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Animal models of Diamond Blackfan Anemia

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Abstract

Diamond Blackfan anemia is a genetic syndrome characterized by red blood cell aplasia in association with developmental abnormalities such as growth retardation, orofacial, hand or limb malformations, urogenital anomalies and heart defects. The only known cause is heterozygosity for mutations in genes encoding ribosomal proteins. Understanding how defective ribosome biogenesis and function, important for all cells, causes defects in erythropoiesis and tissue-specific phenotypes during development is paramount to the evolution of effective treatment protocols. Here, we discuss how animal models based on mammals, insects and fish replicate genetic or developmental aspects of DBA and have led to the identification of pathways and candidate molecules that are important in the pathogenesis of the disease. A recurring theme in many of these models suggests that defective ribosome biogenesis induces a p53-dependent cell cycle checkpoint in cells that require high levels of ribosome production and leads to cell type-specific, whole animal phenotypes.

Introduction

Animal models of human disease are used to investigate questions, test hypotheses and examine therapeutic outcomes that are difficult to address in the clinical setting. A good animal model should (1) accurately reproduce the disease phenotype, response to current therapies and overall prognosis, (2) recapitulate the genetic abnormalities observed in human patients and (3) utilize the same molecular pathways exploited by the disease¹. Although it may be difficult to meet all of these criteria, scientists frequently choose a model to study human disease while recognizing its implicit caveats and limitations. Fortunately, our understanding of Diamond Blackfan anemia (DBA) and other inherited bone marrow failure syndromes has matured in recent years with the help of robust, genetically-tractable animal models.

DBA is human syndrome characterized by erythrocyte aplasia in association with growth retardation, limb, cardiac and/or craniofacial malformations. Most patients exhibit anemia, macrocytosis, reticulocytopenia, and a paucity of red blood cell precursor cells within a normocellular marrow^{2,3}. Other features of the disease include elevated erythrocyte adenosine deaminase activity and an elevated fetal hemoglobin concentration. Current

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therapies include supportive red blood cell transfusion and corticosteroids; stem cell transplantation is the only definitive treatment for the hematologic features of DBA, yet can be complicated by donor suitability and the toxicity of myeloablative regimens⁴. With the current therapeutic armamentarium, the overall survival of DBA patients is 75% at 40 years of age, a number that is negatively affected by a patient's transfusion requirements^{4, 5}.

Ribosomal protein mutations have been identified in individuals with DBA⁶. In most patients, ribosomal protein mutations are heterozygous and sporadic, however, inherited forms of DBA exist in which ribosomal protein mutations segregate in an autosomal dominant manner². Mutations in *Ribosomal Protein S19* (*RPS19*), *RPS24*, *RPS17*, *RPS10*, *RPS26*, *RPS7*, *RPL5*, *RPL11* and *RPL35A* account for approximately 50% of the known genetic alterations in affected individuals^{3,6}. Many mutations result in ribosomal protein deficiency and, thus, implicate ribosomal protein haploinsufficiency in the pathogenesis of DBA³. Recent work in mice suggests, however, that a portion of ribosomal protein mutations may act through a dominant negative mechanism⁷.

Spontaneous and induced ribosomal protein mutations have been identified or created in a number of model organisms^{7–16}. Studies based on these models suggest that ribosomal protein mutations compromise ribosome biogenesis, protein synthesis, cell division and cell survival^{12,15,17,18}. While some DBA phenotypes (such as growth retardation) may be the direct result of impaired cell cycle dynamics, it is not known how mutations that impair translation and cell cycle kinetics in every cell of an organism yield tissue-specific phenotypes, such as limb abnormalities and anemia. Here, we will focus on novel mouse, fly and fish models, and will highlight how these tools have shed light on the pathogenesis of DBA and identified new targets for therapeutic intervention.

Mouse models to study the effects of *RPS19* mutations

Rps19 knockout mice

In 1999, Drapchinskaia and colleagues⁶ identified mutations in *RPS19*, a gene encoding a ribosomal protein, in a proportion of DBA cases. Not surprisingly, their observations posed more questions than they answered: how do mutations in a ubiquitously distributed protein with a well understood role in protein translation lead to a disease with tissue-specific phenotypes such as anemia? Did *RPS19* have a specific role in translation in the red blood cell or did it have an extra ribosomal function? A mouse model might allow these questions to be approached and in 2004, a *Rps19* knockout mouse was reported¹³ (Table 1).

A null *Rps19* allele (*Rps19^{ko}*) was created by replacement of coding exons with a selectable neomycin cassette¹³. As would be expected for an essential gene, no *Rps19^{ko/ko}* animals were recovered from *Rps19^{ko/+}* intercrosses (even when 3.5 day old blastocysts were examined), suggesting that homozygosity for *Rps19* leads to early embryonic lethality. Unfortunately, however, *Rps19^{ko/+}* mice did not recapitulate the DBA phenotype: heterozygous animals did not exhibit developmental defects, differences in hematological parameters, bone marrow composition or hematopoietic cell *in vitro* colony forming ability compared to *Rps19^{+/+}* animals. In addition, *Rps19* hemizygosity had no effect on erythrocyte ADA activity or response to stimulation by erythropoietin¹⁴. Curiously, *Rps19* mRNA and protein levels from spleens of control and mutant animals were similar. Breeding the *Rps19^{ko}* allele into a second genetic background did not alter the phenotype and lead investigators to conclude that *Rps19* mutant mice were able to compensate for the loss of one *Rps19* allele in a way that DBA patients were not.

As well as sounding a warning to those embarking on genetic engineering projects in the mouse, these results suggested that compensation mechanisms observed in the mouse may

be at play in DBA patients. Could these pathways account for the variable penetrance observed in DBA pedigrees where the same genetic lesion can have different phenotypic outcomes? In this light, efforts to understand the compensatory mechanism would be instructive: is *Rps19* mRNA synthesized at a faster rate or is it more stable in these animals?

Xenotransplantation in DBA

Although the genetic data implicating *RPS19* in DBA were compelling, the molecular mechanism underlying the specific phenotypes in affected patients remained puzzling. Definitive proof for a role of *RPS19* in the pathogenesis of DBA was achieved using *in vitro* and *ex vivo* approaches. Knocking down *RPS19* expression in human bone marrow cells (CD34+) impaired erythropoiesis *in vitro*^{19, 20} and could be rescued by ectopic expression of *RPS19* or dexamethasone treatment. Similarly, erythropoietic defects observed in DBA patient cells (CD34+) could be partially corrected by *RPS19* overexpression²¹. Conclusive evidence connecting *RPS19* and DBA came from experiments in which CD34+ cells mobilized from patients with known *RPS19* haploinsufficiency were infected with a *RPS19* retrovirus and transplanted into NOD/SCID immunodeficient mice (a strain of mice that permits the engraftment and proliferation of human blood cells) (Table 1). Infected cells expressing high levels of *RPS19* showed improved engraftment and erythropoiesis compared with uninfected patient cells²² or those expressing low levels of *RPS19*²³. Thus, mounting evidence linked *RPS19* haploinsufficiency and DBA, and proposed a role for gene therapy in the treatment of DBA.

A dominant negative *Rps19* mouse model

Pathogenic deletions, nonsense or frame shift mutations in ribosomal protein genes often lead to reduced protein expression in DBA patients²⁴ and implicate haploinsufficiency in the disease. Intriguingly, however, many DBA mutations are missense²⁵ and posit a role for a dominant negative mechanism. In these patients, mutant proteins are predicted to disrupt ribosome biogenesis and/or the function of mature ribosomes to bring about the phenotype.

To explore a role for a dominant negative mechanism in DBA, a transgenic approach was employed by Devlin and colleagues: a pathogenic *RPS19* mutation, R62W, was introduced into mice⁷. Ubiquitous expression of the transgene was lethal in early embryogenesis, but inducible expression at later time points during development phenocopied DBA. Growth retardation, mild anemia, and reduced erythroid progenitors and circulating red blood cells characterized transgenic animals. In addition, ribosomal RNA processing defects were detected in bone marrow cells. Gene expression analysis showed, among other changes, a significant decrease in *Kit* mRNA. Remarkably, the expression of the *Rps19*^{R62W} was only 10% of the expression of the endogenous *Rps19* mRNA, and thus a more robust phenotype may be observed if expression of the transgene is titrated. These results suggest the R62W mutation may produce its effect by a dominant negative mechanism, acting to inhibit or delay ribosome biogenesis.

Knocking down *Rps19* in mouse erythropoiesis

A number of investigators have proposed that erythrocyte development is particularly sensitive to mutations affecting ribosomes because developing red blood cell progenitors produce substantial amounts of hemoglobin, a process that requires a large pool of mature ribosomes and biosynthetic capacity. Sieff and colleagues explored these ideas using a culture system in which embryonic mouse liver cells are harvested at day 14.5 (E14.5, a time point in which the mouse liver is enriched for erythroid progenitors) and cultured under conditions which support erythroid development²⁶. During the first 24 hours of culture, erythroid cells divide rapidly and RNA production exceeds the increase in cell number. Knocking down *Rps19* in these cells impairs proliferation, but does not affect

differentiation. Cells also accumulate in G1 and are characterized by increased p53 (mouse protein, also known as TRP53) and, as previously observed in DBA patient cells²⁷, decreased *Myb* and *Kit* expression compared to control cells. This system recapitulates several features of DBA and suggests that ribosomal protein mutations impair erythropoiesis via p53-induced cell cycle arrest. In addition, these studies point to a role for KIT signaling in DBA pathogenesis.

An *Rps19* missense mutation in mice with dark skin

During the course of a forward genetic screen in mice for defects in skin color, McGowan and colleagues¹⁶ identified a dark skin mutant (*Dsk*) that harbored a missense mutation in *Rps19*. The *Rps19*^{*Dsk3/+*} animal is characterized by a dark tail, ears and feet, a white belly spot, growth retardation and mild, macrocytic anemia (a ~5–10% reduction in red blood cell count). Based on transcriptional changes in the skin, p53 activation was identified as a critical event linking ribosomal protein mutations to changes in skin color: p53 is both necessary and sufficient for dark skin. Intriguingly, deletion of p53 from the bone marrow of *Rps19*^{*Dsk3/+*} (by crossing to the *Trp53*^{*ko*} allele) also mitigated the anemia phenotype. Thus, stabilization of p53 (human protein, also known as TP53) is a plausible mechanism to account for anemia in DBA and highlights a new pathway and set of molecules that could be the target for therapeutic intervention in affected individuals.

Other mouse strains relevant to DBA pathogenesis

Since the original description of *RPS19* mutations⁶, genetic alterations in at least eight other ribosomal proteins have been identified in DBA patients affecting both the 40S and 60S ribosomal subunits³. In addition, *RPS14* has been implicated in the pathogenesis of 5q-myelodysplastic syndrome (MDS), an adult onset disorder characterized by interstitial deletion of the long arm of chromosome 5, macrocytic anemia and a low rate of progression to acute myeloid leukemia^{28, 29}. Thus, a spectrum of ribosomal protein mutations brings about similar phenotypes and underscores the rationale for studying mice that carry ribosomal protein mutations other than *Rps19*.

Genetically engineered and spontaneous ribosomal protein mouse mutants have been particularly useful for dissecting the molecular pathways leading to the tissue-specific phenotypes observed in DBA patients. Mouse models with conditional deletion of *Rps6*^{15, 16, 30, 31}, heterozygous deletion of *Rpl24*^{12, 32} or conditional deletion of a set of genes found on human 5q (including *Rps14*)³³ have been generated (Table 1). Work based on these animal models has characterized a new cell cycle checkpoint and also highlight a role for activation of the transcription factor p53 in effecting *in vivo* phenotypes in individuals that carry ribosomal protein mutations.

***Rps6* inducible knockout**

Studies using a conditional *Rps6* knockout allele in mice have provided considerable insight into ribosome biology and have defined a cell cycle checkpoint that responds to the state of ribosome biogenesis. Under *in vivo* conditions that require cell proliferation, heterozygosity for *Rps6* leads to cell cycle arrest: conditional deletion of *Rps6* in the mouse liver prevents hepatocytes from dividing after partial hepatectomy, and hemizygosity for *Rps6* during gastrulation induces a proliferative defect that contributes to early embryonic lethality^{15, 30}. In addition, T-cells lacking one copy of *Rps6* fail to proliferate when activated³¹. Thus, cells with reduced ribosomal protein gene dosage may generate sufficient ribosomes under homeostatic conditions, but exhibit defects in ribosome biogenesis in a setting that requires rapid cell division. Checkpoint control mechanisms that sense defects in ribosome synthesis

may prevent cell cycle progression and contribute to the tissue-restricted phenotypes observed in ribosomal protein mutants.

The emerging role of p53

p53, the extensively studied transcription factor, is known for its ability to stem cancer development in response to genotoxic stress by regulating genes involved in processes such as cell cycle progression, senescence and apoptosis. Evidence from a number of model systems suggests that p53 is also poised to respond to stress signals induced by ribosomal protein mutations and phenotypic outcomes depend upon the underlying cell type in which p53 activation occurs^{16, 30, 31}. For example, haploinsufficiency for *Rps6* in mouse skin induces melanocytosis and dark skin, a phenotype that is completely abrogated in the absence of *Trp53*¹⁶. In addition, coat color, skin and retinal defects that characterize the spontaneous, semidominant mouse mutant, *Belly spot and tail* (*Bst*, *Rpl24* mutation) are mitigated by the loss of p53^{12, 32}(Figure 1).

Activation of p53 has also been linked to hematopoietic phenotypes in the mouse³³ (Figure 1). Recently, Barlow and colleagues generated mice with conditional deletion of the syntenic region in 5q- MDS (including *Rps14*). Like human patients, mutant mice exhibit macrocytic anemia, megakaryocyte dysplasia, increased apoptosis of bone marrow cells and progenitor cell defects. To evaluate p53-dependence, mutant mice were crossed into the *Trp53^{ko/ko}* background and hematologic parameters evaluated: an absence of p53 ameliorated many of the hematologic phenotypes. Thus, ribosomal protein haploinsufficiency triggers p53-dependent phenotypes in a number of organ systems including the bone marrow, thus providing a molecular connection between mutations that lead to a generalized impairment of ribosome biogenesis and tissue-specific manifestations observed in DBA (and MDS) patients.

Mutation in the ribosomal protein binding motif of MDM2

p53 is maintained at low levels by MDM2 (human protein, also known as HDM2)-mediated ubiquitylation in normal cells. Accumulating evidence suggests that certain ribosomal proteins (including RPL11, RPL5 and RPL23) are bound and sequestered by MDM2 in cells with defective ribosome biogenesis and inhibit its activity towards p53. Thus, p53 accumulates and downstream pathways are activated. Macias and colleagues³⁴ introduced a base pair substitution into the zinc finger domain of the mouse *Mdm2* locus (knock in allele, *Mdm2^{C305F}*) that disrupts ribosomal protein binding (Table 1). While *Mdm2^{C305F/C305F}* mice exhibit a normal p53 response to DNA damage, they fail to activate p53 after perturbations in ribosome biogenesis. Homozygous mutant mice also develop c-MYC induced lymphomas more rapidly than nonmutant animals, suggesting that the ribosomal protein-MDM2 interaction serves as a barrier against tumor formation. Interestingly, *p19Arf* was not required for the *Mdm2*-mediated response to ribosomal stress. The *Mdm2^{C305F}* knock in mice provide *in vivo* evidence that interaction between ribosomal proteins and the p53-MDM2 pathway is one mechanism by which p53 surveys the integrity of ribosome biogenesis and function.

Flvcr mutant mice

Despite extensive efforts to sequence all ribosomal protein genes in affected individuals, molecular lesions remain elusive in fifty percent of DBA cases. Thus, mutations in genes other than ribosomal proteins should be considered in the pathogenesis of DBA, either as the primary cause of the disease or as genetic factors that influence the penetrance and expressivity of the disease.

Feline leukemia virus induces a DBA-like red cell aplasia in cats by interfering with the activity of its cellular receptor *Flvcr*, a heme exporter. Although efforts to identify causative mutations in the *FLVCR1* gene in DBA families have been unrevealing thus far (*FLVCR1* maps to 1q31.3 near a putative DBA locus)³⁵, *Flvcr* mutant mice are characterized by a striking, DBA-like phenotype³⁶ (Table 2). *Flvcr*-null (*Flvcr^{ko/ko}*) mice die by E16.5 and have impaired definitive erythropoiesis. Embryos also exhibit abnormal limb, hand, and digit development, flattened faces and hypertelorism. Conditional inactivation of *Flvcr* in postnatal mice phenocopies DBA: mutant mice feature severe hyperchromic, macrocytic anemia and a block in erythroid development (at the pro-erythroblast stage). Investigators hypothesize that the failure in red blood cell development is due to accumulation of free heme, which leads to apoptosis and anemia. Intriguingly, alternatively spliced forms of *FLVCR1* have been found in primary cells from DBA patients and lead to altered protein function and/or stability³⁷. Although a causal role for *FLVCR* alterations in DBA is uncertain, the phenotypic overlap is striking and suggests that pathways that control heme accumulation, transport and/or degradation may contribute to the pathogenesis of the disease³⁶.

Mouse models of other ribosomopathies

Treacher-Collins syndrome

Treacher-Collins syndrome is a rare, autosomal dominant disorder caused by haploinsufficiency for *TCOF1* (the gene that encodes TCOF1 or Treacle). Treacher-Collins syndrome is considered a ribosomopathy since TCOF1 is a nucleolar protein present in pre-ribosomal complexes³⁸ and is necessary for ribosome biogenesis³⁹. Although Treacher-Collins patients have normal hematopoiesis, affected individuals exhibit DBA-like craniofacial abnormalities, which reflect abnormal neural crest development.

The *Tcof1* knockout mouse (*Tcof1^{ko/+}*) accurately reproduces the human phenotype: mutant mice exhibit severe craniofacial hypoplasia and dysplasia⁴⁰, a phenotype that is profoundly influenced by genetic background (Table 2)⁴¹. *Tcof1^{ko/+}* embryos have fewer migrating neural crest cells than wild type embryos due to extensive neuroepithelial cell apoptosis, and also exhibit defects in ribosome biogenesis. Blocking p53 function in mutant animals (by crossing into a *Trp53^{ko}* background or by treating with the p53 inhibitor, pifithrin- α) mitigates neuroepithelial cell apoptosis and results in normal craniofacial development, but does not correct abnormalities in ribosome function. Thus, current hypotheses suggest that *TCOF1* mutations trigger defects in ribosome biogenesis that bring about p53 stabilization in Treacher Collins patients, and like DBA, phenotypic features are dependent on the extent of p53 activation and the cell type in which it occurs.

Shwachman-Diamond syndrome (SDS)

SDS is an autosomal recessive disorder characterized by bone marrow failure, neutropenia, exocrine pancreatic dysfunction and a predisposition to leukemia⁴². It is caused by mutations in *SBDS*⁴³ which encodes a nucleolar protein that facilitates assembly of the 60S and 40S ribosomal subunits⁴⁴, and is, thus, classified as a ribosomopathy.

Sbds heterozygous mice (*Sbds^{ko/+}*) mice are indistinguishable from wild type, but homozygotes (*Sbds^{ko/ko}*) die in early embryogenesis (Table 2)⁴⁵. These observations are relevant to the human disease and underscore the essential role of *SBDS*: most patients carry one null allele and one hypomorphic allele (an allele that encodes a protein with some residual function). Two null alleles have never been found in the same patient.

Interestingly, a recent study points to a role for *SBDS* in the bone marrow stem cell niche in MDS and bone marrow failure⁴⁶. Tissue specific deletion of *Sbds* in mouse osteoprogenitor

cells (thought to be a component of the stem cell niche in the bone marrow) induces marrow dysfunction and MDS, and raises the possibility that mutations that disrupt ribosome biogenesis act in the marrow stroma and contribute to the pathogenesis of ribosomopathies like SDS and DBA.

Dyskeratosis congenita (DC)

Dyskeratosis congenita presents during the first decade of life with hyperpigmentation and nail dystrophy. Most patients develop peripheral cytopenias of one or more lineages, and many progress to bone marrow failure. DC is a genetically heterogeneous disease caused by mutations in components of the telomerase complex⁴⁷: *DKC1* (X-linked DC, protein product is DKC1 or dyskerin), *TERT* (autosomal recessive, telomerase reverse transcriptase), *TERC* (autosomal dominant DC, telomerase RNA), *TINF2* (autosomal dominant DC), *NOP10* (autosomal recessive DC) and *NHP2* (autosomal recessive DC). Several DC genes exhibit extra-telomeric functions as well. The most poignant example is *DKC1*; dyskerin is responsible for conversion of uridines to pseudouridines on newly synthesized rRNA molecules, a process that is conserved across species and is central to ribosome assembly. Thus, teasing apart the contributions of telomere biology and ribosome biogenesis to the DC phenotype has been challenging.

Mouse models for DC do not phenocopy the human disease accurately due in part to species specific differences in telomere length⁴⁸. Nonetheless, Ruggero and colleagues⁴⁹ generated a hypomorphic *Dkc1* allele that exhibits impaired rRNA pseudouridylation in early generations, prior to the onset of clinical disease (Table 2). Reduction of telomere length becomes evident in later generations, and thus points to a mechanism in which impaired ribosome function is important for initiation of DC, while telomere shortening modifies the phenotype further. Additional data suggests that defects in pseudouridylation lead to impaired translation of mRNAs containing IRES sequences and posits a mechanism by which defective ribosome activity leads to the DC phenotype^{50, 51}. Other mouse models highlight a role for telomere length in the pathogenesis of DC^{52, 53} (Table 2). Experiments based on mice that carry pathogenic human mutations find that mutant cells have a growth disadvantage compared to wild type cells, a phenotype that occurs in the context of normal telomere length. In the absence of telomerase, the differences in growth are mitigated.

Although it is clear that telomere dysfunction plays a role in the pathogenesis of DC, defects in ribosome biosynthesis and function may contribute to the disease phenotype. Thus, akin to hypotheses for extra-ribosomal functions for ribosomal proteins in DBA, DC genes may be pleiotropic in function and action.

White spotting mutants: an old model revisited

In mice, mutations at either the *Dominant White Spotting* (*W*, encoded by *c-Kit*) or the *Steel* (*Sl*, encoded by *Kit ligand*, *Kitl*) locus result in a clinical syndrome that is similar to DBA (Table 2)⁵⁴⁻⁵⁵. Animals are characterized by macrocytic anemia, reticulocytopenia and impaired erythrocyte development⁵⁶. In addition, hematopoietic stem cell self-renewal, survival and differentiation programs are abnormal^{27, 57}. Akin to human DBA patients, hematologic phenotypes observed in *W* mice can be rescued by stem cell transplantation: mutations in DBA patients and *c-Kit* mutants have a cell autonomous effect on bone marrow stem cells⁵⁴. (*Kitl* acts in the bone marrow environment to produce a non-cell autonomous phenotype)⁵⁴. Thus, investigators have postulated a role for KIT signaling in DBA^{26, 58, 59}.

Several lines of evidence suggest that white spotting mutants may not accurately model DBA, however. First, the anemia in *W* and *Sl* mice does not improve with traditional

corticosteroid therapy⁶⁰. Second, *W* and *Sl* mice manifest phenotypes in other organs that are not apparent in DBA patients: cutaneous abnormalities, mast cell defects and infertility are characteristic of *W* and *Sl* mice since Kit signaling is involved in common biologic processes used by cell types such as skin, mast and germ cells. Finally, extensive efforts to identify molecular alterations in *KIT* or *KITLG* in DBA patients have been unsuccessful^{61–63}.

Despite these observations, KIT signaling may contribute to the pleiotropic phenotypes observed in individuals that carry ribosomal protein mutations. In fact, ribosomal protein mutant mice share non-hematopoietic phenotypes with spotting mutants: white spots, an anatomic location in which pigment cells fail to migrate during development, are observed in adult animals (Figure 2A, B)^{12, 16, 32}. These observations are bolstered by *in vitro* studies showing that erythroid progenitors with reduced *Rps19* gene dosage express lower levels of Kit protein than control cells (see above)²⁶. Thus, *c-KIT* may be an excellent target for new drug development in DBA since the signaling pathway may contribute to erythrocyte development, as well as non-hematopoietic phenotypes in affected individuals.

Minute flies and fish

Drosophila

Dominantly inherited Minute mutants, first described in *Drosophila* more than 80 years ago⁶⁴, represent more than 50 different genetic loci and share phenotypic features including short, thin bristles, delayed larval development and recessive lethality. The genetic basis for this class of mutants puzzled generations of geneticists, but evidence now suggest that all mutations affect ribosomal proteins^{10, 18}.

Studies based on Minute flies have been instructive for scientists and clinicians whose focus is DBA. Early on, investigators focused on shared phenotypes: ribosomal protein mutations in both species are associated with developmental delay and small size. In addition, phenotypes are apparent in tissues and cells that require high rates of protein synthesis – bone marrow cells in human patients and sensory bristles in flies. Thus, current hypotheses suggest that reduced ribosomal protein gene dosage does not compromise cellular process under normal homeostatic conditions, but evokes tissue specific phenotypes in cells that require high protein synthesis (e.g. cell proliferation during development or hematopoiesis).

Phenotypes in Minute flies are, for the most part, due to ribosomal protein haploinsufficiency. Intriguingly, however, the converse is not true; haploinsufficiency for each ribosomal protein does not bring about a Minute phenotype¹⁸. This observation is paralleled in DBA, where *RPS19* is mutated in 25% of cases and *RPL5* is mutated in 7% of cases, but mutations in other ribosomal proteins do not bring about the disease phenotype^{2, 3}. One hypothesis to explain the apparent disparity suggests that ribosomal proteins are expressed at different levels, and phenotypes become evident when the quantity of any one ribosomal protein falls below a critical amount. Thus, variation in expression of different ribosomal proteins (which could be influenced by the rate of transcription, translation and/or transcript/protein stability) may contribute to variable penetrance and expressivity observed in Minute flies and DBA patients.

Zebrafish

Two groups have used antisense morpholino approaches to knockdown *rps19* in zebrafish^{11, 65}. Results from these studies yield similar conclusions: *rps19*-deficiency leads to hematopoietic and developmental abnormalities akin to those observed in DBA patients. Morphants are characterized by defective erythropoiesis and lower hemoglobin concentrations, features that are rescued by *rps19* overexpression. In addition, many animals

exhibit small size, as well as other morphologic abnormalities. Akin to mice that carry ribosomal protein mutations, knockdown of other ribosomal proteins in fish brings about a phenotype similar to the *rps19* morphant⁶⁵ and phenotypes are rescued in the absence of *p53*^{11, 66}.

rps and *rpl* mutations also cause tumors in zebrafish^{67, 68}, a finding that parallels observations suggesting that DBA is a cancer predisposition syndrome. From a forward genetic screen, investigators identified several fish lines that develop malignant peripheral nerve sheath tumors (MPNST) and are heterozygous for different ribosomal proteins. Intriguingly, the presence of tumors correlates with growth impairment (i.e. a “Minute”-like phenotype) and raises the possibility that additional tumorigenic mutations might confer a growth advantage among cells that are growth impaired. These studies also raise the possibility that the cancer predisposition observed in humans that carry ribosomal protein mutations might be the result of a translational defect.

Conclusions

Animal models for DBA have been pivotal in advancing our understanding of the disease. Approaches based on xenotransplantation and genetic engineering in the mouse have delineated potential genetic mechanisms: ribosomal protein mutations can act through haploinsufficient or dominant negative mechanisms to bring about red blood cell aplasia, an idea consistent with observations that the structure of the genetic mutation (e.g. deletion vs. missense) does not predict clinical outcome in DBA patients⁶⁹. Thus, additional efforts to delineate the pathogenesis of DBA can focus on molecular pathways common to all DBA patients.

Although hemizyosity for different ribosomal proteins has been recognized for decades to cause developmental delay and reduced organismal growth, ribosomal protein mutations also cause remarkably specific phenotypes, including white spotting in mice, short bristles in flies and anemia in humans. Hypotheses based on extra ribosomal functions for certain ribosomal proteins have been put forward since it is unclear how mutations in ubiquitously expressed genes with a well-known role in protein translation would manifest as tissue-restricted phenotypes. Two additional, complementary (and not mutually exclusive) mechanisms linking ribosomal protein mutations and bone marrow failure in DBA have emerged from studies based on animal models.

First, defective ribosome biogenesis may activate p53-dependent pathways in many (if not all) cells, and distinct phenotypic outcomes depend upon variation in the type of response. For example, p53 may activate a distinct set of genes in the skin to effect changes in skin or hair color and an alternate transcriptome in maturing erythrocytes to bring about anemia. Intriguingly, most phenotypes associated with ribosomal protein mutations are apparent in tissues characterized by rapid cellular proliferation – skin, bone marrow, developing embryos – thus linking ribosome synthesis and function with cell cycling, p53 and whole animal phenotypes.

A second hypothesis suggests that the balance of heme and globin synthesis is fundamental in DBA. During erythropoiesis, immature red blood cells synthesize a massive amount of globin, a process that requires a large pool of mature ribosomes. Erythroid cells compromised for ribosome function may not be able to maintain the level of globin synthesis required for maturation, leading to a relative excess of free heme, apoptosis and anemia. Additional studies using model organisms and built on these ideas would be instructive: assays to measure free heme in developing red blood cell from ribosomal protein mutants would support these ideas, and therapeutic approaches to titrate heme or increase

globin production may be helpful for treating affected patients. Thus, animal models have and will continue to aid efforts to generate new hypotheses, identify pathogenic pathways and pinpoint new targets for therapeutic intervention in DBA.

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Figure 1.

Activation of p53 links ribosomal protein haploinsufficiency to pleiotropic whole animal phenotypes in the mouse. p53 stabilization in keratinocytes (pink) leads to dark skin, in melanocytes (orange) to white coat color and in embryonic tissues to early lethality. Studies based on ribosomal protein mutants posit a mechanism by which activation of p53 induces red cell hypoplasia and anemia in DBA (hashed arrow).

Figure 2.

Ribosomal protein (Rp) mouse mutants share cutaneous phenotypes with white spotting mutants. A. An adult mouse carrying a *Kit* mutation is characterized by a white belly spot (left). The phenotype is enhanced when placed on a ribosomal protein mutant background (right). B. A histochemical marker for pigment cells (a *LacZ* transgene driven by regulatory elements from a pigment cell gene) stains developing melanocytes (blue) in E15.5 mouse embryos. In nonmutant animals, pigment cell precursors have moved to the developing skin in a dorsal to ventral expansion. Ribosomal protein mutant embryos have fewer X-gal positive cells in the skin (denoted by the large hashed oval devoid of blue staining) compared to nonmutant animals.

Table 1

Ribosomal protein mouse mutants model DBA.

Gene	Molecular approach	Whole animal and cellular phenotype	Implications	Ref(s)
<i>Rps19</i>	Knockout allele	Homozygote-early embryonic lethality; Heterozygote-no hematologic or developmental phenotype; normal <i>Rps19</i> expression	Compensation mechanisms in mice	13, 14
<i>Rps19</i>	Xenograft immunodeficient mice with human DBA cells	Improved engraftment and erythropoiesis in <i>RPS19</i> rescued marrow	Invokes <i>RPS19</i> haploinsufficiency in DBA; possible role for gene therapy	22, 23
<i>Rps19</i>	Transgene carrying a pathogenic missense mutation (β -actin promoter)	Ubiquitous expression-embryonic lethal; Inducible expression-growth retardation, anemia, reduced erythroid progenitors and red blood cells, rRNA processing defects	<i>RPS19</i> missense mutations may act through dominant negative mechanism	7
<i>Rps19</i>	Knockdown in erythroid progenitors (<i>ex vivo</i>)	Impaired proliferation, G1 arrest, induction of p53, decreased <i>Myb</i> and <i>Kit</i>	p53-induced cell cycle arrest results in decreased <i>Kit</i> and impaired erythropoiesis	26
<i>Rps19</i>	ENU-induced missense mutation	Homozygote-early embryonic lethality; Heterozygote-dark skin, white belly spot, growth retardation, mild anemia	Activation of p53 mediates tissue specific phenotypes	16
<i>Rps6</i>	Conditional allele	Liver deletion (2 alleles)-proliferation defect after partial hepatectomy; Ubiquitous deletion (one allele)-embryonic lethality, prolonged cell cycle, increased apoptosis; T-cell deletion (2 alleles)-impaired T-cell development; T-cell deletion (one allele)-decreased T-cell survival, proliferation defect in activated T-cells; Keratinocyte deletion (one allele)-dark skin; Melanocyte deletion (one allele)-light skin	Cell cycle checkpoint responds to defects in ribosome biogenesis; activation of p53 mediates tissue specific phenotypes	15, 16, 30, 31
<i>Rpl24</i>	Spontaneous mutation	White spotting, retinal defects, kinked tail, growth retardation; mutant cells have a prolonged cell cycle and a growth disadvantage	Phenotypes are mediated by p53	12, 32
5q deletion (<i>Rps14</i>)	Conditional deletion of ~500kb on 5qsyntenic region (<i>Rps14</i> and 8 other genes)	Macrocytic anemia, megakaryocyte dysplasia, bone marrow apoptosis, progenitor cell defects	Hematopoietic phenotypes are mediated by p53	33

Table 2

Other mouse models of DBA.

Gene	Molecular approach	Whole animal and cellular phenotype	Implications	Ref(s)
<i>Flvcr</i>	Knockout and conditional allele	Homozygote knockout-early embryonic lethality, impaired erythropoiesis, dysmorphicfacies and limbs; Postnatal deletion-anemia, block in erythropoiesis	Phenocopies many DBA features; role for excess heme in DBA	36
<i>Tcof1</i>	Knockout	Heterozygote-craniofacial hypoplasia, neural crest apoptosis	Phenotypes are mediated by p53	40
<i>Sbds</i>	Knockout	Homozygote-early embryonic lethality; Heterozygote-no phenotype	Gene dosage influences disease penetrance	45
<i>Dkc1</i>	Hypomorphic allele	rRNA modification defects occur early, telomere shortening occurs later	Impaired ribosome function is important for disease initiation, telomere shortening modifies the phenotype	49
<i>Dkc1</i>	Knockin pathogenic mutations	Mutant cells have a growth disadvantage	Growth disadvantage is independent of telomere length	52
<i>Kit</i>	Spontaneous and engineered	White belly spot, macrocytic anemia	Abnormal Kit signaling may contribute to DBA	54, 56
<i>Kitl</i>	Spontaneous and engineered	White belly spot, macrocytic anemia	Abnormal Kit signaling may contribute to DBA	54, 55
<i>Mdm2</i>	Knockin (base pair substitution, C305F)	Mutation impairs mdm2-ribosomal protein binding; perturbations in ribosome biogenesis do not lead to activation of p53	May correct phenotypes in ribosomal protein mutants and DBA patients	34