Modeling Diamond-Blackfan Anemia in the Mouse: Disease Pathogenesis and Evaluation of Novel Therapies

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2012

Citation for published version (APA):
Jaako, P. (2012). *Modeling Diamond-Blackfan Anemia in the Mouse: Disease Pathogenesis and Evaluation of Novel Therapies.* [Doctoral Thesis (compilation), Division of Molecular Medicine and Gene Therapy]. Division of Molecular Medicine and Gene Therapy.

Total number of authors:
1

Modeling Diamond-Blackfan anemia in the mouse

Disease pathogenesis and evaluation of novel therapies

Pekka Jaako

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ABBREVIATIONS

4E-BP	Eukaryotic initiation factor 4E binding protein
5-FU	5-flourouracil
AML	Acute myeloid leukemia
Baso EB	Basophilic erythroblast
BFU-E	Burst-forming unit-erythroid
CD	Cluster of differentiation
cDNA	Complementary DNA
CDR	Common deleted region
CFU-E	Colony-forming unit-erythroid
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
Cre	Cyclization recombination
DBA	Diamond-Blackfan anemia
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
eADA	Erythrocyte adenosine deaminase
eEF-2K	Eukaryotic elongation factor 2K
EF1α	Elongation factor 1α promoter
eIF	Eukaryotic initiation factor
EKLF	Erythroid Krüppel-like factor
Epo	Erythropoietin
EpoR	Erythropoietin receptor
EB	Erythroblast
Env	Envelope glycoprotein
ES	Embryonic stem

FACS	Fluorescence-activated cell sorting
FLI-1	Friend leukemia integration 1
Flp	Flippase
FOG1	Friend of GATA1
FRT	Flp recognition target
Gag	Group-specific antigen
GC	Glucocorticoid
GCR	Glucocoirticoid receptor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-Macrophage colony-stimulating factor
GMP	Granulocyte macrophage progenitor
HIF-1α	Hypoxia-induced factor 1α
HIV-1	Human immunodeficiency virus 1
HSC	Hematopoietic stem cell
HDM2	Human double minute 2
IBMFS	Inherited bone marrow failure syndrome
IL-3	Interleukin 3
IRES	Internal ribosome entry site
JAK2	Janus kinase 2
loxP	Locus of X-over of P1
LSK	Lineage-negative Sca-1-positive c-Kit-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 cell	Natural killer cell
Ortho EB	Orthochromatic erythroblast
pA	Polyadenylation signal
PGK	Phosphoglycerate kinase
PI3K	Phosphoinositide-3-kinase
Pol	Polymerase/reverse transcriptase
Poly EB	Polychromatic erythroblast
preGM	Pre granulocyte-macrophage
Pre-miRNA	Precursor micro RNA
Pre-rRNA	Precursor ribosomal RNA
preMegE	Pre megakaryocyte-erythroid
pri-miRNA	Primary micro RNA
Pro EB	Proerythroblast
Rev	Regulation of viral RNA-splicing and export
RNAi	RNA interference
RNPC	Ribonucleoprotein complex
RP	Ribosomal protein
RRE	Rev-responsive element
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
S6K	Ribosomal protein S6 kinase
SA	Splice acceptor
SD	Splice donor
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
TIRAP	Toll-interleukin-1 receptor domain-containing adaptor protein
TOP	Terminal oligopyrimidine tract
Tpo	Thrombopoietin
TRAF6	Tumor necrosis factor receptor-associated factor 6
tRNA	Transfer RNA
VSV-G	Vesicular stomatitis virus glycoprotein

ARTICLES INCLUDED IN THE THESIS

I. **Jaako P**, Flygare J, Olsson K, Quere R, Ehinger M, Henson A, Ellis S, Schambach A, Baum C, Richter J, Larsson J, Bryder D, Karlsson S. Mice with ribosomal protein S19 deficiency develop bone marrow failure and symptoms like patients with Diamond-Blackfan anemia. *Blood*. 2011;118(23):6087-6096.

II. **Jaako P**, Debnath S, Olsson K, Bryder D, Flygare J, Karlsson S. Dietary L-leucine improves the anemia in a mouse model for Diamond-Blackfan anemia. *Blood*. 2012;120(11):2225-2228.

III. **Jaako P**, Debnath S, Olsson K, Schambach A, Baum C, Flygare J, Karlsson S. Gene therapy corrects the anemia and lethal bone marrow failure in mouse model for RPS19-deficient Diamond-Blackfan anemia. *Manuscript*. 2012.

PREFACE

It has been quite a while since I started my graduate studies involving Diamond-Blackfan anemia (DBA), a rare blood disorder of newborn children characterized by a failure of bone marrow to produce red blood cells. At that time, ribosomal proteins S19 and S24 were the only identified DBA genes and their role in the pathogenesis of DBA was highly speculative. Today, five years later, there are at least nine confirmed DBA genes. Numerous animal models, including mice and zebrafish, have been generated and characterized, resulting in major advances in our understanding of DBA disease mechanisms. More important, these advances have provided rationale for novel diagnostic tools and therapeutic strategies that are currently being validated. It has been an exiting journey and I feel privileged to have been able to be a part of it.

The first part of this thesis gives an introduction to DBA and its two main elements, the hematopoietic system and the ribosome biogenesis. The second part summarizes the present studies and is followed by general discussion with the emphasis on therapeutic approaches.

I hope you enjoy reading this thesis!

HEMATOPOIESIS

Mature blood cells

Blood is composed of liquid (plasma) and several cell types with highly specialized functions. Red blood cells (erythrocytes) mediate the transport of oxygen to all tissues in the body. Platelets, small fragments derived from megakaryocytes, are essential for normal blood clotting. White blood cells form the immune system, which can be divided into innate and adaptive systems. Cells of the innate immune system, such as granulocytes, macrophages and dendritic cells, recognize foreign substances in a generic manner and mediate immediate responses involving the phagocytosis of foreign pathogens and induction of inflammatory responses. In contrast to the innate system, the adaptive immune system involves the generation of highly effective response against specific pathogens. Lymphocytes are the effector cells of the adaptive system and they function by directing immune responses and killing infected cells (T-lymphocytes), or by producing antibodies (B-lymphocytes). Importantly, the adaptive immune response generates long-lived memory cells that evoke a rapid and efficient immune response upon re-exposure to the specific pathogen.

The hematopoietic hierarchy

Most blood cells have a relatively short life span and need continuous replenishment to be maintained in constant numbers. Remarkably, humans are estimated to produce close to one trillion (10^{12}) blood cells a day (Ogawa, 1993). Furthermore, under stress conditions, such as bleeding or infection, the cellular output may be increased. Hematopoiesis, the formation of blood cells, takes place in the bone marrow and is maintained by the hematopoietic stem cells (HSC) that have the capacity to both self-renew and differentiate into all mature blood cells (Figure 1). In this hierarchical process HSCs give rise to progenitor cells that progressively loose their self-renewal ability and become restricted in terms of their lineage potential. Given the small number of HSCs, the differentiating progenitor and precursor cells undergo extensive proliferation, which ultimately amplifies the cellular output originating from each HSC division.
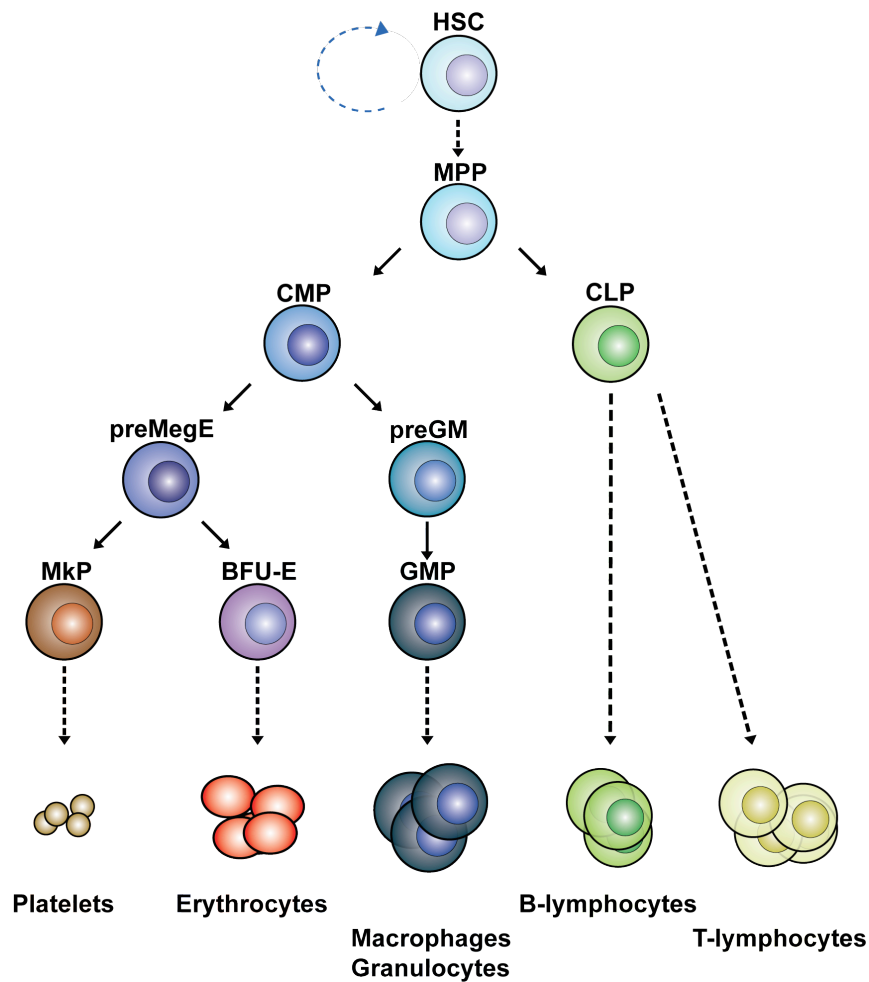
Figure 1. Schematic overview of the hematopoietic system. Hematopoiesis is a hierarchical process in which HSCs give rise to all mature blood cell types through a series of increasingly lineage-restricted progenitor and precursor cells.

Development of the fluorescence-activated cell sorting (FACS) technology has enabled the delineation of the hierarchical relationship of HSCs and progenitor cells. This technology is based on the use of fluorochrome-conjugated monoclonal antibodies to isolate subsets (populations) of bone marrow cells depending on the presence or absence of cell surface proteins. Prospectively isolated cell populations can be subjected to *in vivo* and *in vitro* assays for functional and molecular characterization. Establishing such a precise hierarchical relationship between the progenitor intermediates has provided a useful framework to study a variety of disease processes. In mice, all HSC activity resides in the lineage

marker-negative, Sca-1-positive and c-Kit-positive (LSK) population (Ikuta and Weissman, 1992; Okada, 1992). However, the LSK cells are still heterogeneous in terms of their self-renewal potential, being mostly multipotent progenitors (MPP), and HSC activity can be further enriched based on the expression of CD34 (Osawa, 1996) and Flt3 (Adolfsson, 2001), or the signaling lymphocytic activation molecule (SLAM) markers (Kiel, 2005). The first lineage restriction step occurs following the MPPs and involves the decision between the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) cells (Kondo, 1997; Akashi, 2000). CMPs give rise to the granulocyte-macrophage progenitor (GMP) cells through the pre-GM intermediate, and to the pre-megakaryocyte-erythroid (preMegE) progenitor cells (Pronk, 2007).

ERYTHROPOIESIS

The erythrocyte is the most common cell type in blood. Mature erythrocytes have a limited life span, approximately 120 days in humans and 40 days in mice, and they must be continuously produced in order to renew the red cell mass. During ontogeny, erythropoiesis occurs at distinct anatomical sites (the yolk sac, the fetal liver and the bone marrow), and the generated erythrocytes show distinguishable characteristics that reflect the gestation time and microenvironment (Mikkola and Orkin, 2006; Palis, 2010). In adult humans, erythropoiesis takes place mainly in the bone marrow. However, under chronic stress erythropoiesis may be expanded into extramedullary sites, such as the spleen and the liver, to maximize the production of erythrocytes.

The erythroid compartment

The erythroid lineage consists of erythroid progenitor and precursor cell compartments (Figure 2). Erythroid progenitor cells are relatively infrequent and can be divided into the early and late progenitor cells based on their colony-forming potential *in vitro*. The early progenitor cells (burst-forming unit-erythroid; BFU-E) are the first solely erythroid-restricted cells and give rise to large multi-clustered colonies (Testa, 2004). BFU-Es also possess a limited self-renewal capacity. The late progenitor cells (colony-forming unit-erythroid; CFU-E) give rise to smaller colonies compared to BFU-Es. In reality, BFU-E and CFU-E represent the two ends of a continuum with a hierarchical relationship, but these entities can be distinguished based on their differential growth factor requirements. The proliferation and survival of BFU-Es is mainly dependent on stem cell factor (SCF) and interleukin-3 (IL-3) signaling, while erythropoietin (Epo) alone is sufficient to support CFU-Es.

CFU-Es differentiate into morphologically distinguishable erythroid precursor cells. The first recognizable precursor, proerythroblast, undergoes 3-5 cell divisions giving rise to basophilic, polychromatic and orthochromatic erythroblasts. These differentiation divisions are characterized by a rapid G1 cell cycle phase, which results in a progressive decrease in the cell size (von Lindern, 2006). Simultaneously, maturing precursor cells undergo alterations in morphology that reflect the accumulation of erythroid-specific proteins and nuclear condensation. Orthochromatic erythroblasts withdraw from the cell cycle and form reticulocytes by extruding their nuclei. Reticulocytes loose their

mitochondria and ribosomes within a couple of days and mature into erythrocytes (Testa, 2004).
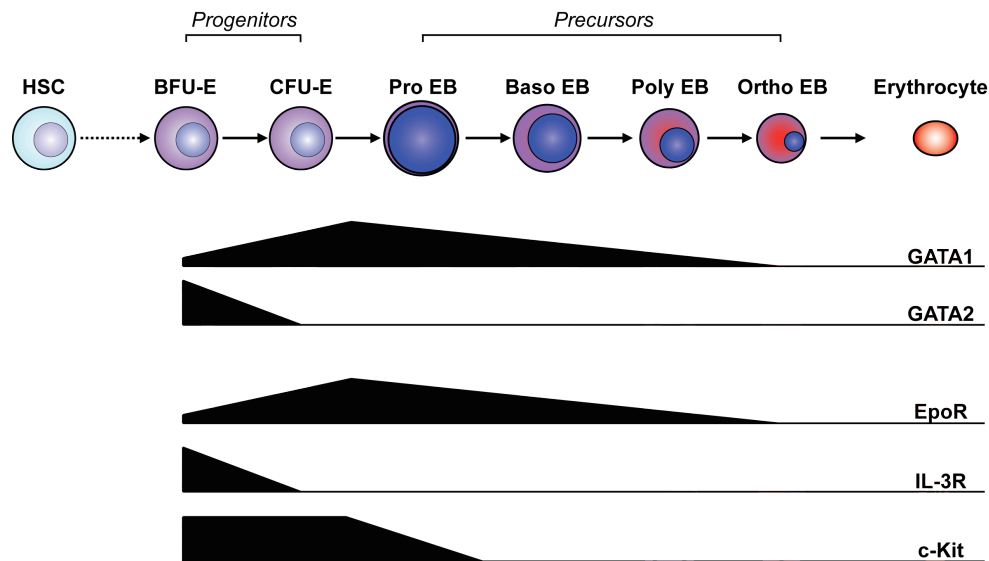
Figure 2 Outline of the erythroid differentiation. Cells of the erythroid lineage can be divided into erythroid progenitor and precursor cells. Erythroid progenitor cells are distinguished based on their differential growth factor requirements and colony-forming capacity *in vitro*. BFU-E progenitor cells are dependent on SCF and IL-3 signaling, while CFU-E progenitor cells are solely dependent on Epo. In contrast to the erythroid progenitor cells, erythroid precursor cells are recognized based on their morphology, which reflects the accumulation of erythroid-specific proteins, decrease in size and nuclear condensation. GATA1 is the main transcription factor driving the terminal erythroid maturation.

Cellular dynamics and extrinsic regulation

Erythropoiesis is a dynamic process that responds to the need for oxygen. The main hormone involved in the regulation of erythropoiesis is Epo. It is synthesized in the kidney in response to hypoxia, and it interacts with the cells bearing its receptors, EpoR, With the exception of reticulocytes, all cells of the erythroid lineage express EpoR, although the receptor density peaks at the CFU-E and proerythroblast stages (Broudy, 1991). Epo signaling is not required for the

commitment to erythroid lineage or the generation of CFU-Es, but it is essential for the survival of the CFU-E progenitor cells (Wu, 1995a; Koury, 1990). Epo signaling involves the homodimerization of EpoR, the activation of pre-bound cytoplasmic Jak2 kinase, phosphorylation of the cytoplasmic domain of EpoR and the subsequent activation of multiple downstream pathways (Constantinescu, 1999). The Jak2/Stat5 pathway appears to be exceptionally important in mediating the anti-apoptotic effect of Epo through the induction of Bcl-xL (Socolovsky, 1999). Furthermore, Dolznig *et al* demonstrated that the exogenous expression of Bcl-xL in primary murine erythroblasts allowed these cells to undergo terminal erythroid differentiation in the absence of cytokines (Dolznig, 2002).

Epo signaling is largely sufficient to fine-tune the erythrocyte production during normal erythropoiesis and to increase the erythroid output in response to hypoxia. However, severe stress response involves the expansion of the erythroid progenitor cell compartment, which results in increased influx of cells into the Epo-responsive stages. The flexibility between the progenitor self-renewal and differentiation is achieved mainly through the interplay between Epo, SCF and glucocorticosteroid (GC) hormone (von Lindern, 1999). SCF is the ligand for c-Kit receptor, which is expressed on a broad spectrum of hematopoietic stem and progenitor cells. Both BFU-E and CFU-E progenitor cells express high levels of c-Kit, and the expression declines at the proerythroblast stage. Disruption of SCF signaling impairs the erythroid recovery following stress (Broydy, 1996). Furthermore, c-Kit and EpoR have been shown to physically interact and cross-phosphorylate each other (Wu, 1995b). GCs are lipophilic hormones that act through binding and modulating the transcriptional activity of nuclear GC receptors (GCR). GCs induce erythroid progenitor self-renewal *in vitro*, and mice deficient for GCR exhibit normal erythropoiesis, but fail to increase erythrocyte production upon stress (von Lindern, 1999; Bauer, 1999). One of the important transcriptional targets of GCR is c-Myb, which has been implicated in the regulation of c-Kit expression (Ratajczak, 1998). Recently, Flygare *et al* demonstrated that many of the genes regulated by GCR contain binding sites for hypoxia-induced factor 1α (Hif-1α), and these factors mediated a synergistic effect on promoting the BFU-E self-renewal (Flygare, 2011).

Transcriptional regulation of erythropoiesis

Commitment of hematopoietic stem and progenitor cells to the erythroid lineage is orchestrated by a series of transcription factors. Targeted disruption of these factor in mice blocks the erythroid differentiation at distinct stages, providing information about their hierarchical relationship and requirement in the embryonic and definitive erythropoiesis (Cantor and Orkin, 2002).

GATA1 is one of the key transcription factors in erythroid and megakaryocytic development and its binding motifs are present in the transcriptional regulatory regions of virtually all erythroid genes. It is expressed at low levels in multipotent progenitor cells where it is involved in the megakaryocyte-erythroid lineage commitment, but later accumulates to high levels during the erythroid differentiation (Zhang, 1999; Zhang, 2000). Complete deficiency of GATA1 in mice results in embryonic lethality due to maturation block and apoptosis at the proerythroblast stage (Pevny, 1995; Fujiwara, 1996). Furthermore, the function of GATA1 in erythropoiesis is dose-dependent as the mice with a hypomorphic GATA1 regulatory mutation (~80 % reduction in messenger RNA; mRNA) show impaired erythroid maturation (McDevitt, 1997). GATA1-mediated erythroid-specific transcriptional program is modulated through critical protein-protein interactions in multiprotein complexes (Cantor and Orkin, 2002). Such interacting proteins include Friend of GATA1 (FOG1) and Erythroid Krüppel-like factor (EKLF). Similarly to GATA1, their targeted disruption results in embryonic lethality.

Another member of the GATA family, GATA2, is also important for erythroid differentiation. It is expressed at high levels in hematopoietic stem and progenitor cells, and GATA2-deficient mice exhibit an early hematopoietic defect characterized by the absence of virtually all hematopoietic lineages (Tsai, 1994).

INHERITED BONE MARROW FAILURE SYNDROMES

Bone marrow failure syndromes consist of diverse disorders characterized by the dysfunction of bone marrow to produce cells of one or more blood lineages. In the majority of patients the underlying cause for bone marrow failure is unknown, although in a subset of patients this can be associated with viral infections or exposure to physical and chemical toxins. However, in approximately one third of the pediatric marrow failure cases the disease is inherited involving a genetic component causing the bone marrow dysfunction (Shimamura and Alter, 2010). Inherited bone marrow failure syndromes (IBMFS) usually present in childhood and are associated with physical abnormalities and cancer predisposition (Table 1).

Recent progress in the genetics has revolutionized the understanding of IBMFS pathophysiology. Surprisingly, many of the genes mutated in these disorders encode components of fundamental cellular processes such as DNA damage repair, telomere maintenance and ribosome biogenesis. Furthermore, an accumulating body of evidence demonstrates that the disruption of these pathways leads to the activation of p53, which may underlie the primarily hematopoietic manifestation of these disorders.

Table 1. Inherited bone marrow failure syndromes.

Disease	Pathogenesis	Hematopoiesis	Other abnormalities	Cancer
Fanconi anemia	DNA damage repair Oxidative damage	Aplastic anemia	Short stature Skin pigmentation Upper limb and skeletal defects	AML Solid tumors
Diamond-Blackfan anemia	Ribosome biogenesis and function	Anemia	Short stature Craniofacial and upper limb defects	AML Sarcoma
Dyskeratosis congenita	Telomere maintenance rRNA modification	Aplastic anemia	Skin pigmentation Leukoplakia Nail dystrophy	AML Solid tumors
Shwachman-Diamond syndrome	Ribosome biogenesis?	Neutropenia	Pancreatic exocrine dysfunction Skeletal abnormalities Short stature	AML

DIAMOND-BLACKFAN ANEMIA

Clinical features

Diamond-Blackfan anemia (DBA) is a congenital disorder that manifests early in life. It classically presents at 2-3 months of age, and the majority of patients (~90 %) are diagnosed during their first year of life. However, in some rare cases DBA may present in adulthood (Willig, 1999; Lipton, 2006).

The main hematopoietic findings at presentation include macrocytic anemia, reticulocytopenia and selective absence of erythroid precursors in otherwise normocellular bone marrow (Diamond, 1976). Together with the early onset of symptoms (<1 year), these criteria have remained the accepted standard for DBA diagnosis. As a supporting hematological feature, the vast majority of patients have elevated erythrocyte adenosine deaminase (eADA) activity (Glader, 1983; Orfali, 2004). Elevated fetal hemoglobin is also often observed. In addition to anemia, some patients present with a modest neutropenia, thrombocytosis or thrombocytopenia (Willig, 1999). Furthermore, neutropenia and thrombocytopenia become increasingly common during the course of the disease (Giri, 2000).

Similarly to other IBMFS, physical defects and cancer predisposition are characteristic for DBA. Congenital abnormalities are present in approximately 40-50 % of the patients (Ball, 1996; Willig, 1999; Ramenghi, 1999; Lipton, 2006). Majority of these involve head and eyes, upper limbs, heart and genitourinary system. Furthermore, one third of cases show retarded growth. The relative risk of cancer in DBA is 5.4-fold higher compared with the general population (Vlachos, 2012). The types of malignancy include mainly solid tumors such as osteogenic sarcoma but also acute myeloid leukemia (AML) (Vlachos, 2012; Vlachos, 2008).

The differential diagnosis of DBA includes an array of acquired disorders, mainly transient erythroblastopenia of childhood (Vlachos, 2008).

Management and outcome

Corticosteroids form the main therapeutic regimen in DBA and approximately 80 % of the patients initially respond to this treatment. However, because of the progressive loss of response or unacceptable side effects, only half of these patients can be sustained on corticosteroids (Lipton, 2006). The remaining patients required chronic transfusion therapy to maintain sufficient hemoglobin levels that

allows for adequate growth and development, while not suppressing the endogenous red blood cell production. Chronic transfusion therapy must be combined with chelation to avoid the accumulation of iron in the liver, heart and other organs. Approximately 20 % of the patients enter spontaneous remission in which physiologically acceptable hemoglobin level is maintained without therapeutic interventions.

Allogeneic bone marrow transplantation is the only curative treatment for the hematopoietic manifestation of DBA, and it is normally considered among the young patients (<10 years) who are transfusion-dependent and have access to a matched sibling donor (Lipton, 2006; Vlachos, 2008). However, although matched sibling donor bone marrow transplantations have been reported with satisfactory results, transplantation using a matched alternative donor is associated with a poor outcome.

Numerous alternative therapies have been applied in the treatment of DBA. After becoming clinically available a number of recombinant human hematopoietic growth factors, such as IL-3 and Epo, have been tested in patients. However, no consistent benefit was observed in these studies (Niemeyer, 1991; Dunbar, 1991; Gillio, 1993; Olivieri, 1994). A few patients have shown a sustained response to the immunosuppressant cyclosporine (Alessandri, 2000; Bobey, 2003; Vlachos, 2008). Furthermore, several other immunomodulatory agents have been tested and found largely ineffective (Vlachos, 2008). Despite the early promise, only two patients showed a partial response to metoclopramide, a known prolactin-releasing drug, in a prospective study of 33 patients (Abkowitz, 2002; Leblanc, 2007). Finally, recent case reports demonstrated a complete remission in response to the histone deacetylase inhibitor valproic acid or amino acid L-leucine (Jabr, 2004; Jabr, 2006; Pospisilova, 2007).

With the current therapies, the overall survival at greater than 40 years is 75.1 % (Lipton, 2006). A high proportion of deaths are treatment-related and corticosteroid-responsive patients have a significant survival advantage compared to transfusion-dependent patients.

Inheritance and genetics

The incidence of DBA is estimated to be 5-7 cases per million live births without ethnic predilection or biased sex ratio (Ball, 1996; Willig, 1999; Ramenghi, 1999). Almost a half of DBA cases are familial and inherited as an autosomal dominant trait with variable penetrance (Orfali, 2004). Indeed, even family members who share a common genetic alteration may show dramatic variation in the severity of anemia and treatment response.

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Mutations in genes encoding ribosomal protein (RP) S19, RPS24, RPS17, RPL5, RPL11, RPS7, RPS10, RPS26 and RPL26 collectively explain the genetic basis for approximately 60-70 % of DBA cases (Draptchinskaia, 1999; Gazda, 2006; Cmejla, 2007; Farrar, 2008; Gazda, 2008; Doherty, 2010; Farrar, 2011; Gazda, 2012). Furthermore, alterations in additional RP genes have been identified in isolated patients, although the pathogenic significance of these rare variants is not clear (Gazda, 2008; Doherty, 2010; Gazda, 2012). All reported mutations are heterozygous, which is consistent with the dominant inheritance pattern.

Twenty-five percent of the patients have mutations in the gene coding for RPS19 making it the most common DBA gene. More than 120 unique alterations have been identified that can be divided into two classes (www.dbagenes.unito.it, accessed in July 2012; Boria, 2008). Mutations in the first class (nonsense mutations, altered splicing, small insertions and deletions) completely disrupt the expression of *RPS19* resulting in a haploinsufficient state in which the intact allele fails to compensate for the defective one. The second class includes missense mutations that interfere with the folding of RPS19 or its assembly into 40S ribosomal subunit, and thus result in a functional haploinsufficiency. Nearly all mutations in the other DBA genes are predicted to cause premature termination, splicing disruption or frame shifting, supporting functional haploinsufficiency as the basis for the disease pathology (Farrar and Dahl, 2011).

Recently, patients with *GATA1* mutations were identified in two unrelated families (Sankaran, 2012). However, the identification and phenotypic characterization of additional DBA patients with *GATA1* mutations will eventually determine whether these patients present true DBA or will be considered as a distinct clinical entity.

Pathophysiology of DBA

Characterization of the hematopoietic defect in DBA

The success of bone marrow transplantation and studies using cultured cells from patients demonstrate the cell intrinsic nature of the hematopoietic defect in DBA. Erythroid progenitor cells are usually present, often in normal numbers, in the marrow of young patients suggesting that the main erythroid failure of DBA results from impaired terminal differentiation of erythroid progenitor cells rather than from their absence (Lipton, 1986; Casadevall, 1994). Consistent with these studies, Ohene-Abuakwa *et al* used a liquid erythroid culture system in order to locate the erythroid defect at the onset of Epo-dependent terminal erythroid differentiation (Ohene-Abuakwa, 2005).

Some patients develop hypocellular bone marrow over time and this is often associated with neutropenia and thrombocytopenia (Giri, 2000). Consistently, age-related decrease in the number of erythroid progenitors and GMPs has been reported (Casadevall, 1994). Although the frequency of immature hematopoietic stem and progenitor cells in patients appears normal, their proliferative capacity is significantly lower compared to controls (Giri, 2000; Hamaguchi, 2003). These findings suggest that the hematopoietic defect in DBA involves hematopoietic progenitors or even HSCs resulting in bone marrow failure.

DBA model systems

Generation of animal models for DBA is pivotal in order to understand the disease pathophysiology and to evaluate novel therapies. Following the identification of *RPS19* as the first DBA gene, Mattson *et al* created a mouse model with targeted disruption of *Rps19* (Mattson, 2004). However, while the homozygous loss of *Rps19* resulted in embryonic lethality, heterozygous mice exhibited no phenotypic abnormalities including normal hematopoiesis. Furthermore, the expression of Rps19 was similar to controls suggesting that these mice, all derived from a single targeted embryonic stem (ES) cell clone, had acquired a way to compensate for the lack of one functional *Rps19* allele (Matsson, 2004; Matsson, 2006).

In 2005, two independent groups generated cellular models for RPS19-deficient DBA by taking advantage of RNA interference (RNAi). Flygare *et al* demonstrated that the downregulation of RPS19 in human CD34-positive hematopoietic cells significantly impaired the erythroid proliferation and differentiation (Flygare, 2005). Furthermore, the severity of the defect correlated with the level of *RPS19* knockdown and was rescued by the overexpression of *RPS19*. Ebert *et al* reported similar results and additionally showed that the defect in erythroid differentiation could be restored by dexamethasone treatment (Ebert, 2005). Finally, Miyake *et al* created hematopoietic cell lines with inducible downregulation of RPS19 (Miyake, 2005).

In 2008, McGowan *et al* reported a novel mouse model for RPS19-deficient DBA that presents a missense mutation resulting in a single amino acid substitution in the Rps19 protein (McGowan, 2008). Similarly to the *Rps19* knockout model discussed above, this mutation was embryonic lethal when homozygous. However, the heterozygous mice exhibited dark skin, retarded growth and a mild reduction in erythrocyte number. Importantly, all of these features were rescued in a p53-deficient background. In addition to the mouse models, two groups generated of rps19-deficient zebrafish models using morpholino technology (Danilova, 2008; Uechi, 2008). Similar to patients, these models showed developmental and hematological abnormalities. Furthermore, the loss of p53 completely rescued the

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phenotypic abnormalities observed upon rps19 haploinsufficiency (Danilova, 2008).

To test the theory that some *RPS19* missense mutations cause DBA by a dominant-negative mechanism, Devlin *et al* generated transgenic mice expressing a common pathogenic *RPS19* mutation (*RPS19R62W*) from a ubiquitous promoter (Devlin, 2010). Constitutive expression of *RPS19R62W* was lethal during early embryonic development. However, conditional expression of *RPS19R62W*, bypassing the embryonic lethality, resulted in a mild macrocytic anemia, reticulocytopenia and decreased numbers of erythroid progenitor cells in the bone marrow. As the expression of endogenous *Rps19* was intact in these mice, this study demonstrates the dominant negative nature of this mutation.

Other mouse models relevant to DBA

Identification of multiple RP genes as disease genes strongly suggests DBA as a disorder of ribosome biogenesis or function, and underscores the rationale for studying animal models with mutations in additional ribosomal proteins. A lot of our understanding of impaired ribosome functions *in vivo* originates from the studies using the conditional *Rps6* knockout mouse model generated in the Thomas laboratory. In 2000, Volarević *et al* demonstrated that the livers of fasting Rps6-deficient mice grew normally in response to nutrients, but failed to proliferate in order to recover from a partial hepatectomy (Volarević, 2000). The phenotype was associated with a distinct ribosomal RNA (rRNA) processing defect and abnormal G1/S cell cycle progression. In a following study, Sulic *et al* deleted *Rps6* selectively in T-lymphocytes (Sulic, 2005). While no thymus could be recovered after the complete deletion of *Rps6*, the heterozygous mice showed normal thymus cellularity, but the T-lymphocytes failed to proliferate in response to T-lymphocyte receptor stimulation. Both *in vivo* and *in vitro* phenotypes were rescued upon the loss of p53, providing evidence for p53 as a sensor for aberrant ribosome biogenesis. Finally, comprehensive characterization of the hematopoietic system of Rps6-deficient mice revealed a macrocytic anemia, thrombocytosis and leukocytopenia (McGowan, 2011; Keel 2012). Bone marrow analysis of these mice revealed a profound reduction in total cellularity together with a relative decrease in the number HSCs, CFU-Es and erythroid precursor cells. Furthermore, abnormalities in peripheral blood and bone marrow were reversed in the p53-null background (McGowan, 2011).

Barlow *et al* generated a mouse model for 5q- syndrome by deleting the *Cd74-Nid67* region in the mouse chromosome 18 that is syntenic with a region within the 5q- common deleted region (CDR) in human and contains eight known genes, including *Rps14* (Barlow, 2010). Heterozygous deletion of the *Cd74-Nid67* region

resulted in macrocytic anemia, modest thrombocytopenia and reduced number of granulocytes. Examination of the bone marrow revealed a 50-60 % reduction in total cellularity with depressed hematopoietic stem and progenitor cell numbers, and erythroid dysplasia. The loss of p53 normalized the stem and progenitor cell defects, erythroid dysplasia, macrocytosis and thrombocytopenia.

Role of p53 in the erythroid failure of DBA

As the studies using DBA animal models demonstrate an activation of p53 in response to ribosomal protein deficiencies, it is tempting to speculate that the erythroid failure of DBA is caused through p53-dependent mechanisms. Recently, downregulation of RPS19 or RPS14 in primary human bone marrow cells was shown to result in the erythroid-pronounced activation of p53 (Dutt, 2010). Furthermore, the treatment of bone marrow cells with nutlin-3, a compound that activates p53 by preventing its interaction with human double minute 2 (HDM2; Mdm2 in mice and hereafter), led to an erythroid-biased activation of p53. Finally, inhibition of p53 with a small molecule pifithrin alpha rescued the erythroid defect in RPS19-deficient and RPS14-deficient human bone marrow cell cultures. Immunohistochemistry for p53 in the bone marrow biopsies from DBA patients demonstrated elevated levels of p53, although variation was observed in terms of the intensity and cell type-specificity of p53 staining (Dutt, 2010).

Translational defects

Ribosomal protein haploinsufficiency has been shown to result in reduced rate of protein synthesis (Cmejlova, 2006). However, whether the global reduction in translation contributes to the severe anemia of DBA is not known. Studies on mice deficient for Flvcr, a heme exporter protein, have led to a hypothesis that defective globin synthesis contributes to the erythroid defect of DBA (Keel, 2008). Conditional deletion of *Flvcr* leads to a severe macrocytic anemia with reticulocytopenia. Furthermore, Flvcr-deficient embryos fail to develop properly showing defective growth, craniofacial and limb abnormalities, and a block in erythroid differentiation at the CFU-E – proerythroblast stage. These findings suggest that the accumulation of free heme in proerythroblasts is toxic, raising a hypothesis that the dysregulation of heme synthesis and globin translation, resulting in a transient excess of free heme, could explain the erythroid defect of DBA. Additionally, Rey *et al* suggested the presence of abnormal *FLVCR1* splice-variants coding for functionally impaired proteins in patient cells (Rey, 2008).

Recently, Horos *et al* suggested that impaired internal ribosome entry site (IRES)-mediated translation contributes to the erythroid defect of DBA (Horos, 2011).

The authors identified a set of mRNAs, including *Bag1* and *Csde1*, with reduced polysomal recruitment and translation in Rps19-deficient and Rpl11-deficient mouse erythroblasts. This finding is interesting given the defect in IRES-dependent translation in a mouse model for X-linked dyskeratosis congenita, caused by a hypomorphic mutation in the *Dkc1* gene encoding pseudouridine synthase that is involved in rRNA modification (Yoon, 2006).

Other ribosomopathies: the 5q- syndrome

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal disorders characterized by dysplastic bone marrow and peripheral cytopenia. The 5q- syndrome is a distinct subtype of MDS, defined by an isolated interstitial deletion of chromosome 5q, and is characterized by macrocytic anemia, normal or elevated platelet counts, dysplastic megakaryocytes and elevated risk of AML (Giagounidis, 2004). Most patients respond to the treatment with lenalidomide, resulting in reduced transfusion requirement that is often combined with a complete cytogenetic response (List, 2006).

The 5q- CDR encompasses forty protein-coding genes (Boultwood, 2002). By a systematic targeting of each gene using the short hairpin RNA (shRNA) technology, Ebert *et al* identified *RPS14* as the critical gene for the erythroid phenotype (Ebert, 2008). RPS14 haploinsufficiency impairs the rRNA biogenesis resulting in the activation of p53 (Ebert, 2008; Pellagatti, 2010; Dutt, 2011). However, multiple genes are likely to contribute to the complex 5q- syndrome phenotype. For instance, Starczynowski *et al* identified two miRNAs, miR-145 and miR-146a, whose expression was reduced in patients with 5q deletion (Starczynowski, 2010). Concurrent loss of these miRNAs led to the activation of innate immune signaling through the elevated expression of *TIRAP* and *TRAF6* genes, respectively, resulting in thrombocytosis, megakaryocytic dysplasia and mild neutropenia. Furthermore, *FLI-1*, a gene coding for a transcription factor affecting megakaryocyte differentiation, has been reported as an additional miR-145 target (Kumar, 2011).

RIBOSOME BIOGENESIS

Ribosome assembly

Ribosome biogenesis takes place in a specialized nuclear compartment, the nucleolus, which is formed around the actively transcribed rRNA genes. Transcription of rRNA genes by RNA polymerase I gives rise to a 47S precursor rRNA (pre-rRNA), which simultaneously associates with trans-acting factors to form the 90S pre-ribosome. After a series of remodeling and pre-rRNA processing, 90S pre-ribosome splits into pre-40S and pre-60S subunits that are exported into the cytoplasm where the final maturation steps occur (Figure 3A) (Hadjiolova, 1993; Rouquette, 2005).

The newly transcribed pre-rRNA undergoes highly specific chemical modifications that result in the methylation or pseudouridylation of approximately 200 nucleotides (Maden and Hughes, 1997). These modifications are guided by the small nucleolar RNAs (snoRNA) and they are concentrated in functionally important regions (Decatur and Fournier, 2002; Yoon, 2006).

The modified pre-rRNA undergoes hierarchical endonucleolytic and exonucleolytic cleavages, eventually giving rise to 18S, 28S and 5.8S mature rRNAs (Figure 3B) (Hadjiolova, 1993). These rRNA processing steps involve hundreds of trans-acting factors, including small nucleolar ribonucleoproteins and ribosomal proteins. Ribosomal proteins assemble with pre-rRNA in a hierarchical manner and facilitate its processing, nuclear export and cytoplasmic maturation (O'Donohue, 2010).

Coordinated expression of rRNA and ribosomal proteins

Coordinated responses of all three RNA polymerases are required in order to produce equimolar amounts of the mature rRNAs and ribosomal proteins that compose the eukaryotic ribosome (Warner, 1999). Production of rRNA is the rate-limiting step in ribosome biogenesis (Mayer and Grummt, 2006). As rRNA itself is the final gene product, active cells must synthesize high amounts of rRNA. This is possible as the human genome contains approximately 200 rRNA gene copies.
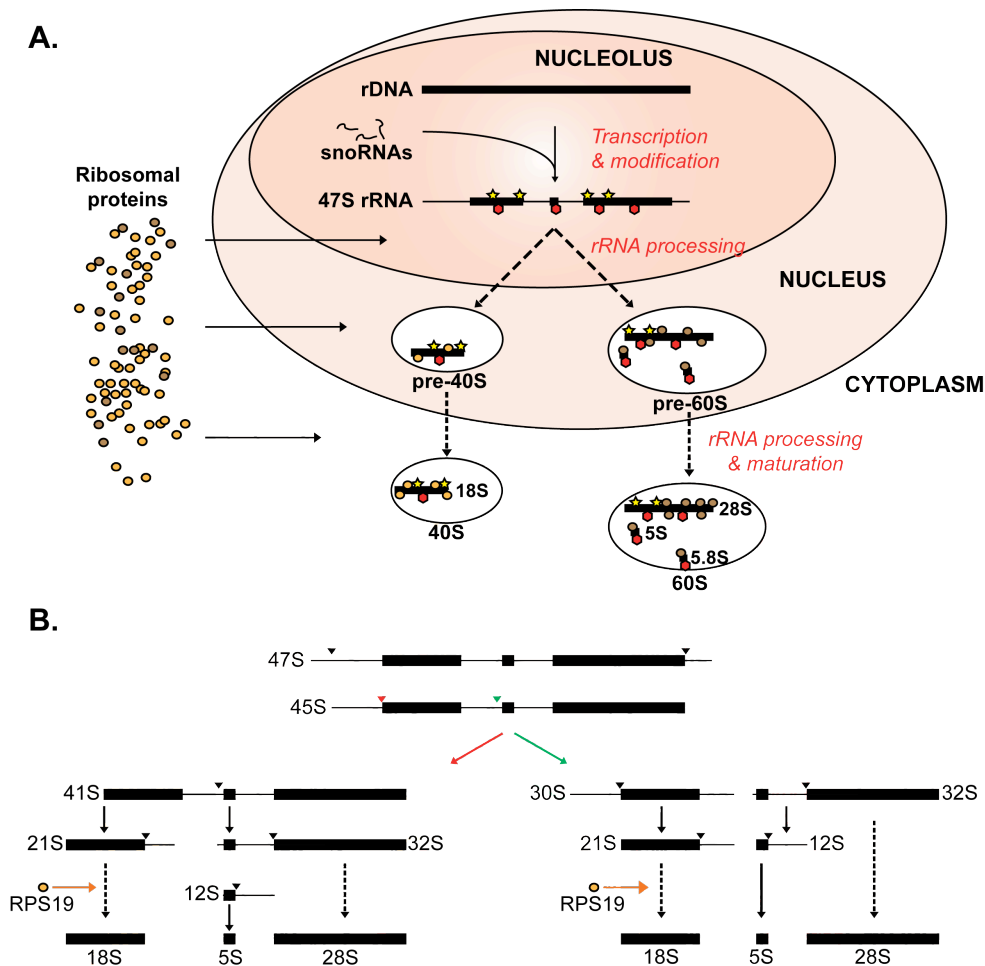
Figure 3. Ribosome biogenesis. (A) Overview of the ribosome assembly. Transcription of rDNA by RNA polymerase I gives rise to a 47S rRNA precursor, which associates with trans-acting factors that mediate a series of chemical modifications and nucleolytic cleavages. This results in the formation of pre-40S and pre-60S ribosomal subunits that are exported into the cytoplasm where the final maturation takes place. (B) Outline of the two alternative rRNA processing pathways. Ribosomal proteins associate with pre-rRNA in a hierarchical manner and facilitate its processing, nuclear export and maturation. Deficiency of ribosomal proteins impairs the rRNA processing at distinct stages; as an example, RPS19 is required for the processing of 21S pre-RNA into 18S rRNA, and RPS19 deficiency leads to a processing block at this stage (Flygare, 2007).

Most ribosomal proteins in humans are encoded by single-copy genes that are located on different chromosomes and expressed from distinctive promoters (Kenmochi, 1998; Perry, 2005). With the exception of the transcriptional start site, mammalian ribosomal protein gene promoters share limited homology (Perry, 2005). Furthermore, a study by Angelastro *et al* suggests high variation in the relative abundance of individual ribosomal protein mRNAs in a given cell (Angelastro, 2002). Ribosomal protein synthesis is controlled mainly at the translational level, which allows a rapid and reversible adaptation to changes in growth conditions. This is achieved through the 5'terminal oligopyrimidine tract (TOP) sequence, which allows the simultaneous translational activation or repression of ribosomal proteins and other components of the translational machinery (Meyuhas, 2000). Ribosomal proteins appear to be synthesized in excessive amounts and the degradation of unassembled ribosomal proteins presents the final level at which ribosomal protein stoichiometry is achieved (Lam, 2006).

Regulation of ribosome biogenesis: the mTOR pathway

Ribosome biogenesis is one of the major energy-consuming processes of the cell and must be tightly coupled to extracellular conditions, such as the availability of nutrients and growth factors, and intracellular stress. The mechanistic target of rapamycin (mTOR) pathway has a key role in sensing and transducing these signals into ribosome biogenesis.

mTOR is a serine/threonine protein kinase that belongs to the phosphoinositide-3-kinase (PI3K)-related kinase family (Laplante and Sabatini, 2012). It can be found in two distinct protein complexes, mTOR complex (mTORC) 1 and 2, which differ in terms of regulation and function. mTORC1 is the primary nutrient-sensitive complex and can be inhibited by the compound rapamycin (Figure 4). It regulates ribosome biogenesis by modulating rRNA gene transcription and ribosomal protein translation. This is achieved mainly through the phosphorylation of two downstream substrates, the eukaryotic initiation factor (eIF) 4E-binding proteins (4E-BP) and the ribosomal protein S6 kinases (S6K).

The mTORC1-4E-BP axis is involved in the initiation of cap-dependent translation. 4E-BP binds to and represses eIF4E, which is the rate-limiting factor in translation initiation. However, the phosphorylation of 4E-BP by mTORC1 leads to its dissociation from eIF4E, allowing eIF4E to participate in the formation of eIF4F cap-binding complex. Recently, two research groups used ribosome profiling to assess the mTORC1-mediated translational control (Thoreen, 2012; Hsieh, 2012). The acute inhibition of mTORC1 specifically suppressed the

translation of mRNAs characterized by 5'TOP or related sequences, and this response was abrogated by the loss of 4E-BPs. These studies demonstrate the close connection between the extracellular conditions and the synthesis of the components of translational machinery.

The effect of the mTORC1-S6K axis on ribosome biogenesis is complex and mediated through multiple downstream substrates (Sengupta, 2010). Phosphorylated S6K regulates the activity of transcriptional initiation factor IA (TIF-1A), a cofactor required for the function of RNA polymerase I, and thus directly promotes the expression of rRNA genes (Mayer and Grummt, 2006). Another example of the S6K substrates is the eukaryotic elongation factor 2 kinase (eEF-2K). S6K-mediated phosphorylation of eEF-2K alleviates its negative effect on eEF-2, which results in enhanced translation elongation (Wang, 2001).

Finally, by phosphorylating Maf1, a RNA polymerase III repressor, mTORC1 directly promotes the transcription of 5S rRNA and transfer RNAs (tRNA) (Shor, 2010).
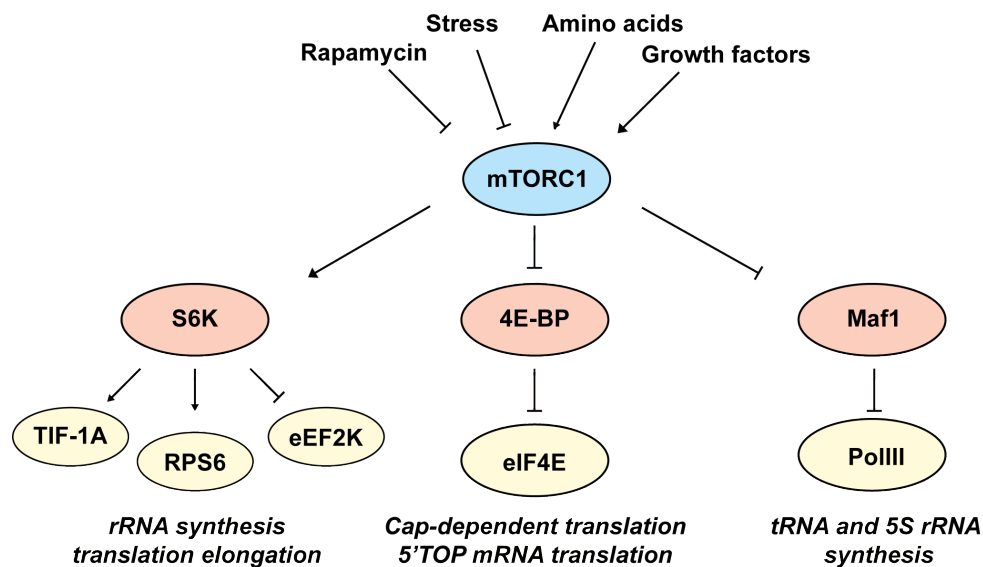

Figure 4. The mTORC1 pathway. mTORC1, the nutrient-sensitive mTOR complex, regulates ribosome biogenesis by promoting rRNA and ribosomal protein synthesis and enhancing translation initiation and elongation.

Ribosomal stress: The RP-Mdm2-p53 pathway

Ribosome biogenesis is central to the regulation of overall protein synthesis and thus coupled to cell growth and division. Perturbations to the dynamics and flow of this process has been associated with alterations in the regulation of cell size and cell cycle progression, leading to developmental defects and increased cancer susceptibility (Ruggero and Pandolfi, 2003). Experimental disruption of rRNA gene transcription using low dose actinomycin D or 5-flourouracil (5-FU) results in the activation of the p53 pathway (Andera and Wasylyk, 1997; Pritchard, 1997). Similar p53-mediated cell cycle arrest and apoptosis is observed upon the genetic deletion of the RNA polymerase I cofactor *TIF-1A* in mouse embryonic fibroblasts (MEF) (Yuan, 2005). Defects in rRNA processing may evoke the p53 response (Pestov, 2001). Finally, numerous *in vitro* and *in vivo* models have demonstrated the activation of p53 in response to ribosomal protein deficiencies (Sulic, 2006; Danilova, 2008; McGowan, 2011).

The p53 tumor suppressor is a central component of the stress response machinery that has evolved in higher organisms to prevent malignant transformation (Vousden and Lu, 2002). Cellular stress stimuli result in the stabilization and accumulation of p53, which acts mainly as a transcription factor that orchestrates genetic programs leading to cell cycle arrest, senescence and apoptosis. The functional outcome of the p53 response depends on the level of p53 and is influenced by the cellular context.

During normal growth conditions, the activity of p53 is kept low by the oncoprotein Mdm2. In the absence of stress, Mdm2 binds to p53 and functions as an ubiquitin ligase, targeting p53 for proteosomal degradation. Various cellular stresses disrupt the interaction between Mdm2 and p53, resulting in the stabilization and activation of p53. However, different forms of stress employ distinct signaling pathways to inhibit the Mdm2-p53 interaction. Oncogenic insults, such as the deregulated expression of *c-Myc* and *Ras*, stimulate the expression and stability of the nucleolar protein Arf, which binds to Mdm2 and inhibits its function (Vogelstein, 2000). Alternatively, DNA damage triggers the ATM-Chk2 pathway, resulting in the phosphorylation of both p53 and Mdm2 and thus preventing their interaction.

The activation of p53 upon impaired ribosome biogenesis employs yet another signaling pathway that prevents the Mdm2-p53 interaction (Figure 5). The first evidence for the existence of this pathway was provided by two research groups who demonstrated the ability of RPL11 to bind Mdm2, inhibiting the degradation of p53 (Lohrum, 2003; Zhang, 2003). Importantly, the RPL11-Mdm2 interaction was enhanced following the inhibition of rRNA synthesis by actinomycin D, suggesting the activation of this pathway in response to defective ribosome

biogenesis. A subsequent study by Bhat *et al* demonstrated a similar response upon serum depletion, which was attenuated by the downregulation of RPL11 (Bhat, 2004). Several other ribosomal proteins, including RPL23, RPL5 and RPS7 have currently been shown to interact with Mdm2 in response to impaired ribosome biogenesis (Jin, 2004; Dai, 2004; Dai and Lu, 2004; Chen, 2007). These ribosomal proteins bind to the central acidic region of Mdm2 in a similar, but non-identical manner (Zhang and Lu, 2009). The RP-Mdm2 interaction inhibits the Mdm2's ubiquitin ligase function towards p53, leading to the accumulation of p53.

Initially, the ribosomal stress response was attributed to the disruption of the nucleolus, releasing free ribosomal proteins into the nucleoplasm (Rubbi and Milner, 2003). However, Fumagalli *et al* demonstrated that the disruption of 40S ribosome subunit biogenesis resulted in ribosomal stress in the absence of nucleolar disruption (Fumagalli, 2009). Based on this study, defective 40S biogenesis leads to the active recruitment of 5'TOP mRNAs, including *RPL11*, to polysomes, resulting in a translational increase in the free RPL11. In contrast, impaired 60S biogenesis suppressed the translation of *RPL11*, but because of the inactive large subunit production, the level of free RPL11 was sufficient to inhibit Mdm2. This study, together with a recent report by the same authors, demonstrates that the deficiency of either 40S or 60S subunit ribosomal proteins elicits a common pathway to activate p53 (Fumagalli, 2012). Furthermore, only RPL11 and RPL5, in a mutually dependent manner, are required to inhibit Mdm2.
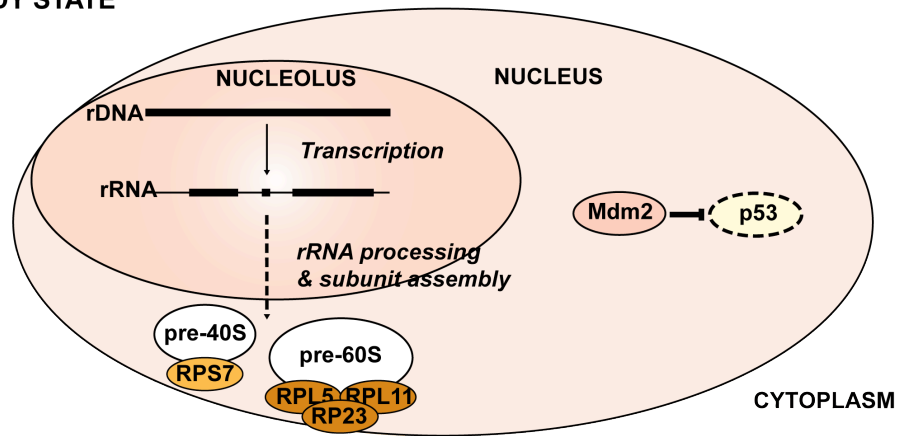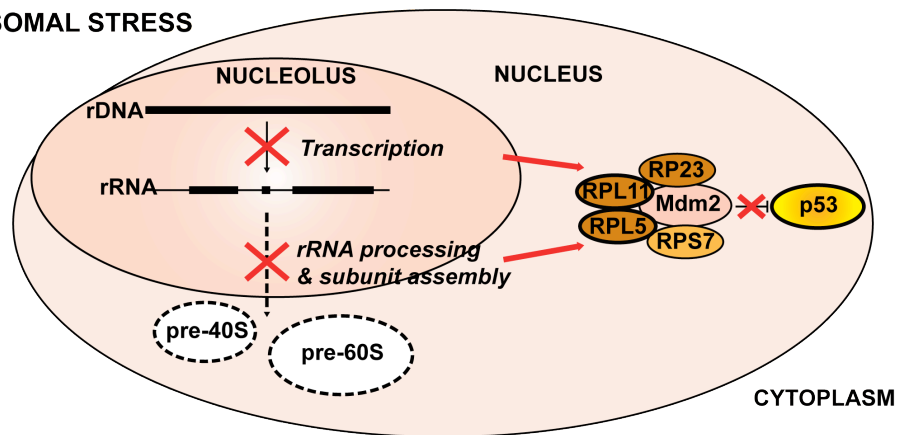
Figure 5. Ribosomal stress. During steady state conditions, the levels of p53 are kept low by Mdm2, which binds to and ubiquitinates p53 resulting in its proteosomal degradation. Impaired rRNA synthesis or processing leads to nuclear accumulation of free ribosomal proteins, which are able to bind to Mdm2 and inhibit its ubiquitin ligase function, resulting in the accumulation of p53. Although multiple ribosomal proteins have been shown to interact with Mdm2, the recent evidence suggests that only RPL5 and RPL11, in a mutually dependent manner, are required for Mdm2 inhibition (Fumagalli, 2012).

GENE THERAPY

Gene therapy of hematological disorders

The concept of gene therapy involves the correction of a disease process by restoring or modifying cellular functions through the introduction of genetic material into target cells. Because of the high prevalence and potentially fatal outcome, the majority of clinical gene therapy trials have involved cancer (Edelstein, 2007). However, based on the rationale of replacing a defective gene with its functional counterpart, monogenic disorders form the most suitable candidates for gene therapy.

Because of their broad relevance for treating variety of human diseases, HSCs are particularly important target cells for gene therapy. Indeed, gene therapy can be applied to all monogenic disorders that are treated with bone marrow transplantation. In this process, patient-derived HSCs are isolated and gene-corrected *ex vivo*, and reinfused back to the patient, where they will reconstitute the hematopoietic system. Most gene therapy studies involving HSCs are based on the use of retroviral vectors because of their ability to integrate into chromosomes of the target cell, allowing a sustained expression of the therapeutic gene in HSCs and in their progeny.

Retroviral vectors

Upon infection, retroviruses are able 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). The integrated provirus exploits the host cell's machinery to propagate itself, while it is stably maintained and replicated as a part of the host genome. The proviral DNA is flanked by long terminal repeat (LTR) sequences, which comprise U3, R and U5 regions. The 5' U3 functions as a viral promoter and enhancer, driving the transcription of the viral DNA. Viral RNA contains a number of sequence elements that are required for its replication and assembly into the virus particle. All retroviruses share three essential genes, *Gag*, *Pol* and *Env*, which encode viral core proteins, enzymes and envelope proteins, respectively. However, more complex retroviruses, such as lentiviruses, code for additional proteins. The profound understanding of retrovirus biology has allowed researchers to design retrovirus-based vectors for gene

transfer. In retroviral vector constructs the viral protein-coding elements have been substituted with a transgene cassette, and thus the vector *per se* is incapable to undergo replication. However, the simultaneous introduction of other constructs encoding the essential viral proteins enables the cell to produce replication-defective viral particles.

Most of the clinical trials have employed gammaretrovirus-based vectors for HSC gene transfer. However, lentivirus-based vectors, such as human immunodeficiency virus 1 (HIV-1) vectors, offer many advantages compared to gammaretroviral vectors. First, in contrast to gammaretroviral vectors, lentiviral vectors are able to integrate into quiescent cells such as HSCs. Furthermore, while the gammaretroviral vectors integrate preferably near transcriptional start sites, lentiviral vectors show a potentially safer integration profile in terms of the probability of oncogene activation (Modlich, 2009). HIV-1 vector constructs are made devoid of all viral elements with the exception of LTRs, packaging signal and Rev-responsive element (RRE) (Figure 6). To abolish the 5' LTR transcriptional/enhancer activity of integrated provirus, a self-inactivating (SIN) deletion has been introduced into the U3 region of the 3'LTR, which serves as a template for the 5'LTR generation during the reverse transcription (Zufferey, 1998). Post-transcriptional regulatory elements are often included in order to increase the transcript stability and translation. To produce lentiviral particles, vector construct plasmid is transfected into 293T cells together with *Gag-Pol*, *Rev* and *Env* plasmids. Vesicular stomatitis virus glycoprotein (VSV-G) *Env* is often used to pseudotype viral particles, allowing the transduction of a broader target cell range and vector concentration.

Clinical gene therapy: Severe combined immunodeficiencies

Severe combined immunodeficiency (SCID) syndromes have been central to the field of gene therapy. SCID includes a variety of genetic defects that impair the differentiation of T-lymphocytes and in some cases affect also B-lymphocytes and natural killer (NK) cells. These conditions possess many qualities that made them suitable for the first clinical gene therapy trials (Qasim, 2009). First, the genetic basis of various forms of SCID was well characterized. Second, clones with rare somatic mutations, which restore the underlying molecular defect, were shown to gain a strong selective advantage and mediate immunological repopulation, suggesting that the gene-correction of only a fraction of HSCs could be sufficient for therapeutic response. Furthermore, because of the strong selective advantage,

only minimal or no pre-conditioning at all was necessary to achieve a stable engraftment.

A.

Wild-type lentivirus

B.
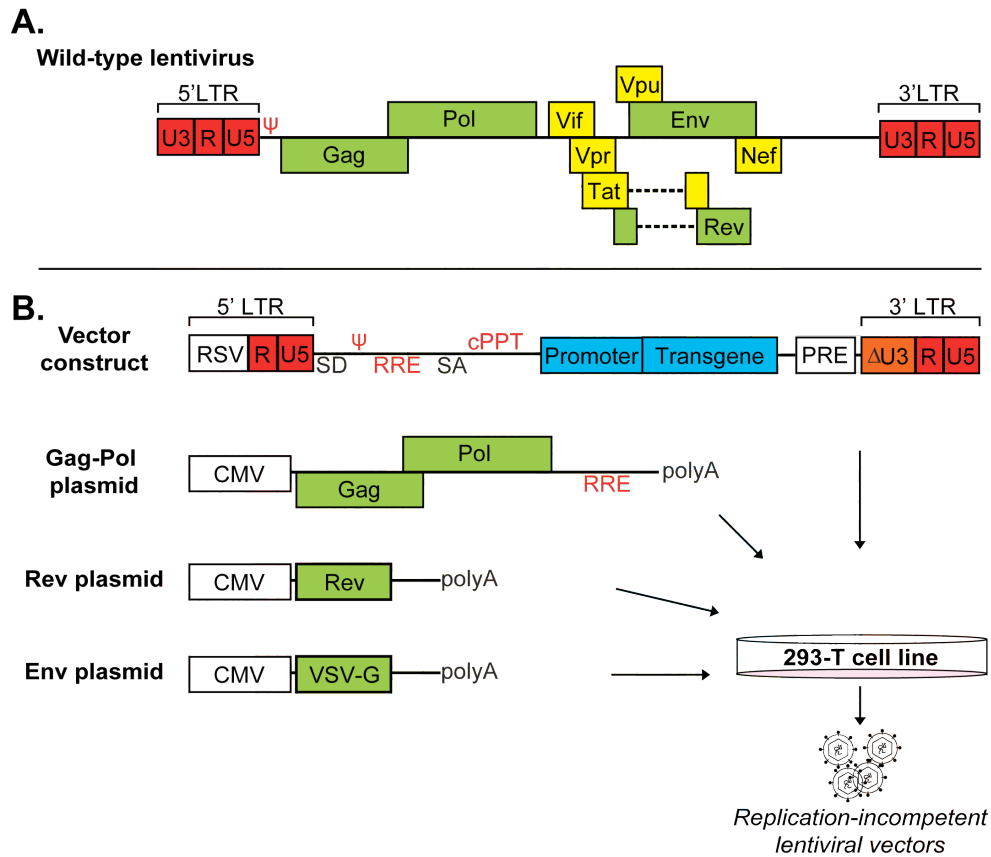
Vector construct

Figure 6. Generation of lentiviral vectors. (A) Schematic representation of wild-type lentiviral provirus. (B) A system to produce third-generation lentivirus vectors. Lentiviral vector constructs are made devoid of all protein-coding genes, and comprise only the transgene of interest, coupled to an internal promoter, and cis-acting elements required for its replication and packaging into viral particles. To abolish the 5'LTR promoter/enhancer activity a SIN deletion is introduced into the U3 region of the 3'LTR. In order to produce replication-incompetent viral particles, the lentiviral vector construct is transfected into a 293-T cell line together with separate plasmids encoding the viral proteins. Essential wild-type lentivirus cis-acting elements and protein-coding genes are shown in red and green, respectively. Wild-type lentivirus genes that are non-essential for the vector generation are shown in yellow. SA, splice acceptor; SD, splice donor; RSV, Rous sarcoma virus promoter; ψ packaging signal; cPPT, polypurine tract; CMV, cytomegalovirus promoter; PRE, post-transcriptional regulatory element.

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After a series of clinical trials with limited success (Kohn, 2003), the complete correction of X-linked SCID (SCID-X1) in two patients in 2000 was the first real success of gene therapy (Cavazzana-Calvo, 2000). Subsequently, successful treatment of two patients with ADA-deficient SCID was reported (Aiuti, 2002). Currently there are a total of 20 SCID-X1 and 27 ADA-deficient SCID patients that have been treated with 85 % and 70 % success rate, respectively (Fischer, 2010). Unfortunately, five out of the twenty SCID-X1 patients developed T-lymphocyte leukemia, caused by the deregulated expression of oncogenes due to insertional mutagenesis, which was fatal in case of one patient. Although these data underscore the need for safer gene transfer systems, they also highlight the potential of HSC-based gene transfer in the treatment of hematological disorders. Indeed, SIN lentiviral vectors have been successfully applied in the clinic, and although the follow-up time for these trials is still relatively short, no severe genotoxic side effects have been reported (Cartier, 2009; Biffi, 2011).

RNA INTERFERENCE

Double-stranded RNAs (dsRNA) trigger conserved responses in eukaryotic cells that result in sequence-specific silencing of gene expression, a phenomenon called RNAi (Meister and Tuschl, 2004). It is a complex process in which dsRNAs are processed into small RNA duplexes by the cytoplasmic endonuclease Dicer (Figure 7). These RNA duplexes can be divided into small interfering RNAs (siRNA) and micro RNAs (miRNA) based on the differences in their biogenesis. siRNAs are processed mainly from the exogenous dsRNA, while miRNAs are derived from the endogenous transcripts containing dsRNA domains.

miRNA biogenesis involves the nuclear endonuclease Drosha, which recognizes and cleaves the stem-loop structure in a long primary transcript (pri-miRNA) forming an approximately 70 base precursor miRNA (pre-miRNA). pre-miRNA is transported into cytoplasm by the nuclear export factor Exportin-5 and processed into miRNA by Dicer (Kim and Nam, 2006). One strand of the miRNA is assembled into ribonucleoprotein complex (RNPC) in which it guides the recognition and silencing of a complementary single-stranded RNA, normally mRNA. The choice of the assembled strand depends mainly on the thermodynamic properties of the RNA duplex (Huppi, 2005). RNPCs are the effectors of the RNAi response and based on the sequence homology between the guide and target RNA, they mediate either degradation or translational repression of the target RNA.

The ability of small RNA duplexes to degrade selected mRNAs has rendered RNAi a valuable tool to study gene functions. Early studies applied synthetic siRNAs in order to knockdown the expression of a gene of interest. The discovery of endogenous miRNAs led to the generation of expression vectors to deliver DNA constructs expressing pre-miRNA-mimicking shRNAs under a ubiquitous RNA polymerase III promoter (Brummelkamp, 2002). Zeng *et al* were able to isolate and modify the target specificity of human miR-30 miRNA backbone (Zeng, 2002). Incorporation of the targeting sequence into the miR-30 miRNA context improved the knockdown efficiency compared to conventional shRNAs and allowed the use of RNA polymerase II promoters (Boden, 2004). Indeed, systems allowing an efficient, stable and regulatable expression of miRNA-styled shRNA, even when present at a single copy in the genome, were generated (Stegmeier, 2005; Dickins, 2005).
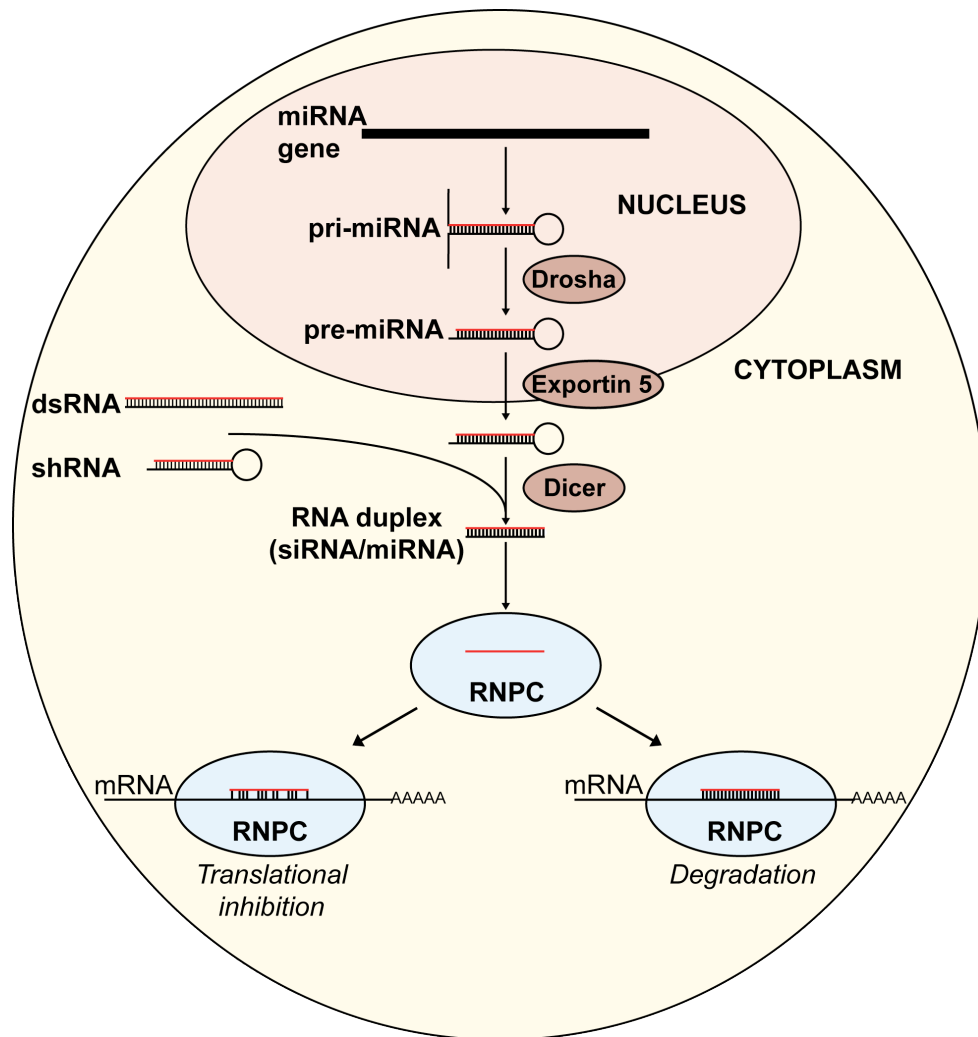
Figure 7. Schematic representation of the RNAi machinery. Endogenous or exogenous dsRNA serves as a substrate for the cytoplasmic endonuclease Dicer, which processes it into a small RNA duplex. One of the RNA strands is integrated into a ribonucleoprotein complex (RNPC), in which it guides the recognition and silencing of a complementary single-stranded RNA. Depending on the degree of sequence homology, RNPC mediates either translational repression or degradation of the target RNA. The RNAi machinery can be exploited to silence genes of interest by introducing synthetic siRNAs, shRNAs or miRNA-styled shRNAs into the target cells.

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DISEASE MODELING IN THE MOUSE

Animal models for a human disease should faithfully reproduce the disease phenotype by recapitulating the genetic and molecular features of the disease, and respond to the existing therapies. Because of its biological and genetic resemblance to humans, feasibility of genetic engineering, short reproduction cycle and low maintenance cost, the laboratory mouse has emerged as a standard organism utilized in biomedical research. There are currently 3776 mouse genotypes modeling a total of 1134 human diseases (Mouse Genome Database, accessed August 2012; Blake, 2011). These models traditionally rely on gene targeting technologies or transgene overexpression.

Loss of function

The gene knockout technology allows the complete inactivation of a gene without the drawbacks of insertional mutagenesis and off-target gene silencing, and thus provides a valuable tool to model diseases with a loss of function gene mutation. This technology relies on homologous recombination to disrupt an endogenous gene in ES cells by replacing it with an exogenous DNA coding for a selectable marker gene. Mice originating from the modified ES cells harbor the genetic alteration in all tissues and are referred to as conventional knockout mice. Combining the knockout technology with Cre/*loxP* and Flp/*FRT* site-specific DNA recombination systems has allowed the generation of mice in which the gene inactivation is conditional and can be induced in a specific tissue and at a specific time *in vivo* (Capecchi, 2005). This is especially advantageous in the case of genes that are essential for normal embryogenesis.

RNAi provides an alternative approach to downregulate gene expression. In contrast to knockout technology, it acts without modifying the genomic locus of the target gene, allowing a reversible gene downregulation. Shortly after the generation of RNA polymerase II driven miRNA-styled shRNAs, Dickins *et al* reported a proof of principle that transgenic RNAi could be used to generate mouse models with tetracycline-regulatable and tissue-specific downregulation of a given gene (Dickins, 2007). Premsrirut *et al* further optimized this technology to allow a rapid generation of single-copy transgenic RNAi mice (Premsrirut, 2011).

PRESENT STUDIES

Article I.

Mice with ribosomal protein S19 deficiency develop bone marrow failure and symptoms like patients with Diamond-Blackfan anemia.

<u>**Aim**</u>

To generate and characterize mouse models for RPS19-deficient DBA using transgenic RNAi.

<u>**Questions addressed in article I**</u>

How efficient is *Rps19* knockdown in the hematopoietic progenitor cells of transgenic RNAi mice *in vivo*?

We generated mouse models for RPS19-deficient DBA by introducing an *Rps19*-targeting shRNA into the downstream of the collagen A1 locus. Two models with different *Rps19*-targeting shRNAs were generated and these mice were bred either heterozygous or homozygous for the given shRNA in order to adjust the level of Rps19 downregulation. To evaluate the *Rps19* knockdown efficiency in these models, we FACS-sorted preCFU-E/CFU-E, preGM/GMP and LSK hematopoietic progenitor cells from adult mice that were administered doxycycline for three days. At this time-point the RNAi response is active, but the bone marrow cellularity is not yet affected allowing the isolation of hematopoietic progenitor cells even from the most severe model. Real-time PCR analysis of *Rps19* mRNA revealed approximately 50 % knockdown in each population. However, the exact knockdown efficiency was dependent on the type of shRNA and its copy number.

Do mice with Rps19 deficiency exhibit an erythroid phenotype?

The onset of Rps19 deficiency resulted in anemia and the severity of the phenotype correlated with the degree of Rps19 downregulation. Majority of the

mice with the most severe Rps19 downregulation failed to recover from the onset of Rps19 deficiency and died due to bone marrow failure. However, the recovered mice were able to compensate for the erythroid defect and with time developed only a mild macrocytic anemia.

At which cellular stage does the erythroid failure occur?

We initially assessed the location of the erythroid failure using flow cytometry. After two weeks of doxycycline administration, Rps19-deficient mice had normal or increased frequencies of hematopoietic stem and progenitor cells compared to control mice. However, at this time-point the frequencies of proerythroblasts and more mature erythroid precursors were decreased. To study the erythroid defect further we took advantage of the inducible nature of our model. We FACS-sorted preMegE and preCFU-E/CFU-E progenitor cells from Rps19-deficient mice into single-cell liquid cultures without doxycycline, and studied to what extent the cellular defect could be restored. Indeed, the removal of doxycycline completely rescued the proliferation of preMegE progenitor cells, while the proliferation of preCFU-E/CFU-E progenitors was only partially restored. Taken together, both experimental approaches indicate that the most severe erythroid defect is located downstream of the preMegE progenitor cell, likely at the CFU-E – proerythroblast transition.

Can the hematopoietic phenotype be rescued by *RPS19* overexpression *in vitro*?

To confirm that the observed hematopoietic phenotype was caused by Rps19 deficiency and not due to off-target gene silencing, we designed a lentiviral vector overexpressing the human *RPS19* cDNA, which is not recognized by the *Rps19*-targeting shRNAs. *RPS19* gene transfer into hematopoietic progenitor cells rescued their proliferative and colony-forming capacity *in vitro*.

What is the role of p53 in Rps19-deficient hematopoiesis?

We initially assessed the role of p53 by quantifying the expression of multiple p53 transcriptional targets using real-time PCR. These studies demonstrated the activation of p53 in preCFU-E/CFU-E, preGM/GMP and LSK progenitor cells. To study the functional relevance of this observation, we crossed the Rps19-deficient mice with mice lacking *Trp53*, the gene encoding p53, and transplanted bone marrow cells from these mice into lethally irradiated wild-type recipients. After two weeks doxycycline administration the loss of p53 rescued the lethal bone

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marrow failure, although the extent of rescue was dependent on the level of Rps19 downregulation.

<u>Summary</u>

This study demonstrates for the first time the feasibility of transgenic RNAi to generate mouse models for human diseases caused by haploinsufficient expression of a gene. Rps19-deficient mice develop a macrocytic anemia and the severity of the hematopoietic phenotype is highly dependent on the level of Rps19 downregulation. We further show that chronic Rps19 deficiency leads to the exhaustion of HSCs. Based on FACS analysis and single-cell cultures of prospectively isolated erythroid progenitor cells, we definitely demonstrate that the most severe erythroid defect is located at the CFU-E – proerythroblast transition. Furthermore, *RPS19* gene transfer rescues the proliferative and colony-forming defect *in vitro* providing evidence for the feasibility of gene therapy in the treatment of RPS19-deficient DBA. Finally, we demonstrate that the loss of p53 ameliorates the hematopoietic defect, implying the central role of p53 in the pathogenesis of DBA.

Article II.

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

Aim

To evaluate the therapeutic potential of the amino acid L-leucine in the treatment of DBA using the mouse models generated in article I.

Questions addressed in article II

Does L-leucine administration improve the anemia in Rps19-deficient mice?

We induced Rps19 deficiency by feeding the transgenic mice with doxycycline-containing food and simultaneously administered 1.5 % (weight/volume) L-leucine in the drinking water, a regimen that doubles the concentration of L-leucine in serum (Macotela, 2011). After two weeks of doxycycline administration, Rps19-deficient mice showed a reduction in the number of erythrocytes and hemoglobin concentration, and both parameters were significantly improved on L-leucine treatment.

Does L-leucine have a general effect on erythroid recovery in wild-type mice?

We assessed whether L-leucine had a stimulatory effect on the erythroid recovery in wild-type mice following sublethal irradiation or phenylhydrazine-induced hemolytic anemia. No differences were observed.

What is the cellular mechanism underlying the therapeutic response?

To investigate the underlying cellular response to L-leucine, we performed immunophenotypic analysis of the myeloerythroid compartment of untreated and L-leucine-treated Rps19-deficient mice. L-leucine treatment decreased the frequencies of hematopoietic stem and progenitor cell compartments. Similarly, the frequencies of all erythroblasts were significantly reduced upon L-leucine treatment. Based on similar reticulocyte counts in untreated and L-leucine-treated Rps19-deficient mice, these findings indicate that L-leucine enhances the

differentiation of Rps19-deficient erythroid precursors into fully functional erythrocytes.

Does Rps19 deficiency affect the mTOR pathway in hematopoietic progenitor cells and is this further modulated by L-leucine treatment?

To study possible activation of the mTOR pathway by L-leucine, we used flow cytometry to quantify phospho-Rps6 and phospho-4E-BP1, the two downstream components of the mTOR kinase, in myeloid progenitor and erythroid precursor cells. However, we observed no differences between the cells from control and Rps19-deficient mice when evaluating these parameters. L-leucine treatment did not influnce the activation of the mTOR kinase.

Does L-leucine treatment affect p53 activity?

We sorted preGM/GMP and preCFU-E/CFU-E progenitor cells from untreated and L-leucine-treated Rps19-deficient mice and quantified a panel of p53 transcriptional targets using real-time PCR. L-leucine treatment resulted in reduced expression of all analyzed p53 transcriptional target genes.

<u>**Summary**</u>

This study demonstrates that the administration of L-leucine improves the anemia and alleviates the stress hematopoiesis in Rps19-deficient mice. Although L-leucine had no effect on the mTOR activity, the therapeutic response was associated with reduced p53 activity in the erythroid and myeloid progenitor cells.

Article III

Gene therapy corrects the anemia and bone marrow failure in a mouse model for RPS19-deficient DBA.

Aim

To provide a proof of principle for the efficacy of *RPS19* gene therapy in the treatment of RPS19-deficient DBA.

Questions addressed in article III.

Does *RPS19* gene transfer reverse the lethal bone marrow failure of Rps19-deficient mice?

We applied the therapeutic (SFFV-RPS19) and control (SFFV-GFP) lentiviral vectors introduced in article I to assess the therapeutic efficacy of gene therapy *in vivo*. Transduced c-Kit-enriched bone marrow cells from the homozygous shRNA-D mice were transplanted into lethally irradiated wild-type recipient mice. Three months after transplantation the recipient mice were administered doxycycline in order to induce Rps19 deficiency. After two weeks of doxycycline administration the recipients transplanted with SFFV-GFP homozygous shRNA-D bone marrow developed a lethal bone marrow failure. Remarkably, the recipients transplanted with SFFV-RPS19 homozygous shRNA-D bone marrow survived and exhibited close to normal blood cellularity.

Does *RPS19* gene transfer confer a reconstitution advantage *in vivo*?

In order to monitor the reconstitution advantage of the gene-corrected Rps19-deficient cells over time, we transplanted lethally irradiated wild-type recipients with heterozygous shRNA-D bone marrow transduced with either SFFV-GFP or SFFV-RPS19 vector. The hematopoietic phenotype in these recipients is modest and non-lethal, allowing the long-term monitoring of the dynamics of transduced cells. Based on the percentage of transduced cells in the peripheral blood with time, the SFFV-RPS19 vector conferred a clear reconstitution advantage over the untransduced Rps19-deficient cells. Similarly, the percentage of gene-corrected Rps19-deficient myeloid and erythroid progenitor cells in the bone marrow was

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significantly higher compared to the recipients with control or SFFV-GFP heterozygous shRNA-D bone marrow.

Summary

This study demonstrates the feasibility of gene therapy in the treatment of RPS19-deficient DBA. *RPS19* gene transfer reversed the lethal anemia and bone marrow failure in Rps19-deficient mice. Furthermore, we show that gene-corrected cells gain a significant reconstitution advantage over the untransduced Rps19-deficient cells.

Importance of the present studies

Knowledge about DBA pathophysiology has been limited due to lack of appropriate animal models. In this study we have successfully generated mouse models for RPS19-deficient DBA. These models recapitulate many of the key phenotypic and molecular features seen in patients, and are therefore well suited for the evaluation of novel therapies for DBA treatment. Using these models we have confirmed the initial finding by Pospisilova *et al* that the amino acid L-leucine can be effective in the treatment of DBA, and thus our results strengthen the rationale for clinical trials of L-leucine (Pospisilova, 2007). Our previous studies have demonstrated the potential of gene therapy to treat RPS19-deficient DBA (Hamaguchi 2002; Hamaguchi, 2003; Flygare, 2008). However, the current study provides a definitive proof of principle that *RPS19* gene transfer can be used to treat RPS19-deficient DBA patients. This finding is of utmost importance as it is expected to lead to a permanent cure.

GENERAL DISCUSSION

Rps19-deficient mouse models

Modeling of haploinsufficient human diseases like DBA in mice is challenging since the phenotype is highly dependent on the level gene downregulation. The complete deletion of essential genes like *Rps19* often results in embryonic lethality, while heterozygosity on the other hand may be compensated efficiently. Indeed, the homozygous loss of *Rps19* is lethal prior to implantation, while the mice heterozygous for *Rps19* exhibit normal phenotype and Rps19 expression (Mattson, 2004).

Transgenic RNAi allowed us to generate mouse models with adjustable degree of Rps19 downregulation, and depending on the type and the number of shRNAs as well as the doxycycline concentration, two different outcomes were observed; the mice with severe Rps19 deficiency developed a lethal bone marrow failure, while the mice with intermediate Rps19 deficiency were able to compensate for the initial reduction in blood cellularity (Figure 9). In the present work, the severe models were employed to assess the role of p53 in DBA pathogenesis and to validate the therapeutic potential of enforced expression of *RPS19*. In parallel, the chronic models with intermediate Rps19 deficiency were used to study the effect of L-leucine treatment.
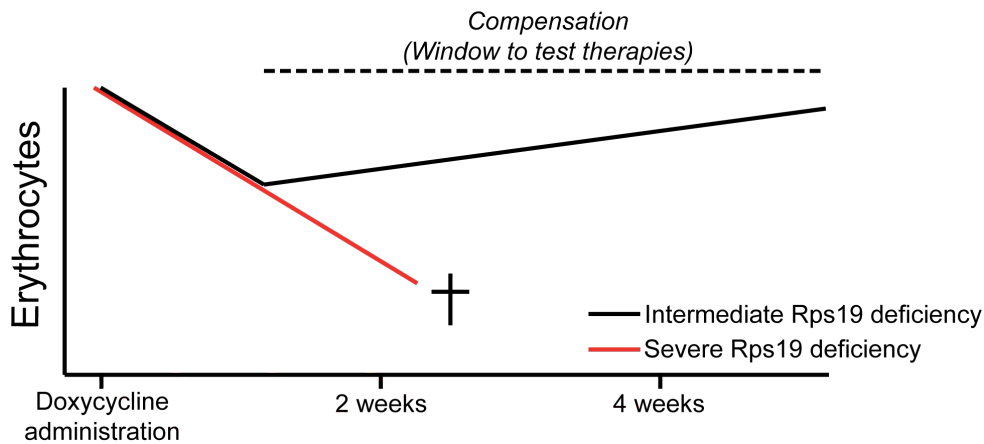

Figure 9. Severe and chronic mouse models for RPS19-deficient DBA.

Similarity to DBA

Patients with premature termination codons in *RPS19* show a 2-4-fold reduction in *RPS19* mRNA in the CD34-positive bone marrow progenitor cells (Gazda, 2004). Similarly, the average *Rps19* knockdown efficiency in the generated mouse models was approximately 50 % and influenced by the type of shRNA and its copy-number. Furthermore, pre-rRNA processing studies in Rps19-deficient cells revealed a defect similar to that seen in RPS19-deficient patients, confirming the functional deficit of Rps19.

The onset of Rps19 deficiency resulted in anemia, the key feature of DBA. The severity of the phenotype correlated with the level of Rps19 and mice with the most severe Rps19 downregulation developed a lethal bone marrow failure. Although rare, aplastic anemia is a real feature of DBA (Lipton, 2006). However, with time the erythroid defect in the surviving mice was considerably milder than in most patients, resembling the non-classical DBA phenotype.

Shortly after the induction of Rps19 deficiency the mice developed thrombocytosis, a feature that is commonly observed in patients at diagnosis (Willig, 1999). This finding suggests that Rps19 deficiency has a relatively minor effect on thrombopoiesis and that the elevation in platelet count merely reflects the homeostatic regulation within the bone marrow. Alternatively, Rps19 deficiency may activate a pathway for megakaryocyte differentiation directly from HSCs (McGowan, 2011). However, Rps19-deficient mice developed thrombocytopenia and neutropenia over time, as is seen in some DBA patients.

Similarity to other mouse models with ribosomal protein deficiency

Our Rps19-deficient mouse models show a similar long-term phenotype as the Rps19-deficient Dsk3 mice, which exhibit growth retardation and mild macrocytic anemia with increased apoptosis in the bone marrow (McGowan, 2008). Just as the Dsk3 mice, Rps19-deficient mouse models also develop dark skin with time (unpublished observation). Interestingly, the erythroid phenotype in both mouse models is considerably mild compared to the robust macrocytic anemia seen in mice with the heterozygous deletion of *Rps6* or *Cd74-Nid67* region containing *Rps14* (McGowan, 2011; Barlow, 2010). This is likely due to a difference in the absolute level of the respective ribosomal protein in the erythroid lineage. Indeed, the Dsk3 mice may present a hypomorphic mutation instead of a true haploinsufficiency. Similarly, the level of *Rps19* knockdown in the erythroid lineage of transgenic mice may not be sufficient or stable enough to cause a more severe anemia. Alternatively, mouse erythroid progenitors may express *Rps19* in relative excess compared to *Rps6* and *Rps14* (Ellis and Massey, 2006). According

to this hypothesis, a 50 % reduction in mRNA levels of the respective ribosomal proteins would not reflect the absolute reduction in protein levels.

Similarly to mice with the heterozygous deletion of *Rps6* or *Cd74-Nid67* region, the generated mouse models developed a hypocellular bone marrow together with a reduction in the number of hematopoietic stem and progenitor cells. The hematopoietic phenotype was rescued upon the loss of p53.

Therapeutic approaches

Remission – the natural therapy

Despite recent advances in understanding the molecular basis of DBA, the natural course of the disease remains largely unpredictable. Approximately 20 % of the patients enter spontaneous remission, often during the first decade of life, in which physiologically acceptable hemoglobin level is maintained without therapeutic intervention. Interestingly, there appears to be no clear correlation between the chance of remission and the type and duration of the therapy. This is further complicated by the failure of the genotype to predict the hematopoietic phenotype, as highlighted by the variable penetrance of the genetic lesion in DBA pedigrees.

In order to understand the nature of spontaneous remission in DBA, it is important to study whether remission is associated with the emergence of clones that have acquired additional mutations, which confer a competitive advantage over the remaining cells. Such a process has been described in some patients with Fanconi anemia and is theoretically possible in DBA, as demonstrated by our own studies involving *RPS19* gene transfer and the loss of p53 (Soulier, 2005). The existence of genetic modifiers of the hematopoietic phenotype is further implied by the presence of non-classical DBA patients. Some of these factors could be cell autonomous and directly modulate ribosome biogenesis or the subsequent p53 response, or alternatively hematopoietic signaling pathways. The GCR has been suggested as one possible factor that may influence the DBA phenotype (Varricchio, 2011).

However, the vast majority of the remitted patients continue to exhibit elevated eADA and macrocytosis, and the same is true for the individuals with non-classical DBA (Willig, 1999; Orfali, 2004). These findings suggest a continuous presence of the erythroid defect, which is compensated through extrinsic factors that stimulate the hematopoietic stem and progenitor cells, leading to increased influx of cells into the Epo-responsive stage. Indeed, Ohene-Abuakwa *et al* demonstrated a consistent erythroid defect of patient cells *in vitro* regardless of the

clinical severity (Ohene-Abuakwa, 2005). Intriguingly, a similar defect was observed when culturing cells of first-degree relatives exhibiting only an isolated elevation in eADA activity. Furthermore, the use of such a sibling as a donor for allogeneic bone marrow transplantation may result in engraftment without red cell reconstitution (Orfali, 1999). Finally, the recent study characterizing Rps6-deficient mice demonstrated that the elevation in eADA activity is dependent on p53 (McGowan, 2011).

Dynamics of the hematopoietic system appears important, as relapses tend to occur under conditions of hematopoietic stress such as pregnancy (Faivre, 2006). Presentation of anemia in DBA normally coincides with the neonatal decline in HSC turnover (Rufer, 1999). Dynamics of the hematopoietic system could also directly influence the severity of the cellular defect of DBA. This is supported by the fact that the chance of relapse in remitted patients appears low, except during stress conditions. How can this hypothesis then be applied in the treatment of DBA? Human hematopoietic growth factors, such as IL-3, have been used to treat patients. However, no consistent benefit was observed in these studies and the outcome could not be predicted on basis of the *in vitro* response. Dunbar *et al* treated six transfusion-dependent patients by sequentially administrating granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 with an interim rest period (Dunbar, 1991). Although a limited response was observed following the GM-CSF period, the treatment with IL-3 eventually resulted in remission in two patients. Interestingly, the therapeutic outcome appeared to be associated with a relatively high pre-treatment level of erythroid precursors and reticulocytes. Similarly, in the trial reported by Olivieri *et al*, involving nine transfusion-dependent patients without detectable reticulocytes, the treatment with IL-3 failed to induce erythropoiesis (Olivieri, 1994). These studies suggest that the dynamics of the hematopoietic system could influence the therapeutic response to growth factor treatment. Therefore, growth factor therapy should not be considered as a replacement therapy, but as a regimen to relieve the stress hematopoiesis and chronic bone marrow failure, possibly to increase the chance of remission. Furthermore, strategies involving the combinatorial use of growth factors, together with corticosteroids, should be explored. One interesting growth factor could be the granulocyte colony-stimulating factor (G-CSF). This growth factor is used routinely in clinical medicine to mobilize HSCs from the bone marrow, and in combination with Epo, it has also been reported to improve the anemia in patients with MDS (Negrin, 1996; Hellström-Lindberg, 1998). Besides its role in granulocytopoiesis, G-CSF administration results in the expansion of hematopoietic stem and progenitor cell pool, which could enhance the generation of erythroid progenitor cells (Morrison, 1998). Another interesting drug for a possible clinical trial could be Eltrombopag, an orally delivered small molecule thrombopoietin (Tpo) agonist (Erickson-Miller, 2009). In addition to its role in megakaryopoiesis, Tpo stimulates hematopoiesis at the HSC level (Qian, 2007;

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Kimura, 1998). Recently, Olnes *et al* reported improved hematopoiesis in patients with refractory aplastic anemia using Eltrombopag (Olnes, 2012). In this study, 11 of 25 patients showed hematologic response in at least one lineage at 12 weeks with six patients having improved hemoglobin levels.

Corticosteroid therapy

Corticosteroids form the main therapeutic regimen in DBA with approximately 80 % of the patients initially responding to the therapy (Lipton, 2006). However, only a half of these patients maintain the therapeutic response and there is no reliable way to predict the responsiveness of a given patient. The fact that the percentage of initial responders is so high suggests that patients do not differ in corticosteroid sensitivity *per se*, but may reflect the dynamics of the hematopoietic system (the rate at which corticosteroid-responsive BFU-Es are formed). This could also explain the high variation in the required corticosteroid maintenance dose. Based on this hypothesis even the initially non-responding patients may show a therapeutic response later in life.

Although their role in the stimulation of BFU-E self-renewal is well established, it is not clear whether corticosteroids have an additional DBA-specific effect. Indeed, GCR has been reported to inhibit p53 function through a physical interaction *in vivo* (Sengupta, 2000).

Lenalidomide

Lenalidomide has proven to be highly effective in the treatment of patients with 5q- syndrome, causing both hematological and cytogenetic responses (List, 2006). Although the underlying mechanism remains elusive, lenalidomide has been reported to promote the erythroid differentiation of human CD34-positive bone marrow cells and the production of fetal hemoglobin (Moutouh-De Parseval, 2008). This is due to its ability to stimulate CFU-E progenitor cells, possibly through the modulation the EpoR turnover (Narla, 2011, Basiorka, 2011). As corticosteroids and lenalidomide promote erythropoiesis at distinct stages, combinatorial use of these agents could provide a more profound therapeutic effect in DBA (Narla, 2011).

L-leucine

L-leucine is an essential branched chain amino acid that plays an important role in the regulation of protein synthesis, and this response involves the mTORC1 pathway (Stipanuk, 2007). Recently, based on the theory of inefficient translation as the underlying cause for the severe anemia in DBA, Pospisilova *et al* reported one patient who became transfusion-independent in response to treatment with the amino acid L-leucine (Pospisilova, 2007). Similarly, L-leucine administration alleviated the developmental defects and in some cases also the anemia of rps19-deficient and rps14-deficient zebrafish models (Payne, 2012). Our studies corroborate these findings as dietary L-leucine improved the anemia of Rps19-deficient mice.

In contrast to the zebrafish models, there was no difference in mTOR activity between the hematopoietic cells from control and Rps19-deficient mice and this was not modulated upon L-leucine treatment. The discrepancy between these results may be partly explained by the high L-leucine concentration used in the zebrafish study, as L-leucine is absorbed by diffusion during early zebrafish development. In contrast to studies assessing the effects of L-leucine in fasting animals, chronic supplementation of leucine has been reported to increase protein synthesis in rats without adaptive changes in the mTOR activity (Stipanuk, 2007; Lynch, 2002). Furthermore, L-leucine appears to stimulate protein synthesis in some but not all tissues, and whether this applies to hematopoietic cells is not known.

The role of mTOR in DBA is controversial. Based on the studies by Fumagalli *et al* the impaired 40S ribosomal subunit biogenesis leads to active translation of 5'TOP mRNAs, including *RPL5* and *RPL11*, which results in the activation of p53 (Fumagalli, 2009; Fumagalli, 2012). According to this model the compounds that partially inhibit the mTOR pathway and thus the translation of 5'TOP mRNAs could potentially provide a therapeutic benefit. However, the enhanced translation of 5'TOP mRNAs should similarly stimulate the translation of *RPS14* and *RPS19*, which could underlie the therapeutic effect of L-leucine in these two animal studies.

The therapeutic response to L-leucine in Rps19-deficient mice may also be partly systemic, as L-leucine broadly affects multiple metabolic and signaling pathways. For instance, the administration of L-leucine to mice has been shown to increase the serum concentration of corticosterone, the main murine glucocorticoid hormone (Macotela, 2011).

Targeting the p53 pathway

Mouse models deficient for ribosomal proteins clearly demonstrate the central role of p53 mediating the hematopoietic defect (Barlow, 2010; McGowan, 2011). Furthermore, as demonstrated by McGowan *et al* the level of p53 correlates with the severity of hematopoietic phenotype (McGowan, 2011). Our studies corroborate these findings as the loss of p53 restored the lethal bone marrow failure in the Rps19-deficient mice. Based on these findings it is tempting to speculate that the erythroid defect in DBA is largely caused through a p53-dependent mechanism. Interestingly, the downregulation of RPS19 or RPS14 in primary human bone marrow cells suggested that ribosomal protein deficiency results in an erythroid-pronounced activation of p53, which could partly explain the differential hematopoietic manifestation between humans and mice (Dutt, 2011). Immunohistochemistry for p53 in the bone marrow biopsies of patients with DBA and 5q- syndrome generally confirms the elevated level of p53, although high variation is observed in terms of the intensity and cell type-specificity of p53 staining (Pellagatti, 2010; Dutt, 2011).

The identification of p53 could provide a novel therapeutic avenue for the treatment of DBA and related disorders. Inhibition of p53 with a small molecule pifithrin alpha rescues the erythroid defect of RPS19-deficient and RPS14-deficient human bone marrow cell cultures (Dutt, 2011). Indeed, a transient dampening of the p53 pathway could provide a therapeutic benefit in patients. However, direct interference with p53 raises concerns because of its role as a tumor suppressor. Strategies targeting disease-specific factors either upstream or downstream of p53 could provide a more promising alternative. One such a target could be the zinc finger of the central acidic domain of Mdm2 that is essential for the Mdm2-induced p53 degradation in response to ribosomal stress. Small molecules preventing the binding of RPL5 and RPL11 into this domain could potentially inhibit the activation of p53 in DBA without interfering with its tumor suppressor function. Indeed, the Mdm2^{C305F} knockin mice that harbor a mutation that disrupts the interaction between RPL5 and RPL11 with Mdm2 retain a normal p53 response to DNA damage, but not to perturbations in the ribosome biogenesis (Macias, 2011).

Gene Therapy

Our results presented in article III provide strong evidence for gene therapy as a curative treatment for RPS19-deficient DBA. However, as the current therapies, especially the corticosteroid therapy, have a relatively good outcome, moving gene therapy to the clinic will require a careful assessment of the risk-benefit ratio of

this approach. We envision that the first clinical trials could be applied to patients with a chronic transfusion-dependent DBA.

In the current study we applied SIN lentiviral vectors, in which the potent spleen focus-forming vector (*SFFV*) promoter drives the expression of codon-optimized human *RPS19* cDNA. However, for the future clinical application moderate promoters must be validated, as they are potentially safer with regards to the probability of insertional mutagensis. One such a promoter could be the human phosphoglycerate kinase (*PGK*) promoter. Clinical trials for Fanconi anemia employing similar SIN lentiviral vectors, in which the *PGK* promoter drives the expression of *FANCA* cDNA, are being conducted (Tolar, 2012). However, the elongation factor 1α (*EF1α)* short promoter may prove to be even more viable alternative (Zychlinski, 2008). Furthermore, a lentiviral vector utilizing the *EF1α* promoter combined with the locus control region of β-globin has been shown to allow a constitutive but erythroid-pronounced transgene expression (Montiel-Equihua, 2012).

The safety and efficacy of ongoing clinical trials using the SIN lentiviral vectors will largely determine the future of DBA gene therapy. Although the follow-up time for these trials is still relative short, no severe genotoxic side effects have been reported (Biffi, 2011). The development of a human gene therapy protocol for RPS19-deficient DBA is estimated to take approximately five years.

GATA1

The identification of *GATA1* as a DBA disease gene is highly interesting in terms of its role in DBA pathogenesis (Sankaran, 2012). *GATA1* normally gives rise to both full-length and short protein isoforms due to alternative splicing. However, the mutation observed in two DBA families (three individuals) completely blocks the production of the full-length form. Interestingly, an identical mutation has been previously reported to cause macrocytic anemia (Hollanda, 2006).

This finding raises questions about the role of GATA1 in the patients with ribosomal protein mutations. Ribosome protein deficiency could bias the ratio of how the two *GATA1* isoforms are translated. On the other hand, GATA1 and p53 have been shown to physically interact and antagonize each other (Trainor, 2009).

FUTURE PLANS

Physiological relevance of the RP-Mdm2-p53 pathway in DBA

As discussed above, numerous studies have demonstrated the importance of the RP-Mdm2 pathway in the activation of p53 upon ribosomal protein deficiency *in vitro* (Fumagalli, 2009; Fumagalli, 2012). However, evidence for the physiological role of this pathway in DBA is lacking. To study the relevance of the RP-Mdm2-p53 pathway upon Rps19 deficiency, we will cross the generated mouse models with the $Mdm2^{C305F}$ knockin mice (Macias, 2011). If the activation of p53 in the Rps19-deficient mice is solely mediated through the Rpl5/Rpl11-mediated inhibition of Mdm2, the hematopoietic phenotype of these mice will be rescued to a similar degree observed in the p53-null background. This experiment will answer the question whether the RP-Mdm2 pathway presents a therapeutic target in the treatment of DBA.

Development of clinical gene therapy protocol

Our current study demonstrates the proof of principle that *RPS19* gene transfer can be used to treat RPS19-deficient DBA patients. The next step in the development of a clinical gene therapy protocol will involve the evaluation of weaker and thus potentially safer promoters. Our previous studies suggest that relatively high expression of *RPS19* is needed for the full phenotypic correction. However, weaker promoters may prove to be sufficiently effective when combined with the codon-optimized *RPS19* cDNA. In order to validate these vectors similar experiments to the current study will be performed and these will be combined with studies on primary cells from patients with RPS19-deficient DBA.

Our results demonstrate that enforced expression of *RPS19* confers a profound reconstitution advantage over the untransduced cells *in vivo*. Therefore it is possible that relatively low gene transfer efficiency may be sufficient enough for therapeutic benefit over time. Furthermore, for similar reasons minimal pre-conditioning regimen may be required. In addition to the optimization of the therapeutic vector construct *per se*, we will assess the extent of pre-conditioning required for the engraftment of gene-corrected HSCs.

SVENSK SAMMANFATTNING (SUMMARY IN SWEDISH)

Diamond-Blackfan anemi (DBA) är en sjukdom som normalt drabbar nyfödda barn. Dessa patienter har nedsatt nybildningen av röda blodkroppar vilket medför ett lågt hemoglobin värde och orsakar symptom som trötthet. Ungefär hälften av patienterna uppvisar medfödda missbildningar. DBA patienter har dessutom en ökad risk för att drabbas av cancer. Kortison är den främsta behandlingsstrategin och den lindrar anemin i ungerfär 40% av patienter. Resten av patienterna kräver ständiga infusioner av röda blodkroppar. De här behandlingsmetoderna kan dock eventuellt ge upphov till livsfarliga biverkningar. Benmärgtransplantation är för tillfället den enda botande terapin men kan medföra en rad komplikationer och är dessutom inte tillgänglig för alla patienter. Av en okänd anledning blir ungerfär 20% av patienterna friska av sig själva.

En stor del av DBA patienter (60-70%) har visats ha mutationer i gener som kodar för ribosomala proteiner som är komponenter i ribosomen, cellens proteinfabrik. Ribosomalt protein S19 (RPS19) är den vanligaste sjukdomsgen och den är muterad i en fjärdedel av alla patienter.

Målet med vår forskning är att utvecka nya botmedel för DBA. I arbetet som presenteras i den här avhandlingen har vi konstruerat musmodeller som har en brist på proteinet RPS19 precis som en del av DBA patienter har. Dessa möss visar symptom som anemi och kan användas till att studera både sjukdomsmekanismer och utveckla nya terapier för DBA. Enligt en teori orsakas anemin i DBA av en bristfällig proteinsyntes (translation). Genom att använda våra musmodeller har vi kunnat visa att stimulering av proteinsyntes med en aminosyra leucin lindrar anemi hos dessa möss. Våra resultater är viktiga eftersom de både bekräftar de preliminära fynden som tyder på att leucinrik kost lindrar symptomer hos patienter och motiverar ett behov av en större klinisk studie. I den sista delen av den här avhandlingen använder vi våra musmodeller för att bevisa att genterapi har potential att bota DBA hos patienter med en brist på RPS19. Vi har konstruerat ett modifierat virus som kan föra med sig en normal RPS19 gen in i mössens blodbildande stamceller. Eftersom de blodbildande stamcellerna ger upphov till alla blodceller, inklusive röda blodkroppar, räcker det att korrigera RPS19 genen i dessa celler för att bota anemi. Nästa steg i det här projektet är att upprepa dessa eksperiment med en kliniskt relevant virus vektor för att förbättra säkerhetsaspekterna av denna terapi.

TIIVISTELMÄ (SUMMARY IN FINNISH)

Diamond-Blackfan anemia on harvinainen vastasyntyneiden verisairaus, jossa potilaan kyky muodostaa uusia punasoluja on vajavainen. Tämän seurauksena veren hemoglobiinipitoisuus laskee epätavallisen alhaiseksi, mikä ilmenee tavallisesti kalpeutena ja väsymyksenä. Monet potilaista ovat lyhytkasvuisia ja lähes joka toisella on synnynnäisiä epämuodostumia. Sairauteen kuuluu lisäksi kohonnut syöpäalttius. Diamond-Blackfan anemian ensisijainen hoitomuoto on kortikosteroidihoito, joka korjaa punasolujen tuotantoa noin 40 prosentilla potilaista. Lopuilla potilaista riittävää punasolumäärä ylläpidetään toistuvien verensiirtojen avulla. Molemmat hoitomuodot voivat kuitenkin ajan kuluessa aiheuttaa hengenvaarallisia sivuoireita. Luuydinsiirto on nykyisin ainoa parantava hoitomuoto. Lisäksi noin joka viides potilas paranee itsestään, mutta syytä tälle ilmiölle ei toistaiseksi ymmärretä.

Suurimmalla osalla Diamond-Blackfan anemia potilaista (60-70%) on löydetty mutaatioita ribosomaalisia proteiineja koodaavista geeneistä. Ribosomaaliset proteiinit ovat ribosomin eli solun proteiinitehtaan komponentteja. Ribosomaalinen proteiini S19 (RPS19) on yleisin sairausgeeni, ja sen mutaatiot selittävät noin neljänneksen kaikista tapauksista.

Tutkimukseni ensisijainen tavoite on kehittää uusia Diamond-Blackfan anemian hoitomuotoja. Tämän väitöskirjan ensimmäisessä osiossa olemme kehittäneet uusia hiirimalleja, jotka potilaiden tapaan eivät kykene muodostamaan riittävästi RPS19-proteiinia. Kehitetyt hiirimallit muistuttavat oireiltaan potilaita ja täten ne soveltuvat hyvin sekä tautimekanismien tutkimiseen että uusien hoitomuotojen testaamiseen. Erään hypoteesin mukaan potilaiden punasolujen vajaatuotanto on seuraus tehottomasta proteiinisynteesistä. Käyttämällä kehitettyjä hiirimalleja olemme kyenneet osoittamaan, että proteiinisynteesin stimulointi leusiini-aminohapon avulla tehostaa punasolujen muodostusta. Tuloksemme ovat merkittäviä, sillä ne vahvistavat käsityksen, jonka mukaan leusiini saattaa lieventää potilaiden anemiaa, ja täten ne tukevat suunnitteilla olevia potilastutkimuksia. Väitöskirjan kolmannessa osiossa käytämme kehitettyjä hiirimalleja tutkiaksemme geenihoidon tehokkuutta Diamond-Blackfan anemian hoitomuotona. Tätä tarkoitusta varten olemme kehittäneet geenimuunnellun viruksen, joka kykenee siirtämään mukanaan toimivan RPS19-geenin hiiren verta muodostaviin kantasoluihin. Koska kaikki verisolut, mukaan lukien punasolut, ovat peräisin verta muodostavista kantasoluista, RPS19-geenin korjaus näissä soluissa korjaa myös punasolujen vajaatuotannon. Tuloksemme osoittavat

geenihoidon parantavan hiirimallien anemian pysyvästi ja täten ne todistavat periaatteen, jonka mukaan geenihoito soveltuu RPS19-puutteellisten Diamond-Blackfan anemia -potilaiden hoitoon.

LIST OF PUBLICATIONS NOT INCLUDED IN THIS THESIS

I.   Singbrant S, Karlsson G, Ehinger M, Olsson K, **Jaako P**, Miharada K, Stadtfeld M, Graf T, Karlsson S. Canonical BMP signaling is dispensable for hematopoietic stem cell function in both adult and fetal liver hematopoiesis, but essential to preserve colon architecture. *Blood*. 2010;115(23):4689-4698.

II.   Siva K, **Jaako P**, Miharada K, Rörby E, Ehinger M, Karlsson G, Karlsson S. SPARC is dispensable for murine hematopoiesis, despite its suspected pathophysiological role in 5q- myelodysplastic syndrome. *Leukemia*. Prepublished April 5, 2012; DOI 10.1038/leu.2012.97.

ACKNOWLEDGEMENTS

Science is really a team sport and there are many people who deserve acknowledgement and thanks for their contributions to this thesis and making my time as a PhD student so memorable.

First and foremost, I am deeply grateful to Stefan Karlsson, my supervisor, for all the guidance and support over these years. I highly appreciate the independence and trust you have given me that has allowed me to develop as a scientist.

Johan Richter, my co-supervisor, for always having time for discussions and sharing your clinical expertise.

Johan Flygare, for introducing me to DBA and for your friendship and inspiration. I wish I had a bit of your scientific optimism…

Karin Olsson, for all the help and patience over these years. I truly appreciate it.

David Bryder, for all the practical and intellectual input on my work. Your enthusiasm for science is contagious.

Jonas Larsson, for all the great discussions and input. To me you are the true Drömelvan champion.

Jörg Cammenga, for the discussions that always make me confused on a higher level and for your great humor.

Shubhranshu Debnath, for all the help and taking over the DBA project.

I would like to thank all of my present and past co-workers at the Division of Molecular Medicine and Gene Therapy for creating such an exceptional working environment; my ASH crew Roger, Ken-ichi and Ronan for all the good times; Jens, Kristian and Alex for the gym (sauna) sessions; Matilda and Carmen for introducing me to Malmö nightlife; Göran and Christine for your friendship; Lina, Talia, Zsuzsanna, Praveen, Valli and Roman for being such a nice roommates.

Thanks to all members of the Bryder group; Gudmundur, Martin and Gerd for all the help and fun.

Stefan Karlsson, Johan Flygare and Lina Jansson, for critical reading of this thesis.

Axel Schambach and Christopher Baum, at the Department of Experimental Hematology, Hannover Medical School, for your invaluable contribution to this thesis.

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