. However, L-leucine treatment caused a reduction in the expression

of p53 transcriptional targets genes which suggested that the therapeutic response is due to reduction in p53 activity in erythroid and myeloid progenitors.

Article- III

Gene therapy cures the anemia and lethal bone marrow failure in a mouse model of RPS19-deficient Diamond-Blackfan anemia.

Aim

To evaluate proof-of-principle for the efficacy of RPS19 gene therapy in the treatment of RPS19 deficient Diamond Blackfan Anemia.

Summary

In this study, we assessed the therapeutic efficacy of gene therapy using a mouse model for RPS19-deficient DBA. Here, we designed lentiviral vectors harboring a codon-optimized human RPS19 cDNA driven by the spleen focus-forming virus (SFFV) promoter, followed by IRES and GFP (SFFV-RPS19). A similar vector without the RPS19 cDNA was used as a control (SFFV-GFP). To assess the therapeutic potential of the SFFV-RPS19 vector *in vivo*, transduced c-Kit enriched bone marrow cells from control and homozygous shRNA-D mice were injected into lethally irradiated wild-type mice. Based on the percentage of GFP-positive cells, transduction efficiencies varied between 40 % and 60 %. Three months after transplantation, recipient mice were administered doxycycline in order to induce Rps19 deficiency. After two weeks of doxycycline administration, the recipients transplanted with SFFV-RPS19 or SFFV-GFP wild type control cells showed normal blood cellularity. Remarkably, at the same time-point the recipients transplanted with the SFFV-GFP transduced homozygous shRNA-D bone marrow showed a dramatic decrease in blood cellularity that led to death, while the recipients transduced with SFFV-RPS19 shRNA-D bone marrow exhibited close to normal blood cellularity. These results demonstrate the potential of enforced expression of RPS19 to reverse the severe anemia and bone marrow failure in DBA. To assess the reconstitution advantage of transduced hematopoietic stem and progenitor cells with time, we performed similar experiments with heterozygous shRNA-D bone marrow cells. We monitored the percentage of GFP-positive myeloid cells in the peripheral blood, which provides a dynamic read-out for bone marrow activity. After four

months of doxycycline administration, the mean percentage of GFP-positive cells in the recipients with SFFV-RPS19 heterozygous shRNA-D bone marrow increased to 97 % of all the myeloid cells in the blood, while no similar advantage was observed in the heterozygous shRNA-D bone marrow recipients transduced with the SFFV-GFP vector. Consistently, SFFV-RPS19 conferred a reconstitution advantage over the non-transduced Rps19-deficient cells in the bone marrow. Furthermore, SFFV-RPS19 reversed the hypo-cellular bone marrow observed in the SFFV-GFP heterozygous shRNA-D recipients. Taken together, using mouse models for RPS19-deficient DBA, we demonstrate that enforced expression of RPS19 rescues the lethal bone marrow failure and confers a strong reconstitution advantage *in vivo*. These results provide a proof-of-principle for gene therapy for the treatment of RPS19-deficient DBA.

Article IV

Lentiviral Vectors with Cellular Promoters Correct Anemia and Lethal Bone Marrow Failure in a Mouse Model for Diamond-Blackfan Anemia.

Aim

To study the efficacy of RPS19 vectors driven by cellular promoters to cure RPS19-deficient DBA.

Summary

In this study, we demonstrate the efficacy of RPS19 lentiviral vectors using clinically relevant promoters to correct the lethal bone marrow failure in Rps19 deficient mice. In the current study, we decided to utilize ubiquitously expressed EFS promoter with or without the Locus Control Region (LCR) of the beta globin gene for treatment of RPS19-deficient DBA. Our data has shown that these vectors rescue the proliferation defect and erythroid development of transduced c-Kit⁺ Rps19 deficient bone marrow cells *in vitro*. The induction of Rps19 deficiency in mice which are homozygous for the shRNA D/D constructs generated lethal bone marrow failure. Remarkably, the bone marrow failure generated by shRNA D/D bone marrow was cured with EFS-RPS19. Since quite high levels of RPS19 are needed to correct the RPS19 deficiency by transgenesis we were concerned that the EFS promoter might not generate sufficient levels of RPS19 in erythroid progenitors to correct the anemia. Therefore, we included vectors containing the EFS plus the

beta globin locus control region. However, the findings show that the EFS promoter without the beta globin locus control region generates sufficient levels of RPS19 to cure the anemia and bone marrow failure in RPS19 deficient mice.

Additionally, we demonstrated that RPS19-deficient bone marrow cells can be transduced and these cells survived the transduction procedure and had the capacity to repopulate the bone marrow. However, most of the studies were performed with transduced shRNAD/D bone marrow and transplanted into normal recipients. The RPS19 deficiency was induced once the recipients had a stable graft. This is a justified since we have previously shown that the anemia and bone marrow failure in the induced mice is due to the deficiency in the hematopoietic cells and not due to a failure of the niche cells. If the recipients have Rps19 deficiency in all cells before transplantation of the transduced cells, some of the Rps19 deficient mice will not tolerate the combined toxicity of the doxycycline Rps19 downregulation and the radiation. However, the majority of the Rps19 deficient mice survived this procedure as mentioned above.

In this study, we have shown that our RPS19 deficient mouse model is a valuable and suitable model to test gene therapy using viral vectors with the RPS19 gene. In the mice used here, the haploinsufficiency is generated by RNAi which is induced postnatally. The haploinsufficiency in the mice generates most of the hematological symptoms found in DBA but not the physical abnormalities found in a large fraction of patients. The haploinsufficiency in the mice causes reduced proliferation and erythroid development, which can be corrected by RPS19 overexpression. The data presented shows that the RPS19 vectors increase the production of HSC and early progenitor cells after overexpression in RPS19 deficient hematopoietic cells.

Significantly, by designing a codon-optimized RPS19 cDNA, driven by the EFS promoter, we have succeeded in generating a clinically relevant vector system that allows high enough RPS19 expression for functional correction of the anemia and BM failure in Rps19-deficient mice. Our studies assessing the efficacy of clinically relevant EFS promoters show less likely risk to cause insertional oncogenesis. Further our studies using EFS-RPS19 or LCR.EFS-RPS19 vectors with these promoters are safer vectors that can generate sufficient RPS19 expression to correct the pathophysiology of Diamond Blackfan anemia. In normal cells, ribosomal protein production is a tightly regulated physiological and excess protein is subjected to proteasomal degradation. Because of this mechanistic regulation of ribosomal proteins, ectopic expression of RPS19 possesses a very low risk to promote uncontrolled growth. In our study, we did not observe any hematologic abnormalities due to enforced expression of RPS19. Our results collectively demonstrate the feasibility of clinical gene therapy to cure RPS19-deficient DBA.

Conclusions

Article I

- 5S RNP–Mdm2 interaction has a dominant role in mediating the p53 activation upon Rps19 deficiency.
- Disruption of 5S RNP–Mdm2 interaction causes mild anemia by triggering a p53 signature in erythroid progenitor cells
- 5S RNP-Mdm2-p53 pathway may play vital role in the functional decline of the hematopoietic system.

Article II

- Erythrocytes and Hemoglobin concentrations were significantly improved on L-leucine treatment in Rps19-deficient mice.
- L-leucine enhances the differentiation of Rps19-deficient erythroid precursors into fully functional erythrocytes.
- L-leucine treatment resulted in reduced expression of all analyzed p53 transcriptional target genes.

Article III and IV

- RPS19 deficient DBA can be cured with gene therapy.
- Proof of principle study with SFFV-RPS19 vector reversed the lethal anemia and bone marrow failure in RPS19 deficient mice.
- The SIN lentiviral vectors with EFS and LCR-EFS promoter along with codon optimized RPS19 efficiently reverses the bone marrow manifestation symptoms in RPS19 deficient mice.
- The SIN lentiviral vectors with EFS promoter employed in our study performed as predicted by the previously reported lentiviral studies, and can be considered as having a safe integration profile

General Discussion

The mouse model

Rps19 deficiency causes DBA with anemia and bone marrow failure. In order to study the molecular mechanism and therapeutic potential in DBA, we took advantage of our in house generated mouse model for RPS19-deficient DBA (Jaako, Flygare et al. 2011). The mouse models are engineered to contain a doxycycline-regulatable Rps19-targeting shRNA (shRNA-D) located downstream of the Collagen A1 locus allowing dose-dependent down regulation of Rps19 expression (Jaako, Flygare et al. 2011). Transgenic animals were bred to be either heterozygous or homozygous for the shRNA-D in order to generate two models with intermediate or severe Rps19 deficiency. RPS19 deficiency was induced by feeding the mice with doxycycline in the drinking water. Administration of doxycycline to homozygous transgenic Rps19 knockdown mice (DD) results in lethal anemia and rapid reduction in bone marrow cellularity. Heterozygous transgenic Rps19 knockdown mice (D+) show an initial reduction in red blood cell count. However, over time the mice recover or have considerably milder anemia.

Pathophysiological mechanism in DBA - “RP-Mdm2-p53” pathway

Studies conducted in primary human hematopoietic cells and animal model suggested that p53 activation may be an underlying cause of cell death and pathogenicity in DBA (Willig, Draptchinskaia et al. 1999, Danilova, Sakamoto et al. 2008, Dutt, Narla et al. 2011, Jaako, Flygare et al. 2011, Gazda, Preti et al. 2012). Our laboratory had previously demonstrated that the hematopoietic phenotype upon Rps19 deficiency is reversed in the absence of p53 (Jaako, Flygare et al. 2011). In our study Mdm2C305F mutated mice having wild-type p53, gave us the tool to address more accurately the influence of p53 on Rps19-deficient hematopoiesis

in lack of the physiological compensatory mechanisms present in the p53-deficient background.

Our *in vitro* studies shown an activation of the p53 pathway upon induction of Rps19 deficiency in c-Kit enriched HSPCs. The gradation of the p53 response was strictly reliant on the level of Rps19 downregulation.

Our results showed that the Mdm2C305F mutation caused a significant decrease in the level and activity of p53, irrespective of the degree of Rps19 downregulation resulting in a significant increase in proliferation, thus, demonstrating a prevailing role of the 5S RNP–Mdm2 interaction for p53 activation upon Rps19 deficiency. Our previous data showed that the most severe erythroid defects occurs in Rps19-deficient mice at the CFU-E stage (Jaako, Flygare et al. 2011). Together with previous findings our collective data indicate that both activation of p53 and disruption of the 5S RNP-Mdm2 resulted in defective erythropoiesis, to a large extent at the CFU-E level. Erythroid progenitor cells consequently appear sensitive to aberrations in p53 homeostasis mediated by the 5S RNP–Mdm2 interaction. Taken together, our study demonstrates the first physiological evidence that the 5S RNP-Mdm2-p53 pathway contributes to the anemia in DBA.

Therapeutic approaches for DBA

L-leucine

L-leucine is an essential branched chain amino acid that plays an significant role in the regulation of protein synthesis through the mTROC1 pathway (Stipanuk 2007). Pospisilova and co-workers reported one transfusion dependent patient who became transfusion free in response to L-leucine (Pospisilova, Cmejlova et al. 2007). It has also been reported that L-leucine improves the developmental defects and underline anemia in Rps19 and Rps14 deficient zebrafish models. In Article II we have shown that dietary administration of L-leucine improve the anemia in Rps19 deficient mice and this is similar to the findings from the zebrafish.

In mice treated with L-leucine both erythrocyte numbers and the hemoglobin concentration, were significantly improved. In addition, our findings also show that L-leucine had no effect on erythroid recovery in wild-type mice after sublethal irradiation or phenylhydrazine-induced hemolytic anemia. The hypocellular bone marrow in Rps19-deficient mice was significantly improved after L-leucine treatment. We have also shown that L-leucine administration led to diminished expression of known transcriptional targets of p53, signifying the reduced activity of p53 after L-leucine treatment.

In conclusion, our findings demonstrate that the administration of L-leucine improves the anemia in the mouse model for RPS19-deficient DBA, and the therapeutic effect is at least in part due to reduced p53 activity in hematopoietic progenitors. Our study thus supports the role of L-leucine as a possible therapeutic agent in the treatment of DBA.

Gene therapy

Our recent proof of principle study shows that the hematological manifestations in RPS19 mice could be cured by using lentiviral vectors harboring SFFV promoters for overexpressing codon optimized human RPS19 cDNA (Article III). In the steady state condition, ribosomal proteins are produced in excess quantities to the needs of the ribosome assembly. This excess protein is subject to proteasomal degradation. As a consequence of this physiological regulation, it is unlikely that the ectopic expression of RPS19 would promote uncontrolled growth. This conception is reinforced by findings from transgenic mice overexpressing the normal RPS19 cDNA in addition to the endogenous Rps19 gene (Devlin, Dacosta et al. 2010). In our gene therapy approach with the SFFV promoter to drive the transgene cassette, we did not find signs of evident insertional mutagenesis in our experiments. However, we cannot completely exclude that the use of a strong internal promoter, such as SFFV, may results in a residual risk factor.

To minimize the residual risk of insertional mutagenesis from strong internal promoter we assess the efficacy of RPS19 lentiviral vectors using clinically relevant elongation factor 1 α short (EFS) promoter with or without Locus control region of the beta-globin gene (LCR) in Rps19 deficient mice (Article IV). Our findings demonstrated that EFS-RPS19 vectors can produce sufficient transgene expression to cure the anemia and bone marrow failure in RPS19 deficient mice. We also demonstrated that RPS19-deficient bone marrow cells can be transduced and these cells survived the transduction procedure and had the capacity to repopulate the bone marrow. Our studies assessing the integration profile of clinically relevant lentiviral vectors with EFS promoters demonstrated reduced risk of insertional mutagenesis.

Our findings presented in article III and IV provide strong support for the use of gene therapy to cure RPS19-deficient DBA. We predict that the first gene therapy trials could be applied to transfusion-dependent patients. Taking gene therapy to the clinic will require careful assessment of risks-to-benefit ratio as well as long term follow up in these trials.

Future studies

Development of protocol for clinical gene therapy

Corticosteroids and blood transfusion are common therapeutic options for DBA. These therapies possess a high risk of therapy-related complications and are non-curative. Allogenic BM transplantation is the only curative therapeutic option available for DBA patients. With respect to findings from previous experiments, it was shown that RPS19 deficient mice exhibit HSC exhaustion which led to BM failure. Also, our findings show that RPS19 deficient BM cells exhibit less competitive repopulation capacity when transplanted into wild type recipient mice (Jaako, Flygare et al. 2011).

Based on our findings, the results suggest that a third generation lentiviral vector with codon optimized RPS19 driven by EFS promoter in a SIN lentiviral backbone will be effective for DBA clinical gene therapy. These vectors will be single gene vectors that lack the selection marker gene and IRES and can be pseudotyped with VSV-G. Additionally, large quantity production of clinical vectors will require Good manufacturing practice standard. In order to progress to clinical gene therapy future studies should include the efficacy of clinical vectors in DBA patient's CD34+ BM cells and evaluation of the efficacy and safety of ectopic expression in xenograft mice. Pre-clinical research will also involve optimization of the mouse model used in xenotransplantation. The vector will be then analyzed for efficiency of gene transfer and for integration site analysis and clonal proliferation to study its safety aspect. These will further clarify the potential for clinical gene therapy for treating DBA.

The clinical gene therapy trials will be offered to patients who are non-responsive to corticosteroids therapy and are blood transfusion dependent where a suitable HLA matched donor is not available for allogenic BM transplantation.

With reference to the findings, gene-corrected cells will have a competitive advantage over non-corrected cells. Therefore, it may be feasible to develop non-myeloablative conditioning regime together with gene therapy to treat DBA. Non-myeloablative conditioning does not require complete ablation and is less toxic and has fewer side effects than fully myeloablative conditioning. The non-myeloablative conditioning approach has been effectively applied in gene therapy trials for ADA-SCID (Aiuti, Slavin et al. 2002, Gaspar, Björkgren et al. 2006).

Alternative gene therapy strategies

New alternative methods for gene delivery have emerged in recent years, such as Zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR) associated 9 (Cas9) system. These are genetically engineered endonuclease with the capability to introduce site specific double stranded break (DSB), thereby offering potential control over the integration site over randomly integrated lentiviral/retroviral vectors. However, these gene correction tools require rigorous testing for safety and efficacy (Zhang and McCarty 2016). At this time, gene editing in HSC is not efficient enough to generate a therapeutic benefit. However, advances in gene editing technical development may allow this approach to be successful in the future for the treatment of genetic disorders.

Populärvetenskaplig sammanfattning

Hematopoes (“att skapa blod” på grekiska) är den kontinuerliga och dynamiska process av blodbildning i kroppen. Blod kan liknas vid ett flytande organ, där det hematopoietiska systemet förser blodet med alla nödvändiga funktioner för att trygga individens överlevnad. En vuxen människa producerar en biljon nya blodceller varje dag.

De flesta blodceller har en relativt kort livslängd och det krävs kontinuerlig ersättning av dessa för att kunna bibehålla ett konstant antal. Hematopoietisk utveckling är en hierarkisk process, på toppen av hierarkin finns de hematopoietiska blodstamcellerna (HSC) vilka utgör en sällsynt population av celler som definieras utifrån deras kapacitet att bilda kopior av sig själva samt skapa nya, specialiserade, blodceller. Blod består huvudsakligen av plasma och ett flertal olika celler med specialiserade funktioner. Mogna blodceller kan grovt beskrivas som att vara involverade i antingen immunförsvaret, sårläggning eller transport av viktiga molekyler.

Röda blodceller (erythrocyter) är den vanligaste celltypen som finns i blod. Hos vuxna människor finns ungefär 25 biljoner röda blodkroppar. Mogna erythrocyter har en livslängd på 120 dagar i friska, vuxna, människor och 55 dagar i möss. Varje sekund genereras ungefär 2 miljoner nya erythrocyter, detta är en mycket hög hastighet av cellnybildning och kräver kontinuerlig tillgång av järn för hemoglobinsyntes. Processen där erythrocyter bildas kallas erytropoes. Denna process sker främst inuti benmärgen hos friska, vuxna individer. Erytropoesen är påverkad vid kronisk stress och sjukdom. Vid sådana tillstånd sker erytropoes även i andra organ såsom mjälte och lever.

Diamond-Blackfan anemi (DBA) är en ärftlig sjukdom där så kallad benmärgssvikt uppträder tidigt i livet. DBA upptäcks ofta vid 2-3 månaders ålder och patienterna diagnosticeras vanligen under deras första levnadsår. I ovanliga fall uppträder symtomen först vid vuxen ålder. DBA är en sällsynt sjukdom som drabbar 5-7 personer av 1 miljon individer, både män och kvinnor drabbas i lika hög utsträckning. Patienterna föds frekvent med missbildningar i ansiktet och händerna och har ofta kortare kroppslängd. DBA-patienter har också högre risk att utveckla cancer under deras livstid. För behandling används i första hand kortison. Endast hälften av patienterna kan över tid fortsätta behandlas med kortison eftersom det uppträder en progressiv förlust av respons men även kraftiga bieffekter av

behandlingen. Dessa patienter blir i sin tur beroende av kroniska blodtransfusioner. Problemet är att järnförgiftning kan uppkomma vid kroniska blodtransfusioner och därför måste behandling ges för att minska ansamling av järn i levern, hjärtat, njurarna och andra vitala organ. Det enda sättet att bota DBA idag är att göra en så kallad allogen benmärgstransplantation. Det innebär att benmärgsceller från en immunkompatibel donator ges till patienten.

Sjukdomsmekanismerna bakom DBA har knutits/associerats till fel hos ribosomer, vilka är små enheter i cellerna som har till uppgift att producera proteiner. Mutationer i de proteiner som i sin tur bygger upp ribosomer har identifierats hos 60-70% av patienterna. Den vanligaste mutationen förekommer hos en gen som kodar för ribosomalt protein S19 (RPS19), ungefär en fjärdedel av DBA-patienterna har mutationer i denna gen.

Denna avhandling har som fokus att förstå sjukdomsopatogenesisen i DBA och utvecklingen av nya behandlingsmetoder för denna sjukdom. I artikel I var målet att förstå den fysiologiska relevansen av signaleringsvägen

5S RNP-Mdm2-p53 för den erytroida defekt som uppkommer vid brist på RPS19. Föregående studier har visat att aktiveringen av p53 triggas av inhiberingen av Mdm2, vilket i sin tur har till uppgift att inhibera p53 genom 5S RNP. I denna studie visar vi att 5S-RNP-Mdm2-interaktionen har en dominant roll vid aktiveringen av p53 vid Rps19-brist och att avbrytningen av denna aktivering partiellt återställer erytropoesen hos möss med Rps19-brist. Något oväntat upptäckte vi också att avbrytning av interaktionen mellan 5S RNP och Mdm2 orsakar mild anemi genom att aktivera en p53-signatur i erytroida celler vilket leder till en selektiv defekt i erytropoes.

I artikel II var målet att utvärdera den terapeutiska effekten av aminosyran L-leucin för behandling av DBA. Vår studie visar att L-leucin inte påverkar aktiveringen av mTOR. Detta gjordes genom att kvantifiera två komponenter nedströms i aktuell signaleringsväg i både myeloida och erytroida progenitorceller med hjälp av flödescytometri. Behandling med L-leucin orsakade en reduktion i transkriptionen av gener reglerade av p53, vilket tyder på att den terapeutiska responsen beror på reduktion i p53-aktivitet i såväl erytroida som myeloida progenitorceller.

I artikel III och IV undersöker vi genterapibehandling som möjlig framtida behandling av DBA orsakad av RPS19-brist. Genterapi innebär korrigering av sjukdomsprocessen genom att återställa eller ändra cellulära processer, vilket åstadkoms av att genetisk information introduceras i cellerna. Därmed erbjuder genterapi ett terapeutiskt alternativ för monogena hematologiska sjukdomar när det inte finns någon immunkompatibel benmärgsdonator. Metoden kräver genetisk korrektion av patientens egna stam- och progenitorceller utanför kroppen. Cellerna ges efter korrektion tillbaka till patienten genom transfusion. Eftersom stamceller

har den unika förmågan att dels bilda kopior av sig själva, men även ge upphov till mer specialiserade blodceller, och eftersom korrigeringen sker genom stabil integration av den terapeutiska genen i dessa celler, åstadkommer korrigeringen en livslång bot och upprättande av ett friskt blodsystem hos patienten.

I denna studie har vi visat att vår musmodell med RPS19-brist är en användbar och passande modell för att studera genterapi genom att använda virala vektorer med RPS19-genen. Våra resultat visar att konstant uttryck av RPS19 räddar mössen från att utveckla den annars letala benmargssvikten, samt bidrar till en stark tillväxt av de transplanterade cellerna. Dessa resultat ger så kallat proof-of-principle för genterapi som behandling av DBA orsakad av RPS19-brist.

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Last 6 years have been an exciting journey of my life. I spent this time studying the process of blood formation and its regulation. My work is a small fragment in understanding molecular mechanism and evaluating novel therapies for rare blood disorder- "Diamond-Blackfan anemia".

Apart from Science I also enjoy cooking, traveling, hiking and photography. Since it is all about getting insight, creativity, challenge and change. I hope you will get some insight into this rare disorder and enjoy your journey through this book.

Shubhranshu Debnath, Lund, September 2017



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