Studies of the Ribosomal Protein S19 in Erythropoiesis

BY

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Abstract

Ribosomal proteins are components of the ribosome, the protein synthesis machinery. The ribosomal protein S19 gene (RPS19) is mutated in Diamond-Blackfan anemia, DBA, which is a rare congenital anemia with absence or reduction of erythroid precursors in bone marrow. In this thesis, the role of RPS19 in erythropoiesis is investigated.

A genetic analysis of RPS19 in 24 DBA cases was performed. Four novel RPS19 mutations were identified with evidence of wide clinical expression of the disease.

Due to the clinical overlap in Transient Erythroblastopenia of Childhood, TEC, and DBA, the two diseases may be caused by a common genetic factor. In a study of seven TEC families, all affected shared at least one parental haplotype in the RPS19 gene region. Coding exons of RPS19 were normal for all affected, although mutations in intronic and regulatory sequences are not excluded. This indicates a genetic factor behind TEC and a possible association between RPS19 and TEC.

To investigate the role of RPS19 in erythropoiesis in a mammal, we created a mouse model for the targeted disruption of the homologue Rps19 on the C57BL/6J genetic background. Null mutants are embryonic lethal prior to implantation. The Rps19<sup>−/−</sup> mice, however, are viable with normal development including the hematopoietic system. The Rps19 transcript level in Rps19<sup>−/−</sup> mice is normal. Accordingly, RPS19 protein levels are similar in Rps19<sup>−/−</sup> and Rps19<sup>−/+</sup> mice. This argues for a transcriptional up-regulation to compensate for the loss of one Rps19 allele.

Peripheral blood is normal in Rps19<sup>−/−</sup> mice also on the FVB/NJ strain which argues against strain-specific effects of the Rps19 disruption. Preliminary results indicate a reduced erythroid proliferation in response to erythropoietin in Rps19<sup>−/−</sup> mice, suggesting the requirement of both Rps19 alleles for normal erythroid proliferation under stress. This would support a mechanism by which haplo-insufficiency for RPS19 causes DBA.

Keywords: RPS19, Rps19, erythropoiesis, erythroblastopenia, Diamond-Blackfan, anemia

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“I have little patience with scientists who take a board of wood, look for its thinnest part, and drill a great number of holes where drilling is easy”
–Albert Einstein
List of publications

**Paper I.** Truncating ribosomal protein S19 mutations and variable clinical expression in Diamond-Blackfan anemia  

**Paper II.** Familial transient erythroblastopenia of childhood is associated with the chromosome 19q13.2 region but not caused by mutations in coding sequences of the ribosomal protein S19 (RPS19) gene  

**Paper III.** Targeted disruption of the ribosomal protein S19 gene is lethal prior to implantation  

**Paper IV.** Ribosomal protein S19 in erythropoiesis: Reduced response to erythropoietin in mice with a disrupted *Rps19* allele  
**Matsson H**, Davey EJ and Dahl N. *Manuscript*

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Abbreviations

5’UTR  5’ untranscribed region
A    Adenine
ADA  Adenosine deaminase
BFU-E Burst forming unit – erythroid
bp   Base pair
C    Cytosine
cDNA Complementary deoxyribonucleic acid
CFU-E Colony forming unit – erythroid
CFU-GEMM Colony forming unit – granulocyte erythocyte monocytes macrophage
CFU-GM Colony forming unit – granulocyte/macrophage
CFU-S12 Colony forming unit – day 12 on spleen
DBA  Diamond-Blackfan anemia
DNA  Deoxyribonucleic acid
EPO  Erythropoietin
FACS Fluorescent activated cell sorter
FGF2 Fibroblast growth factor 2
G    Guanine
hrEPO Human recombinant erythropoietin
mRNA Messenger RNA
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
RNA  Ribonucleic acid
rRNA Ribosomal ribonucleic acid
RPS19 Ribosomal protein S19
T    Thymine
TEC Transient Erythroblastopenia in Childhood
TGM2 Transglutaminase type 2
Introduction

The information that determines the characteristics of all living organisms is stored in the DNA, or deoxyribonucleic acid. DNA is composed of a sugar-phosphate backbone with attached bases. The complexity of DNA is based on the order of the four bases guanine (G), adenine (A), thymine (T) and cytosine (C). The structure of the DNA was determined in 1953 by Watson and Crick as an alpha-helical structure (1). Nuclear DNA in man consists of 3.2 billion bases and is stored in an elaborate way to minimize the size of the genome in the cell nucleus. The DNA is wrapped around histone proteins and compressed into structures known as chromosomes. The mitochondria, however, carry its own genome in the form of a single double stranded circular structure. In humans, there are 23 different chromosome pairs including the chromosomes X and Y. The sex of the offspring is determined by the combinations of X and Y in the fertilized egg. The XX combination gives rise to a female and XY to a male. Before cell division the two sets of chromosomes are duplicated by the replication event and are separated from each other. Germ cells harbor only one set of chromosomes and their genetic material have been reduced by half through the process of meiosis.

All the information required to create and sustain the organism is contained within the DNA. This information is stored in approximately 30,000 – 40,000 genes. The near complete sequence of the bases in our genome were presented simultaneously in 2001 by the International Human Sequencing Consortium (2) and Celera Genomics (3). The coding regions of the genes, exons, must be decoded to mediate a specific function in the cell. A gene is processed to pre-messenger RNA, pre-mRNA, by the enzyme RNA polymerase. The pre-mRNA is modified through splicing to excise non-coding DNA sequences between exons to produce the mRNA. The mRNA is transported from the nucleus to the ribosome, the protein producing unit in the cell. The complete protein is assembled in the ribosome by translation of a set of three adjacent bases, codons, into an amino acid. Each codon is recognized by a transfer RNA, tRNA, which will connect the amino acids to each other and complete the protein. The function of a protein depends on the order of the specific amino acids, the two- and three dimensional structures of the protein and modifications of the protein structure after translation. Proteins do not always act alone; they can also function as macro-molecules containing several identical or different types of proteins.
Erythropoiesis

In the bone marrow, red and white blood cells are formed by the process known as the hematopoiesis. The uncommitted stem cells in bone marrow have the potential to give rise to any hematopoietic cell type. During erythropoiesis, stem cells receive signals to undergo cell division and to start differentiation into cells of the erythroid lineage. The different stages of erythropoiesis are represented by specific cell types with different properties. Erythrocytes are developed from the myeloid precursor cell also named CFU-GEMM. During differentiation the primitive erythroid cells will produce hemoglobin, lose their nuclei and change the cell morphology to become mature red blood cells, erythrocytes. The hemoglobin in the erythrocytes has the ability to take up oxygen from the lungs and release it in tissues with low oxygen pressure. The average lifespan of the erythrocyte in human is 120 days and the erythrocytes normally comprise approximately 45% of the peripheral blood volume.

The erythropoiesis is regulated by a variety of factors that determine the survival of cells and the differentiation of premature blood cells into erythrocytes. The commitment of hematopoietic cells to undergo erythroid development is regulated by several transcription factors including TAL1, LMO2 and GATA2 (4). Evidence of the importance of GATA2 in erythropoiesis comes from the analysis of mice lacking GATA2 (GATA2-/-) who dies at embryonic day 10.5 due to a severe reduction in erythroid progenitor cells (5). The cytokine interleukin-3 (IL-3) (6), granulocyte monocytes – colony stimulating factor (GM-CSF) (7), thrombopoietin (TPO) (8) and stem cell factor (SCF) (9,10) are essential for the expansion of primitive erythroid cells known as burst-forming units (BFU-E). It has been demonstrated by in vitro studies that TPO has little effect on erythroid proliferation but rather enhances erythropoiesis by preventing BFU-E cells from programmed cell death, apoptosis (11). A further differentiation into erythroid colony forming unit (CFU-E) is primarily dependent on erythropoietin (EPO) (12,13) see figure 1.

Figure 1. A general schematic representation of erythropoiesis in man. Different cell types and important factors in erythroid differentiation are indicated in the figure.

Stimulation of red blood cell production will normally take place when cells in the kidneys sense that the oxygen pressure in the blood falls below a critical level. A lowered oxygen level will induce the expression of the hypoxia-
inducible factor 1 (HIF-1), which specifically binds to an enhancer in the 3’ region of the EPO gene (14) and induces its transcription. EPO binds to its membrane bound receptor (EPOR) on the surface of erythroid progenitors. The activated EPOR recruits the Janus family tyrosine protein kinase 2 (JAK2) and thereby triggers a phosphorylation cascade leading to the dimerization and nuclear translocation of STAT5, a member of the signal transducer and activator of transcription proteins (15,16). STAT5 will induce the expression of the anti-apoptotic factor Bcl-X_L (17) thereby promoting differentiation and proliferation of erythroid progenitors by preventing the cells from undergoing apoptosis. The importance of EPO signaling is illustrated in mice lacking EPOR or EPO which have normal or increased numbers of BFU-E and CFU-E cells in fetal liver but die at embryonic day 11-15 due to a failure to produce more mature erythroid cells (8,18). GATA1 and its cofactor Friend of GATA1 (FOG1) are needed at a later stage in the erythroid differentiation to promote the production of more mature erythroid cells. GATA1 activates the EPOR promoter to ensure survival of cells stimulated by EPO (19). Studies of mouse models show that murine erythroid cells from yolk sac lacking GATA1 fail to differentiate beyond the pro-erythroblast stage (20) and FOG1^{−/−} mice die at day 10.5-11.5 due to severe anemia with failure of erythroid maturation similar to the phenotype of GATA1^{−/−} erythroid precursors (21).

A normal erythropoiesis is essential for life since a sufficient number of erythrocytes must be produced to replenish dead cells in the circulation. There is also a need of a rapid response to stress such as blood loss or hypoxia. An anemia occurs when the production of erythrocytes no longer support the need for new blood cells in the body. Causes of anemia include iron deficiency, defective response to erythropoietin, increased red cell destruction, infections and non-functional or reduced levels of hemoglobin. Anemic patients have insufficient oxygen supply to tissues in the body and will therefore suffer from tiredness and weakness and if untreated, a severe anemia will result in death. Some anemic syndromes are inherited, and among the most common types is impaired production of alpha-globin (alpha-thalassemia) or beta-globin (beta-thalassemia). The alpha- and beta globins are essential building blocks of adult hemoglobin.

Diamond-Blackfan anemia

Patients with Diamond-Blackfan anemia, DBA, show a congenital deficiency of red blood cell precursors but normal numbers of cells from other hematopoietic lineages (22,23). One or several additional dysmorphic features are present in approximately 30% of patients including facial- and upper limb malformations, prenatal or postnatal growth retardation, renal abnormalities, mental retardation and congenital heart defects (23-25). The
diagnostic criteria in DBA include normochromic and macrocytic anemia in early infancy, normocellular bone marrow with a selective deficiency of red cell precursors (<5% of nucleated cells), absent reticulocyte response and normal leukocyte and platelet maturation with a normal to increased peripheral platelet count (22,23). The clinical symptoms are revealed early in infancy and most cases are diagnosed within the first year (25). Diamond-Blackfan anemia shares common features with Fanconi anemia but cytogenetic analysis (chromosome fragility test) is usually normal in DBA cases. Viral infections such as parvovirus B19 which may cause chronic erythroblastopenia can be excluded by serology tests and PCR analysis of bone marrow cells. Elevated serum levels of adenosine deaminase (ADA) (26,27) are often detected in DBA patients which could further aid in the diagnosis of DBA.

The estimated incidence, approximately 1/200,000 births, makes DBA a very rare disorder. The majority of reported cases are Caucasians but DBA has been recognized also in other ethnic groups including Africans (28,29), Arabs (30) and East Indians (31). Most DBA cases are identified as sporadic but approximately 10% of the patients inherit the disease and both recessive and dominant inheritance patterns are reported for familial DBA (23,32,33). There are also reports of autosomal dominant inheritance with incomplete penetrance (25,34). Both genders are equally affected and familial and sporadic cases do not differ significantly by clinical criteria (24).

Treatment

Approximately 70% of patients respond to corticosteroid treatment, but 30% are steroid non-responders and require regular blood transfusions (25). Both transfusion- and steroid treatments are known to produce adverse side effects and there is a need for new improved treatment regimes for DBA. Bone marrow allografting is used successfully to restore erythropoiesis (35) but the risks are considerable and equivalent to other allogenic bone marrow transplantations. The clinical expression of the disease is highly variable and a proportion of patients undergo spontaneous remission upon treatment (36,37).

Recently reports suggest novel forms of treatments for Diamond-Blackfan anemia patients. Abkowitz et al described a DBA patient that improved her blood hematocrit values when breast-feeding her children. The improvement correlated with the release of prolactin from the pituitary glands during breast-feeding. Treatment with metochlopramide, a drug known to induce prolactin release, was beneficial and the hematocrit value increased nearly two-fold for seven years of treatment. In a follow-up study, three out of nine DBA patients responded to metochlopramide treatment (38). Hamaguchi et al presented a study in which an onco-retroviral vector carrying the normal human \textit{RPS19} was introduced into CD34$^+$ bone marrow cells from four
DBA patients with either a mutated or a deleted \textit{RPS19} allele. Over-expression of the transgene increased the number of BFU-E and CFU-E colonies almost three-fold \textit{in vitro} suggesting that this treatment may be used as a gene-therapy in the future for \textit{RPS19} mutated DBA patients (39).

Genetics

Because of the pathology of the red cell precursors in DBA patients, genes involved in regulation of erythropoiesis are candidates for the disease. Defects in the EPO gene have been ruled out since urinary and serum levels of EPO are high (40) and no auto-antibodies against EPO have been detected (23). Also the genes for EPOR (41), stem cell factor or its receptor (42,43) and interleukin 9 (44) have all been analyzed and excluded as candidates genes for DBA.

Genetic linkage for the DBA phenotype to the chromosomal region 19q13 was found with evidence of genetic heterogeneity (33). Micro-deletions on 19q and investigation of a balanced chromosomal translocation in a girl with DBA allowed for the identification of the ribosomal protein S19 gene (\textit{RPS19}) as a candidate for further analysis (33,45). A subsequent sequencing analysis of the gene revealed mutations in the protein coding regions in 10 out of 40 DBA patients (46). The mutations found were equally distributed among sporadic and familial cases and there are no clear correlation between the type and position of mutations and severity of symptoms in patients. Recent studies show that \textit{RPS19} is mutated in 15 – 22 % of DBA cases (36,37,47-50).

Transient Erythroblastopenia of Childhood (TEC)

TEC occurs in the first years of life and is characterized by a pure red cell aplasia with absence of reticulocytes in peripheral blood (51). The disorder is transient with a complete recovery, usually within 1 - 2 months after diagnosis. Due to the transient nature of TEC, most patients do not require treatment but in some cases blood transfusion is necessary (52). It has been proposed that parvovirus B19 infection play an important role in the onset of TEC. However, this hypothesis has not been confirmed experimentally or clinically (52). The cellular features resemble DBA but differ in that serum adenosine deaminase (ADA) levels are normal and red blood cells are of normal size (26). In addition, no physical abnormalities have been identified in TEC patients (52,53). TEC is mostly sporadic but a fraction of reported cases have a family history of the disease indicating a genetic defect behind inherited TEC. The molecular mechanism behind TEC is currently unknown.
Ribosomal proteins

The protein synthesis machinery in mammals consists of four ribosomal RNA (rRNA) molecules and 80 ribosomal proteins (RPs) and all RP genes have been mapped in the genome (54,55). Together, they build up the small and large subunits of the ribosome and promote the translation of mRNA templates into proteins. The ribosome is a massive structure and contain 5-10 % of all cellular proteins in a typical mammalian cell (54). The different components in the subunits of the ribosomes are assembled in the nucleolus and transported out to the cytoplasm (56). It is believed that the rRNAs are responsible for the basic biochemistry of protein synthesis. The recruitment of ribosomal proteins to the site of protein synthesis may coincide with the appearance of nucleases (57). The ribosomal proteins could therefore have the function to protect the RNA ribosome from degradation. The ribosomal proteins are also thought to have been recruited to the ribosome to facilitate an optimal configuration and folding of the rRNAs thereby promoting speed and accuracy of the protein synthesis process (58).

The genes for ribosomal proteins show high homology between diverse organisms and similar sequence architecture between mammals. In the prokaryote Escherichia coli, the 53 ribosomal genes are arranged into 20 different operons. In contrast, no clustering of ribosomal genes exist in the genome of eukaryotes and both human sex chromosomes and at least 20 autosomes carry one or more ribosomal protein genes (54,59). The RPs are all encoded by a single gene, but for many ribosomal protein genes, the functional copy has generated a number of transcriptional silent pseudo-genes (47,54,60).

In mammals, the control of ribosomal protein expression is primarily on the translational level (61,62). A common feature of mRNA of ribosomal protein genes is the presence of a 5’oligopyrimidine tract (5’TOP) in the transcriptional start site (63). This structure are also found in the eukaryotic elongation factors 1 and 2 (64). The TOP mRNAs are regulated in a growth-dependent manner at the translational level. This regulation acts by a rapid repression of translation of TOP mRNAs during growth arrest, thereby minimizing the energy consumption of the cell. Indeed, most of the energy consumed in the cell is used for generating compounds in the protein synthesis machinery (65). When TOP mRNAs become repressed they shift from polysomes in growing cells to mRNP particles in quiescent cells. The mRNPs are large ribonucleoprotein complexes which form during premRNA splicing and are needed for the transport of mRNAs into the cytoplasm. These complexes can also mediate quality control by prevent translation by the induction of nonsense-mediated mRNA decay (66,67). When cells receive signals from growth factors, the translational machinery must turn to an active state. One of the earliest events detected upon growth factor stimulation is the phosphorylation of ribosomal protein S6 (RPS6). It is now
known that the S6K1 and S6K2 kinases mediate phosphorylation of RPS6, which results in the release of the repressor and initiate translation of the TOP mRNAs (68). This theory is supported by the study of a dominant negative S6K1 mutant which inhibits translation of TOP mRNA after mitogenic stimulation (69).

Ribosomal protein S19

The human ribosomal protein S19 gene is 11 kb and consists of five protein coding exons and a 5′untranslated region (46). RPS19 share common structural features with other RP genes such as the absence of a canonical TATA-box and the presence of a polypyrimidine stretch in the transcriptional start site. The regulation of transcription of most RP genes is still unknown. Recent studies of human and mouse RPS19 genes revealed several consensus regions with significant sequence identity in the 5′ region upstream of the translational start site (70). Using a luciferase reporter assay, the authors show an additive effect of the promoter and one consensus region on promoter activity. Surprisingly, a deletion of a putative site for the transcription factors c-Rel and Rel-a within the consensus region resulted in a three-fold increase in luciferase activity. This suggests the existing of a yet unknown transcription factor that represses the RPS19 promoter activity when bound to its recognition site.

RPS19 encodes the ribosomal protein S19 which consists of 145 amino acids with a predicted molecular weight of 16 kDa (71). The RPS19 proteins are highly homologous among diverse organisms with a 98 % identity and similarity compared to mouse (46). RPS19 is associated with the small (40 S) subunit of the ribosome and binds together with four other ribosomal proteins the eukaryotic initiation factor 2 (eIF2) (72). As expected for a ribosomal protein, RPS19 is expressed in a variety of tissues including hematopoietic tissues as presented in publicly available gene expression profiles of tissues from normal human individuals [GEO GDS181] and [GEO GDS422], accessible at the National Center for Biotechnology Information (NCBI) public database.

The role of RPS19 in erythroblastopenia is still unclear and it is currently the only ribosomal protein to be associated with a human disease. The association of RPS19 with a phenotype confined to erythropoiesis is surprising and raises the question of whether extra-ribosomal functions exist for RPS19 in erythropoiesis.

Extra-ribosomal functions for ribosomal proteins

The ribosomal proteins are now not only regarded as proteins with specific functions in the protein synthesis machinery. It has been speculated that proteins that existed prior to the incorporation into the ribosome may have re-
tained an original and extra-ribosomal function. In such a case, the RPs would have at least one function apart from their specific function in protein synthesis (57).

In *Drosophila melanogaster*, evidence for extra ribosomal functions of RPs has emerged from the identification of mutations in the genes encoding RPS2, RPS6, RPL19 and RPS21 respectively. The RPS2 (*string-of-pearls*) mutations result in an arrest of oogenesis at stage 5 of development (73). Mutations in RPS6 result in hypertrophied hematopoietic organs (74), RPL19 mutants display abnormal wing blade development (75) and down-regulation of RPS21 result in small body size and hypertrophied hematopoietic organs with reduced number of circulating hemocytes (76). Analogous to the *Drosophila* RPS2, RPS6, RPL19 and RPS21 mutants, the clinical features of DBA could suggest extra-ribosomal and tissue-specific functions for RPS19.

There are several reports in the literature of extra-ribosomal functions for human ribosomal protein genes. The *RPS4X* and *RPS4Y* genes coding for the RPS4 isoforms S4X and S4Y were considered candidate genes for the extra-gonadal phenotypes in Turner syndrome (77) but this hypothesis has been rejected in more recent studies (78,79). Interestingly, the existence of two different isoforms of S4 lead to the formation of “male” and “female” ribosomes with *RPS4X* being 10-15% as abundant as *RPS4Y* in male ribosomes (80). The *RPS4X* and *RPS4Y* are interchangeable in the ribosome and the amino acid sequence differences between the isoforms suggest that *RPS4Y* may play a part in determining the sex specific characteristics of males. The ribosomal protein S3 has been implicated in both DNA repair (81) and apoptosis (82). A recent report describes the release of ribosomal protein L13 from the ribosome in response to IFN-γ. Cytoplasmic L13 was able to bind specifically to the mRNA of ceruloplasmin, thereby silencing its translation (83). The L13 protein has therefore been assigned a role in regulating plasma iron homeostasis. Several reports demonstrate extra-ribosomal functions also for the protein RPS19. Previously published results describe a homo-dimer of RPS19 acting as a chemotactic factor in the process of clearing apoptotic cells in rheumatoid arthritis synovial tissue (84-86). In NIH3T3 cells, free S19 bind to fibroblast growth factor 2 (FGF2), which is involved in the differentiation of many cell types (87). This last finding reveals that a proportion of the S19 exists as free molecules in the cytosol and is thus not associated with the ribosome itself. The functional significance of the S19-FGF2 interaction remains to be clarified.

The existence of multiple functions of several human RP genes is intriguing and an increased knowledge of RP gene regulation and functions may in the future unravel several RPs involved in human diseases.
Aims

The overall aim of the studies described in this thesis was to investigate the role of the ribosomal protein S19 gene in erythropoiesis.

In the first paper, the aim was to study the ribosomal protein S19 gene (RPS19) and surrounding regions for genetic defects in a set of DBA cases. The genotype – phenotype relationships were investigated in DBA cases with RPS19 mutations to relate the type of mutation present with the clinical manifestations of the disease.

In the second study, the aim was to investigate whether DBA and the related disorder Transient Erythroblastopenia in Childhood (TEC) are caused by a common genetic factor.

The aim of the third paper was to investigate the role of RPS19 in erythropoiesis in a suitable animal model. This was addressed by genetic and functional studies of mice with a targeted disruption of the murine ribosomal protein S19 gene (Rps19).

The aim with the studies in the fourth paper is to investigate the requirement for two functional Rps19 alleles in erythropoiesis by testing the response to erythropoietin in Rps19+/- mice. The Rps19+/- genotype is also introduced in a different inbred mouse strain to investigate any strain-specific effects of the Rps19 disruption.
Methods

Sequencing analyses
Analysis of the sequence composition in genes is an important tool for determining the genetic cause of inherited diseases. Automated sequencing analysis is performed on PCR fragments of the DNA of interest by the method described by Sanger et al. (88). Fluorescent dideoxynucleotides are used as chain-terminating inhibitors in the cycle sequencing reaction. This procedure will randomly incorporate the fluorescent nucleotide analogues to produce DNA fragments of different sizes which can be resolved on vertical polyacrylamide gels or capillaries. The used of four different fluorescent dyes, one for each type of base, enable the detection of the base sequence in one well or capillary per sample. Sequencing analysis may readily reveal heterozygous or homozygous base substitutions and small deletions or duplications in the amplified DNA. A larger deletion that spans the region of interest will not be detected since no product will be amplified.

Southern blotting
The Southern blotting method invented by Dr. Ed Southern is often used in order to detect larger rearrangements in the genome (89). Genomic DNA can be digested with one or several restriction endonucleases to generate fragments to be size-separated on agarose gels. After separation, the DNA is transferred to filters by capillary blotting and hybridized with a radioactive DNA probe under stringent conditions. The DNA probe will hybridize to complementary sequences within the genomic DNA fragments on the filter. The result of the experiment can be visualized using X-ray films. When compared to reference samples, genomic deletions, duplications or inversions can be detected as restriction sites are lost or created and thereby alter the size of the fragments produced in the restriction digest.

Haplotype analysis
The segregation of chromosomal regions can be studied in families using markers such as polymorphic microsatellite repeats or informative single nucleotide polymorphisms, SNPs. The different sizes or variants of several markers (alleles) along a chromosomal region can be typed and analyzed as blocks of alleles, or haplotypes. The affected and healthy individuals in a family are investigated for shared haplotypes in the area of interest. A chromosomal region segregating with the disease can be found for instance when
affected family members share identical haplotypes not present in healthy individuals for a disease with autosomal dominant inheritance.

**Generation of mice with targeted disruption of a gene**

Mouse models with disrupted genes, “knock-out” mice, can be very important tools for the study of gene functions or disease mechanisms behind human disorders. A mouse model for the disruption of a gene can be created using homologous recombination in embryonic stem cells (ES cells), see figure 2. A linear DNA construct including a selectable marker surrounded by DNA sequences homologous to the specific gene region is introduced into ES cells by electroporation. The electroporated cells are cultured with selective media to enrich for cells with a stable integrated construct. Surviving cells are grown as individual colonies and analyzed for targeted disruption of the gene of interest. ES cells positive for the correct disruption are injected into mouse blastocysts and transferred into oviducts of pseudopregnant female mice. The foster females are mated with males of a specific genetic background, e.g. C57BL/6J, and the offspring produced will be chimeras of the particular genotype from which the manipulated ES cells and blastocysts are derived. The offspring from chimeras will be genotyped to ensure germ-line transfer of the targeted disruption. Further mating with mice from the desired inbred strain can then be performed to obtain mice with the disrupted gene on the desired genetic background.

**Histological analysis of mouse organs**

Pathological abnormalities such as increased apoptosis, cell death or developmental abnormalities can be detected by microscopic analysis of stained sections of mouse organs. Typically, organs are excised, dehydrated and fixed and cut into five micrometer thin sections. Usually, the organ morphology is well preserved and interpretable with help of different staining procedures including for example Giemsa, hematoxylin – eosin or Van Giesen dyes.

**Hematological analysis of peripheral blood**

The blood can be tested for a set of hematopoietic markers to reveal any abnormalities in the hematological system. Peripheral blood samples from euthanized \textit{Rps19} disrupted mice and controls on the C57BL/6J and FVB/NJ genetic backgrounds were analyzed for hemoglobin concentrations, hematocrit, red- and white blood cell numbers, mean corpuscular volume and mean corpuscular hemoglobin concentration. Mice at both three and seven weeks of age were sampled in order to capture the blood status in young and adult mice respectively.
Figure 2. A simplified procedure for generation of mice with a disrupted gene by homologous recombination in ES cells.

Real-time PCR

The relative abundance of *Rps19* transcripts can be quantified with the real-time quantitative PCR method (90). Total RNA from a tissue such as spleen is prepared and subjected to first strand synthesis to obtain one cDNA molecule for each transcript present in the sample. The cDNA products are subjected to PCR with an *Rps19* specific probe with attached reporter and quencher molecules. A probe specific for a housekeeping gene is used in parallel as an internal reference. Intact probes do not fluoresce when the quencher is in close proximity of the reporter molecule. During PCR the probes will hybridize to its targets and be degraded by the polymerase enzyme. This leads to the physical separation of the quencher from the reporter molecule and the release of a fluorescent signal. The amount of fluorescent light released from the reporters is in linear relationship to the abundance of cDNA products in the sample. The signals from the *Rps19* probe are normalized with the reference signal and can be used to determine the relative number of *Rps19* transcripts in each sample. The abundance of the mRNA can be compared between individuals with different genotype or compared between different tissues from each individual.
Fluorescent-activated cell sorting
The relative number of cells of a specific lineage or differentiation stage can be analyzed according to the presence of cell surface markers. These protein markers can be targeted with specific antibodies coupled with fluorescent dyes and analyzed with fluorescent-activated cell sorting, FACS. Erythroid cells from the proerythroblast stage until the mature erythrocytes express the Ter119 cell surface marker. Ter119 positive cells can therefore be counted and used as markers for erythroid progenitor proliferation in bone marrow of mice.

Antiserum production and testing
Antibodies are powerful tools for the analysis of expression levels of specific proteins in tissue samples. Since no RPS19 antibodies are commercially available, polyclonal antiserum against RPS19 was produced in chicken. IgY antibodies were purified from egg yolk of immunized hens by delipification and extraction using an aqueous two-phase system (91). Specific IgYs were further enriched by passing the antiserum over columns with covalent bound recombinant His-tagged RPS19 protein produced in bacteria. The detection of specific protein species from tissues can be performed using the Western blotting technique. Protein extracts from human and mouse tissues were size-separated on polyacrylamide gels by electrophoresis and transferred to nitrocellulose or vinyl filters. To prevent unspecific binding, the filters were blocked by incubation in a protein buffer over night. The filters were incubated with the column eluate from the IgY purification step. After washing, the filters were incubated with horseradish peroxidase (HRP) conjugated anti-IgY antibodies followed by a second wash. A luminal solution is added to the filters and visible light is emitted by the HRP-catalyzed breakdown of luminol. The signals can be captured on chemiluminescence films which are developed using standard techniques.
Present investigations

Paper I: Truncating ribosomal protein S19 mutations and variable clinical expression in Diamond-Blackfan anemia

The gene \textit{RPS19} was earlier found to be mutated in 10 out of 40 examined DBA patients (46). Three different missense mutations were found together with seven mutations that predicts altered translation or truncation of the RPS19 protein. The study also report variable expression of \textit{RPS19} mutations and incomplete penetrance in one DBA family.

In this study, 24 additional DBA cases were screened for mutations in the protein coding regions of \textit{RPS19} by sequencing analysis to further investigate phenotype-genotype correlations and type of mutations present. Four novel mutations were found which all predict truncated forms of the S19 protein.

First, a G\textsubscript{G}A base substitution was found in a patient corresponding to a transition from Tryptophan to a stop codon at amino acid position 33 (Trp33Stop). The patient was diagnosed with severe anemia at two months of age and requires blood transfusions once every month.

The second mutation identified was a C\textsubscript{C}T substitution resulting in an Arginine to stop codon transition at amino acid position 84 (Arg84Stop). The patient receive blood transfusion every second month and presented with anemia at ten month of age.

The third mutation was identified in a girl with DBA and osteosarcoma. A 1 bp deletion at cDNA position 329 was detected which results in a frame shift from codon 103. No stop codon was generated within the protein coding region of \textit{RPS19} by this deletion but it is likely that the protein will be non-functional or degraded in the cell. The patient was blood transfusion dependent before the discovery of the osteosarcoma.

The fourth mutation was found to segregate with the disease in a family with three affected and one unaffected individual. The father and the elder sister was diagnosed with mild anemia at adult age and the younger sister has a severe and transfusion-dependent anemia. The defect in this family is a
complex mutation including a TT to AA base substitution at cDNA position 157-158 followed by a CT insertion at position 160. This result in a Leucine to Glutamine amino acid transition at codon 45 (Leu45Gln) and a frame shift starting from codon 47. The insertion creates a premature stop at codon 76.

DNA from 17 of the 24 probands was available for further studies and a Southern hybridization was performed in order to detect possible genetic rearrangements involving RPS19, which would otherwise be missed by sequencing analysis. The analysis showed a normal hybridization pattern using a 449 bp probe complementary to the RPS19 5’UTR and a 450 bp probe corresponding to exon 1-5 of the RPS19 cDNA. Genetic rearrangements of the RPS19 gene region were excluded as the cause of DBA for the 17 DBA patients.

The results from this study indicate further evidence of the involvement of the gene RPS19 in DBA. No clear phenotype-genotype correlation was demonstrated among the RPS19 mutated DBA patients. The finding of both mildly and severely affected family members sharing the same genotype indicates that the clinical expression of DBA could be highly variable.

Paper II: Familial transient erythroblastopenia of childhood is associated with the chromosome 19q13.2 region but not caused by mutations in coding sequences of the ribosomal protein S19 (RPS19) gene

TEC is a pure red cell aplasia that occurs most commonly between six months and four years of age (51,52). The clinical manifestations at onset of the disease can be difficult to distinguish from DBA although the anemia in TEC is transient and characterized by a complete recovery. Due to the clinical overlap between TEC and DBA patients, we investigated whether the two diseases are caused by mutations in the same gene.

The segregation of microsatellite markers in the RPS19 region on chromosome 19q13.2 was studied in seven TEC families with affected siblings. The parents in two families had a period of transient anemia in childhood which argues for an inherited factor behind the disease. Within each of the seven families, all affected individuals shared at least one parental haplotype. Healthy siblings, when available, did not share this haplotype. Two-point linkage analysis, assuming a penetrance of 95 %, revealed a maximum LOD score of 1.76 at D19S408 for the TEC families if the parents with history of anemia are regarded as affected. The marker D19S408 is located 1.7 Mb telomeric of RPS19 on chromosome 19. If the parents with a history of anemia are treated as phenotypically unknown, the maximum LOD score is
decreased to 1.63 at D19S408 assuming a penetrance of 70%. The time of onset of the disease differed among the affected individuals. This information contradicts the prior hypothesis of a shared environmental factor behind TEC. A subsequent sequencing analysis of the coding regions of \textit{RPS19} revealed DNA sequences corresponding to the normal allele for all affected individuals.

Mutations located in regulatory or intronic sequences of \textit{RPS19} are not excluded to be involved in the onset of familial TEC and DBA. In addition, complete deletions of \textit{RPS19} cannot be excluded in TEC. However, all affected siblings were heterozygous for a marker located 10 kb from \textit{RPS19} which rules out the presence of large deletions of the \textit{RPS19} gene region. The segregation of haplotypes within the chromosomal 19q13 region follows a similar pattern as in familial DBA caused by \textit{RPS19} mutations (34). A positive, although not significant, linkage to the 19q13 region was demonstrated for the seven TEC families. This argues for a gene in the 19q13 region behind the onset of familial TEC. The results also suggest molecular mechanisms other than structural mutations in \textit{RPS19} behind inherited TEC.

**Paper III: Targeted disruption of the ribosomal protein S19 gene is lethal prior to implantation**

The gene \textit{RPS19} is associated with Diamond-Blackfan anemia in man, suggesting a role of \textit{RPS19} in erythropoiesis. To fully understand the disease mechanisms in which mutated \textit{RPS19} causes DBA, a functional study of a \textit{RPS19} defect similar to what is found in DBA patients is needed. We created mice with targeted disruption of the homologue \textit{Rps19} on the C57BL/6J genetic background in order to investigate the effect of the gene disruption in a mammalian model organism. A mouse model for DBA may also provide a platform for possible future gene therapy experiments. In this report, we present the effects of the first disrupted ribosomal protein gene in a mammalian animal model.

A targeted disruption of protein coding exons 1-5 in \textit{Rps19} was created by homologous recombination in embryonic stem cells. Germ line transfer of the disrupted gene was obtained from one female chimera and \textit{Rps19}+/- mice were continuously bred on the C57BL/6J genetic background in order to investigate the effect of the gene disruption in a mammalian model organism. A mouse model for DBA may also provide a platform for possible future gene therapy experiments. In this report, we present the effects of the first disrupted ribosomal protein gene in a mammalian animal model.

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Analysis of embryos showed that no \textit{Rps19}+/- blastocysts were found on gestation day 3.5 among 14 investigated blasto-
cysts after mating of Rps19+/− mice. This indicates that a complete loss of RPS19 expression is lethal prior to implantation in C57BL/6J mouse.

In order to analyze for gross organ malformations, heart, kidney, liver, spleen and lung from Rps19+/− mice were sectioned and stained with a variety of dyes. Microscopic studies of the stained sections revealed no tissue abnormalities among heterozygous mice when compared to control littermates. Peripheral blood was analyzed for three and seven weeks old Rps19+/− and Rps19+/+ mice respectively. Hemoglobin concentration, red- and white blood cell number, hematocrit, mean corpuscular volume and mean corpuscular hemoglobin concentration were all normal for Rps19+/− mice.

Cells from the proerythroblast stage to mature erythrocytes in bone marrow express the cell surface marker Ter119. FACS analyses of bone marrow samples from femur revealed that Ter119 positive cells of Rps19+/− mice did not differ significant from controls and also the level of apoptosis among Ter119 positive cells were normal. Bone marrow cells from Rps19+/− and wild type littermates were cultured in vitro to study the proliferation ability of primitive hematopoietic cells. Colonies of primitive erythroid cells (BFU-E) and myeloid cells (CFU-GM) were scored after seven days of culture on methylcellulose. Also a test of erythroid progenitor re-population ability was performed. Bone marrow cells from Rps19+/− and Rps19+/+ mice were injected into radiated mice and the number of colony forming units (CFU-S12) on the spleen were counted after 12 days. No significant differences in BFU-E, CFU-GM or CFU-S12 colony numbers were seen for Rps19+/− mice compared to wild type mice suggesting normal erythroid and myeloid progenitor proliferation. Analysis of the relative Rps19 mRNA levels in spleen by real-time PCR showed no clear difference between Rps19+/− and Rps19+/+ mice.

This study describes the creation and analysis of a mouse model for the disruption of Rps19. Absence of Rps19 is early embryonic lethal in mouse. Surprisingly, Rps19 transcript levels in Rps19+/− mice were similar to wild type mice. A disrupted Rps19 allele in mouse does not produce any pathological effect and this could possible be explained by an up-regulation of the level of Rps19 mRNA to compensate for the loss of one Rps19 allele.

Paper IV. Ribosomal protein S19 in erythropoiesis: Reduced response to erythropoietin in mice with a disrupted Rps19 allele

C57BL/6J mice with a targeted disruption of Rps19 have a normal growth and development including the hematopoietic system. The effect of the disrupted Rps19 can however be influenced by polymorphisms or other genetic
alteration specific for the type of mouse strain used. We hypothesize that a transcriptional up-regulation of \textit{Rps19} mRNA can compensate for the loss of one \textit{Rps19} allele. We also hypothesize that absence of an \textit{Rps19} allele may have an effect on erythropoiesis under strong stimulation.

To control for strain specific phenotypes, we introduced the \textit{Rps19}^{+/−} genotype in mice of the FVB/NJ strain. Results from peripheral blood analyses on FVB/NJ \textit{Rps19}^{+/−} mice show no significant difference compared to normal controls. Normal peripheral blood in \textit{Rps19}^{+/−} mice on two distinctly different inbred mouse strains argues against strain specific effects of a disrupted \textit{Rps19} allele.

Polyclonal antiserum against RPS19 was produced in chicken. Specific IgYs were extracted from egg yolk and affinity purified with the help of recombinant RPS19 produced in \textit{Escherichia coli}. The RPS19 antiserum detects a protein with a molecular weight of approximately 18 kDa consistent with RPS19 in protein extracts from both human and mouse tissues. Measurements of the abundance of RPS19 protein by Western blotting reveal similar levels in \textit{Rps19}^{+/−} mice and wild type mice bone marrow. This is analogous to the normal \textit{Rps19} transcript level in \textit{Rps19}^{+/−} mice as previously reported.

Both alleles may be needed in order to produce sufficient RPS19 protein in rapid proliferating erythroid cells. To address this question, the proliferation of erythroid cells in bone marrow was investigated after stimulation by erythropoietin. C57BL/6J \textit{Rps19}^{+/−} and \textit{Rps19}^{+/+} mice were injected with 200 IE human recombinant erythropoietin (hrEPO) for two consecutive days. At day five after the first injection, bone marrow samples were collected and analyzed by FACS analysis for the erythroid specific cell surface marker Ter119. Preliminary results from two independent experiments indicate a reduced response to hrEPO in \textit{Rps19}^{+/−} mice compared to control littermates. The number of Ter119 positive cells in \textit{Rps19}^{+/−} mice is approximately 50% of that of wild type mice. The results show a similar, but less pronounced insensitivity to EPO as described for erythroid cells in DBA.

Previous analyses show normal levels of \textit{Rps19} transcripts in spleen in \textit{Rps19}^{+/−} mice. Also, the RPS19 protein levels are similar in \textit{Rps19}^{+/−} mice compared to wild type controls. These findings support the hypothesis of a gene regulation at the transcriptional level to compensate for the disruption of an \textit{Rps19} allele. The reduced response to EPO indicated by the preliminary results need to be verified with additional experiments. Despite the absence of any measurable differences in \textit{Rps19}^{+/−} levels, both alleles may be required for erythroid cell proliferation in bone marrow under stress. This would support a mechanism by which haplo-insufficiency for RPS19 causes DBA.
Discussion

The results in the first study in this thesis further support \textit{RPS19} as a disease gene in DBA. The clinical expression of DBA was found to be highly variable as both mildly and severely affected family members inherited the same \textit{RPS19} defect.

The overlapping clinical phenotypes of DBA and TEC and the identical inherited haplotypes in the \textit{RPS19} gene region in TEC families may be due to a common genetic factor behind both diseases. Mutations in intronic sequences or regulatory elements of \textit{RPS19} are not excluded in TEC cases. It is therefore possible that TEC and DBA are allelic, although there is to date no clear explanation of why the anemia is transient in TEC cases.

A targeted disruption of an \textit{Rps19} allele in mouse does not produce any detectable abnormalities. One explanation for the lack of phenotype could be that the molecular mechanisms controlling erythropoiesis in mouse differ from that in humans. The RPS19 protein may also possess an extra-ribosomal function in humans confined to erythropoiesis which is not present in the mouse. Another and perhaps more likely explanation could be the existence of compensatory mechanisms for the loss of an \textit{Rps19} allele on a transcriptional or a translational level in mouse. A transcriptional up-regulation of \textit{Rps19} is supported by the normal \textit{Rps19} mRNA and protein levels detected in C57BL/6J \textit{Rps19}+/- mice. Evidence of normal \textit{RPS19} transcript levels is also reported for \textit{RPS19} mutated DBA patients including one patient with a missense mutation and a second individual with a complex mutation involving double base substitutions and an insertion of two bases in \textit{RPS19} which predicts a truncated protein (39).

The ribosomal proteins are known to be regulated on the translational level (63,68,69). There is now also evidence of transcriptional regulation by the binding of RNA transcripts complementary to regulatory sequences at 5′ untranslated regions within ribosomal genes. The anti-sense strand of the first intron of the \textit{RPS14} gene encodes two non-coding RNA molecules, both complementary to a region in the 5′ UTR of \textit{RPS14}. The RNA molecules promote transcription of the gene whereas the S14 protein acts as repressor of transcription (92). The presence of a similar regulation of transcription in \textit{Rps19}+/- mice may explain normal levels of \textit{Rps19} transcripts in \textit{Rps19}+/- mice despite the loss of one allele.
Primitive blood cells from \textit{RPS19} mutated DBA patients do not respond to the erythroid growth factor EPO (39). C57BL/6J \textit{Rps19} \textsuperscript{+-} mice show reduced response to erythropoietin compared to wild type littermates. This result indicate a similar, but less pronounced, insensitivity to EPO as described for erythroid cells in DBA (93,94). If the RPS19 protein is an important regulator of erythropoiesis and involved in the EPO response pathway, the loss of an \textit{Rps19} allele may first result in reduced RPS19 production in bone marrow under stress. A reduced RPS19 expression may influence the EPO-mediated signaling pathway and lead to a reduced production of anti-apoptotic factors. This may in turn result in a block in erythroid differentiation and proliferation due to increased apoptosis. To address this question, we will determine the RPS19 levels in bone marrow of EPO injected \textit{Rps19} \textsuperscript{+-} and control mice in parallel with the analyses of erythroid proliferation. This will enable us to correlate the RPS19 protein level in each animal to their response to hrEPO stimulation. RPS19 may form protein complexes with proteins known to be involved in the signal transduction regulated by EPO. Therefore, proteins such as JAK2, STAT5a and STAT5b will be candidates for expression studies by Western blotting using mouse bone marrow samples from EPO-injected mice.

As a part of the ribosome biogenesis, ribosomal proteins are transported into the nucleus and are together with ribosomal RNA integrated into the ribosomal subunits in the nucleolus. A mutated or truncated RPS19 protein may lose the ability to be integrated into the small ribosomal subunit. This could lead to decreased rate of production of normal ribosomes with a negative effect in tissues with a high proliferating rate. Recently, two nucleolar localization signals (NoS) have been identified for the RPS19 protein in subcellular localization studies using truncated forms of RPS19 produced by C- and N terminal deletion constructs (95). Two mutations, each localized to one of the two NoS, were previously identified in DBA patients and these caused failure to localize RPS19 to the nucleolus. Normally, a vast amount of erythrocytes are produced each day in the bone marrow to compensate for the erythrocytes that have reached the end of their life span. A reduced production of ribosomes due to mutated RPS19 could lead to a decrease in red cell proliferation. Mutations in other ribosomal genes may also reduce ribosome levels in the cell. Apart from RPS19, only the genes for ribosomal proteins S3a, S13, S16 and S24, which all are involved in the binding of the eukaryotic initiation factor 2, have been investigated. No mutations were found by sequencing analysis of cDNA for these ribosomal proteins in 14 DBA patients (96).

Approximately 20\% of DBA patients present with a mutated \textit{RPS19} allele. A number of mutations in intronic or regulatory sequences within the gene may have escaped identification. However, the low incidence of RPS19 mutations in DBA suggests the existence of more than one gene behind DBA. The observed variable clinical expression and the genetic heterogene-
ity also suggest several genetic factors in the etiology of DBA. There are, to date, no other genes associated with DBA apart from \textit{RPS19} and the gene(s) behind DBA in cases without \textit{RPS19} mutations are still unknown. A recent study present genetic linkage in 18 small DBA families to a 8.1 cM region on chromosome 8p23.3–p22 (97). No candidate gene for DBA has yet been identified within this region. The same study also present seven families not linked to either chromosome 19q or 8p suggesting the existence of at least three disease genes behind DBA. Linkage analysis of large DBA families may still present new candidate genes for the disease in the future. This approach is dependent on accurate phenotypic criteria for affected individuals. Therefore, linkage analysis can sometimes be complicated due to incomplete penetrance in families and the highly variable clinical manifestations of the disease.

Recent research has increased our knowledge about the mechanisms behind DBA. It is likely that DBA is caused by defects in several genes. This is supported by the finding of linkage in DBA families to two different chromosomal regions and additional families not linked to any of the regions (34,97). One of the key characteristics of DBA is the specific defect in the development of erythroid progenitor cells. The block in erythroid cell development is demonstrated by the impaired proliferative capability of CD34$^+$ CD38$^-$ cells from DBA patients (98). In this report, the number of hematopoietic progenitor cells from bone marrow of DBA patients was found to be normal. However, the number of erythroid progenitor cells were significant reduced compared to normal. What are then the molecular mechanisms behind the defect of erythroid development in DBA? One scenario could be that absence or reduced expression of transcription factors acting in hematopoietic precursors could fail to promote erythroid cell differentiation. Also, defects in signal transduction leading to increased apoptosis of DBA cells could perhaps explain the EPO resistance of erythroid progenitors.
Future directions

One approach to learn more about the S19 protein functions in erythropoiesis is to study the phenotypic effect of altered *RPS19* expression in an *in vitro* system using cells capable of erythroid differentiation. There are indications of a down-regulation of *RPS19* expression during erythroid differentiation in murine erythroblasts from spleen (70). A construct containing both human *RPS19* cDNA and a drug resistance gene driven by a strong human promoter can be used as expression vector. Cells can be selected for stable expression of the *RPS19* gene. With this tool it is possible to study the effect of *RPS19* over-expression on differentiation and proliferation and investigate possible extra-ribosomal functions of *RPS19* in cells during erythroid differentiation.

Expression of small interfering RNA (siRNA) can effectively inhibit translation by initiating specific degradation of mRNA complementary to the siRNA sequence in cell culture systems (99). A system inducing a strong suppression of *RPS19* mRNA could be used in human cell cultures capable of erythroid differentiation. If a vector coding for *RPS19* siRNA are under the control of an inducible promoter, the cells could be investigated at different time-points during differentiation with or without suppression of *RPS19* mRNA levels. These experiments could provide information on the level of S19 transcript and S19 protein required for cellular survival as well as the role of *RPS19* during erythroid differentiation.

Instead of disrupting one *Rps19* allele at the genomic level, the *Rps19* mRNA could be suppressed with siRNA complimentary to the gene sequence. Suppression of the target mRNA has been successfully performed in mouse ES cells to create a mouse model with stable expression of the siRNA (100,101). A vector including *Rps19* siRNA under the control of an inducible promoter can be integrated into the genome of mouse ES cells. The ES cells are then used to create transgenic mice carrying the siRNA construct. By using an inducible system, the expression of the transgene can be activated in adult mice thereby avoiding the potential lethal effect of a strong suppression of *Rps19* transcripts during embryonic development. Different siRNA sequences can be tested in an appropriate cell system for optimal degree of suppression of *Rps19* mRNA before the use in mouse.

The identification of protein-protein interactions involving RPS19 would be valuable for the identification of binding partners and new cellular pathways involving the RPS19 protein. Genes for RPS19-binding proteins could also be candidates for sequencing analysis to identify mutations or functional
polymorphisms affecting the functions of the proteins. The potential functions of a protein complex involving RPS19 could be facilitated through weak or very brief protein-protein interactions. To circumvent this problem, cellular protein-protein interactions can be stabilized with the membrane soluble chemical crosslinker dithibis-succinimidylproprionate (DSP) as previously reported (102). Cell lysates containing crosslinked proteins can be immuno-precipitated with RPS19 antibodies. The proteins interacting with RPS19 can be further identified with a combination of 2D-gel electrophoresis and mass spectrometry.

Identification of proteins binding to RPS19 mRNA could give new insight in the regulation of the RPS19 protein expression. RNA-protein interactions can be studied using gel mobility shift assays previously described (103,104). Cellular protein extracts from cells capable of erythroid differentiation can be incubated with radioactively labeled RPS19 mRNA. Subsequent addition of RNase T1 and heparin will degrade labeled mRNA bound non-specifically to proteins and displace proteins bound non-specifically to the RNA respectively (104). The RNA-protein complexes can be resolved on native polyacrylamide gels and visualized using X-ray films. By this means, RPS19 mRNA-protein interactions can be studied in cell lines under different erythroid development stages in order to gain information on RPS19 regulation in erythropoiesis. The identity of proteins interacting with RPS19 mRNA can be determined by iso-electric focusing and mass spectrometry. If a protein interacting with RPS19 mRNA is identified in this way, manipulations of the RPS19 mRNA can be done to reveal sequences acting as binding sites for the interacting protein. A series of truncations of the RPS19 mRNA can be made with restriction endonucleases and tested with the method described above to map the protein binding region. This system will also be useful for the study of the impact of previously reported RPS19 missense mutations on the RNA binding ability of the interacting protein.

One important reason for the creation of the mouse model for the Rps19 disruption was to obtain a platform for the development of future gene therapies. Mouse models of human diseases have been important tools for the development of efficient gene therapy treatments (105). If significant reduction of erythroid proliferation in response to EPO is demonstrated with repeated experiments in C57BL/6J Rps19+/- mice, these animals could be used as a model system for gene transfer experiments. The defect in erythropoietin response in Rps19+/- mice may be corrected by a lentiviral-mediated gene transfer of normal Rps19 into hematopoietic stem cells by similar methods previously described for the over-expression of RPS19 in CD34+ hematopoietic progenitors from DBA patients (98). The effects of an Rps19 gene transfer could be investigated in vivo and perhaps lead to the establishment of an efficient and safe gene therapy protocol. Except for bone marrow allografting, no other efficient and safe treatment could establish a normal erythropoiesis in DBA patients. The development of an efficient and safe
gene therapy regimen would be beneficial for RPS19 mutated DBA patients who suffer from severe side effects from their current treatment.

Linkage analysis of DBA families can be complicated by genetic heterogeneity and reduced penetrance in families. Instead of studying one gene at a time, a different approach to identify new genes for DBA is to use DNA microarrays (106,107). With this method, it is possible to detect abnormal under- or over-expression of genes in samples from DBA patients compared to healthy controls. Since the main defect in DBA is confined to erythropoiesis, bone marrow cells from DBA patients will be the most interesting tissue for the expression analysis. Reverse transcription of total RNA is used to produce cDNA labeled with different fluorescent dyes for the test sample and control. The cDNA from both test and control are pooled and allowed to hybridize onto glass slides containing thousands of spotted oligonucleotides, each representing individual genes. The signal strengths from each spot are dependent on the number of corresponding transcripts present in the subject and the control respectively. Expression data can be clustered to arrange genes included on the DNA array according to the cellular functions (108). Analysis of gene expression with DNA microarray can provide valuable data which could aid researchers to identify new DBA genes or identify cellular processes involved in the disease.
Swedish summary

Studier av Ribosom-protein S19 i erytropoesen


I det första arbetet undersöks 24 DBA patienter för förändringar i genen \textit{RPS19}. Fyra tidigare okända typer av förändringar identifierades vilket bekräftar att \textit{RPS19} har en roll i uppkomsten av DBA i dessa patienter. Det påvisades också att svårigheten av symptomen kan variera kraftigt även bland individer inom en familj som nedärver identiska förändringar i \textit{RPS19}.

Individer som har varit drabbade av en typ av anemi (TEC) som liknar DBA, men är spontant övergående, undersöks för samma typ av sjukliga förändringar som identifierades för DBA. I sju familjer med TEC nedärvs ett område på kromosom 19 som innehåller \textit{RPS19} intakt bland alla drabbade. De delar av genen \textit{RPS19} som kodar för proteinet visade sig vara normala men det kan inte uteslutas att en viktig faktor för sjukdomen finns i regionen. Sammantaget visar detta på en sannolik gemensam genetisk faktor bakom TEC och DBA.

För att kunna studera betydelsen av \textit{RPS19} i blodbildningsprocessen i ett däggdjur användes en musmodell där bara en kopia av normalt två av motsvarande gen i mus (\textit{Rps19}) är funktionsduglig. Möss med bara en kopia har en normal tillväxt och utveckling inklusive blodbildningsprocessen. Även blodbildande celler från benmärg återfinns i normala antal samt växer normalt i odling utanför kroppen. Frånvaron av defekter i möss med bara en normal \textit{Rps19} gen kan bero på en kompenserande mekanism som korrigerar mängden av genprodukten till det normala. Möss med en total avsaknad av
genen, däremot, föds inte levande utan dör mycket tidigt i fosterstadiet. Detta betyder att genen är essentiell för överlevnad.

Normalt stimuleras nybildning av blodceller i benmärg i både mus och människa av tillväxtfaktorn erytropoetin, EPO, som utsänds från njurarna vid syrebrist i blodet. Primitiva blodceller från DBA patienter reagerar inte på EPO. Vi testade om en liknande defekt finns hos möss med en utslagen kopia av Rps19. Preliminära resultat visar att möss med en utslagen kopia av Rps19 reagerar sämre än normalt på EPO. Tillväxten av tidiga blodceller i benmärg är bara hälften så stor jämfört med normala möss av samma stam. Denna defekt är inte lika allvarlig men liknar den i celler från DBA patienter där EPO inte har någon effekt alls på blodbildningen. Dessa preliminära resultat antyder att båda genkopiorna av Rps19 behövs för normal blodbildning under kraftig stimulering i mus. Resultaten främjar även hypotesen om att frånvaron av en funktionell genkopia hos människa kan orsaka DBA.

Sammantaget visar dessa studier att genen RPS19 är nödvändig för normal blodbildning. RPS19 är inblandad i sjukdomsförloppet bakom Diamond-Blackfans anemi och det finns indikationer på att genen kan vara inblandad även i den mildare och övergående sjukdomen TEC. En frånvaro av en kopia av motsvarande gen i mus är inte tillräcklig för att utveckla anemi men denna musmodell kan bidra till att öka förståelsen av sjukdomsförloppet bakom DBA och utveckling av framtida terapiformer för sjukdomen.
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine*. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)